



**HAL**  
open science

# Biological Markers For Chronic Obstructive Pulmonary Disease And Asthma

Zeina Akiki

► **To cite this version:**

Zeina Akiki. Biological Markers For Chronic Obstructive Pulmonary Disease And Asthma. Santé publique et épidémiologie. Université Paris Saclay (COMUE); Université Libanaise, 2016. English. NNT : 2016SACLS081 . tel-01571267

**HAL Id: tel-01571267**

**<https://theses.hal.science/tel-01571267>**

Submitted on 2 Aug 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

NNT : 2016SACLS081

THESE DE DOCTORAT  
DE  
L'UNIVERSITE LIBANAISE  
ET DE  
L'UNIVERSITE PARIS SACLAY  
PREPAREE A L'UNIVERSITE PARIS SUD – XI

ECOLE DOCTORALE DE SCIENCES ET DE TECHNOLOGIE - EDST  
ET  
ECOLE DOCTORALE DE SANTE PUBLIQUE – EDSP - N°570

Spécialité de doctorat: Santé Publique - Epidémiologie

Par

Mme Zeina AKIKI

**Marqueurs biologiques  
de la broncho-pneumopathie chronique obstructive et de l'asthme**

Thèse présentée et soutenue à Beyrouth, le 11 avril 2016

**Composition du jury :**

Mme Nadine SALEH, Professeure, Université Libanaise, Présidente du jury  
Mr Denis HEMON, Professeur, Inserm, Rapporteur  
Mr Hani TAMIM, Professeur, American University of Beirut, Rapporteur  
Mr Nicolas ROCHE, PU-PH, Université Paris Descartes, Examineur  
Mr Denis CHARPIN, PU-PH, Université d'Aix-Marseille, Examineur  
Mme Rachel NADIF, HDR, CR1, Inserm, Co-directrice  
Mme Soulaïma CHAMAT, Professeure, Université Libanaise, Co-directrice  
Mme Pascale SALAMEH, Professeure, Lebanese American University, Co-directrice



**Titre :** Marqueurs biologiques de la broncho-pneumopathie chronique obstructive et de l'asthme.

**Mots clés :** Maladies respiratoires, épidémiologie, BPCO, contrôle de l'asthme, SP-D sérique, hs-CRP sérique, profils de cytokines sériques, 8-IsoPs du condensat de l'air exhalé.

**Résumé :** L'étude des marqueurs biologiques dans la broncho-pneumopathie chronique obstructive (BPCO) et l'asthme, deux maladies respiratoires chroniques affectant des millions de personnes dans le monde, pourrait améliorer leur diagnostic, leur traitement et leur prévention.

Cette thèse comprend deux parties. La première visait à évaluer l'association entre un marqueur spécifique des poumons, la protéine surfactant D (SP-D) sérique, et la BPCO, et à trouver un seuil de SP-D capable de discriminer les patients BPCO des témoins. Elle a été réalisée dans le cadre d'une étude cas-témoin au Liban incluant des patients BPCO (n=90), des asthmatiques (n=124) et des témoins (n=180). La deuxième partie visait à évaluer les associations chez les adultes des marqueurs de l'inflammation systémique (protéine C-réactive ultra-sensible, hs-CRP (n=252) et cytokines (n=283)) et des marqueurs de dommages dus au stress oxydant (8-isoprostanes 8-IsoPs (n=258) du condensat de l'air exhalé), avec les phénotypes de l'asthme.

Cette étude a été réalisée dans le cadre de l'étude épidémiologique longitudinale Française des facteurs génétiques et environnementaux de l'asthme (EGEA).

Les résultats ont montré que les niveaux de SP-D sériques étaient associés positivement avec la BPCO et des seuils des niveaux de SP-D chez ces patients ont été identifiés avec d'excellentes valeurs discriminantes. Dans EGEA, aucune association n'a été trouvée entre les niveaux de hs-CRP sériques et le contrôle de l'asthme. Des profils de cytokines sériques (identifiés par analyse en composante principale) avec des niveaux élevés d'interleukine(IL)-1Ra et d'IL-10 ont été associés avec moins de crises d'asthme et un risque plus faible d'un mauvais contrôle de l'asthme sept ans plus tard. Les résultats des analyses préliminaires sur les associations entre les niveaux de 8-IsoPs et les phénotypes de l'asthme sont également présentés.

Globalement, ces résultats ont montré l'utilité d'étudier les marqueurs biologiques en lien avec la BPCO et l'asthme.

**Title:** Biological markers for chronic obstructive pulmonary disease and asthma.

**Keywords:** Respiratory diseases, epidemiology, COPD, asthma control, serum SP-D, serum hs-CRP, serum cytokine profiles, 8-IsoPs of exhaled breath condensate.

**Abstract:** Studying the biological markers in chronic obstructive pulmonary disease (COPD) and asthma, two chronic respiratory diseases affecting millions of individuals around the world, could improve their diagnosis, their treatment and their prevention.

This thesis includes two parts. The first aimed to assess the association between a lung-specific biomarker, serum Surfactant Protein D (SP-D), and COPD, and to find cut-off points able to discriminate COPD patients from controls using SP-D levels. It was performed in a case-control study in Lebanon including COPD (n=90) and asthma patients (n=124) and controls (n=180). The second part aimed to assess the cross-sectional and longitudinal associations in adults between systemic inflammatory biomarkers (high sensitivity C reactive protein hs-CRP (n=252) and cytokines (n=283)) and biomarkers of damage due to oxidative stress (8-Isoprostanes 8-IsoPs (n=258) in the exhaled breath condensate) and asthma outcomes.

This study was performed in the French longitudinal epidemiological study on the genetics and environmental factors of asthma (EGEA).

Results showed that serum SP-D levels were positively associated with COPD and thresholds for SP-D levels in these patients were identified with excellent discriminant values. In EGEA, no association was found between serum hs-CRP levels and asthma control. Serum cytokine profiles (identified by principal component analysis) with high levels of interleukin (IL)-1Ra and IL-10 were associated with less asthma attacks and lower risk of poor asthma control seven years later. The results of the preliminary analyses on the associations between the levels of 8-IsoPs and asthma outcomes are also presented.

Overall, these results have shown the usefulness of studying the biological markers related to COPD and asthma.

## *ACKNOWLEDGMENTS*

---

*After three years of tireless effort and perseverance, I would like to thank the people who have made this achievement possible:*

First and foremost, I would like to give acknowledgments to my advisors, Rachel NADIF in France as well as Soulaima CHAMAT and Pascale SALAMEH in Lebanon, for being tremendous advisors for me and for allowing me to grow as a research scientist.

I am deeply grateful to Rachel NADIF. She is an exceptional mentor, who offers the perfect balance between academic freedom and wise guidance. She has perceptively guided my autonomous reasoning and I was able to finalize this research because of her demanding follow-up. Her comments on the thesis form and contents have significantly improved the work. I learnt a lot from her on many levels; her dedication and perfectionism are inspiring. I'm greatly touched by her availability despite the fact that we live in two different countries. I thank her for the long hours we spent working together by videoconference and for the amicable and hard-working ambiance she maintains in her research unit.

Then, my deepest thanks go to Soulaima CHAMAT for having accepted to supervise my work. Her accurate comments, wise technical and practical advice, and questions on the work performed have significantly contributed to its improvement. I thank her for her support, her encouragement and her perfectionism.

Moreover, my endless gratitude goes to Pascale SALAMEH, the person who has accompanied me during my master studies and my PhD. It is thanks to her professional demeanour that I was able to gain an in-depth understanding of the specialization and thanks to her accurate comments that I was able to conduct this research. I cannot but also acknowledge her intellectual flexibility and wit, her continuous encouragement, her dedication and her openness, for they allowed me to enjoy a sufficient margin of freedom to finalize this research.

I warmly thank the jury members who have done me the honor to participate in this thesis committee. I thank Professor Nadine SALEH for being the jury president. I extend my sincere thanks to Professor Denis HEMON and Professor Hani TAMIM for agreeing to evaluate this work. I also thank Professors Nicolas ROCHE and Denis CHARPIN for accepting to be members in this thesis committee.

Similarly, my recognition goes to everyone who has worked on the study conducted in Lebanon, especially Dr. Mirna WAKED, Dr. Rania JOUNBLAT and Dr. Dalia Fakhri as well as to all the people who have participated in it. I equally thank everyone who worked on the EGEA study.

I thank all the members of the Immunology Laboratory in the Lebanese University, as well as all members of the INSERM unit 1168 (VIMA).

I gratefully acknowledge the funding sources that made my PhD possible, the College des Soeurs du Saint-Coeur.

I also like to thank Professor Jean BOUYER and Audrey BOURGEOIS for their availability and for efficiently and devotedly managing the doctoral school (EDSP). I also thank Professor Fawaz Al OMAR and all EDST staff.

Huge thanks go to all my friends for their understanding and encouragement in my many moments of crisis. Their friendship makes my life a wonderful experience.

I would like to dedicate this thesis to my parents, my sisters, my husband and my newborn baby.

Words cannot express how grateful am I to my fantastic family, my mum, my dad and my sisters, Nawal and Ghada, who have been by my side and supported me all the way, always encouraging me to move forward with confidence and enthusiasm. I thank them for their endless love.

All my love and thanks to my wonderful and encouraging husband Ziad whose patient support in the last two years is much appreciated. I thank him for always being there for me, for encouraging me and always backing my choices, even when it meant dedicating more time to my work.

Last but not least, I owe my adorable newborn, Maria a great deal since her presence in our lives is a real blessing.

This thesis is only the beginning of my journey.



## *PUBLICATIONS AND COMMUNICATIONS*

---

### **Articles:**

- 1- **Akiki Z**, Fakh D, Jounblat R, Chamat S, Waked M, Holmskov U, Sørensen G L, Nadif R, Salameh P. Surfactant Protein D, a clinical biomarker for COPD with excellent discriminant values. *Exp. Ther. Med.* 2016 Mar;11(3):723-30 (published online on January 13, 2016).
- 2- **Akiki Z**, Rava M, Diaz Gil O, Pin I, Le Moual N, Siroux V, Guerra S, Chamat S, Matran R, Montserrat F, Salameh P, Nadif R. Blood cytokine profiles and subsequent risk of asthma symptom control in adults from the EGEEA study (submitted to *Allergy*).
- 3- 8-isopropanes and asthma control in adults from the EGEEA study (in preparation).

### **Articles as co-author:**

- 1- Fakh D, Junker K, **Akiki Z**, Medlej-Hashem M, Chamat S, Waked M, Salameh P, Holmskov U, Sørensen GL, Jounblat R. Surfactant protein D (SP-D) levels, polymorphisms and multimerization in healthy and inflammatory disease state (draft manuscript).
- 2- Salameh P, Salame J, Khayat G, Akhdar A, Ziadeh C, Azizi S, Khoury F, **Akiki Z**, Nasser Z, Abou Abbass L, Saadeh D, Waked M. Exposure to outdoor air pollution and chronic bronchitis in adults: a case-control study. *Int J Occup Environ Med.* 2012 Oct;3(4):165-77.
- 3- Salameh P, Waked M, Khoury F, **Akiki Z**, Nasser Z, Abou Abbass L, Dramaix M; Chronic Bronchitis Study Group. Waterpipe smoking and dependence are associated with chronic bronchitis: a case-control study in Lebanon. *East Mediterr Health J.* 2012 Oct;18(10):996-1004.

### **Communications:**

- 1- Poster discussion, ERS congress, Munich, September 2014:  
**Akiki Z**, Fakh D, Jounblat R, Chamat S, Waked M, Sørensen GL, Nadif R, Salameh P. SP-D as a biomarker for COPD in the Lebanese population.
- 2- LAAS conference, Lebanese University, March 2014:  
**Akiki Z**, Fakh D, Jounblat R, Chamat S, Waked M, Sørensen GL, Nadif R, Salameh P. SP-D as a biomarker for COPD patients in the Lebanese population (preliminary results).
- 3- Poster discussion, doctoral forum, EDST - Lebanese University, July 2013 (2<sup>nd</sup> prize):  
**Akiki Z**, Fakh D, Jounblat R, Salameh P, Chamat S, Nadif R, Sørensen GL, Waked M. Validity of serum Surfactant Protein D as a lung specific biomarker in the diagnosis and in the therapeutic follow-up of patients with Asthma and Chronic Obstructive Pulmonary Disease.



### **My involvement in this thesis work:**

In the first part of the thesis performed in Lebanon, I was involved in the whole process from the data collection till writing the manuscript entitled “*Surfactant Protein D, a clinical biomarker for COPD with excellent discriminant values*”. As for the second article entitled “*Surfactant protein D (SP-D) levels, polymorphisms and multimerization in healthy and inflammatory disease state*”, I performed the data entry and statistical analyses and I will review the article once finalized.

In the second part of the thesis performed on French data, I performed the statistical analyses on data extracted from the EGEA study and I wrote the article entitled “*Blood cytokine profiles and subsequent risk of asthma symptom control in adults from the EGEA study*”. Moreover, I’m currently finalizing the analyses and the article on 8-isoprostanes and current asthma.

*UNIT OR LAB: NAME AND ADDRESS*

---

**France:**

**Since 2015**

Inserm, UMR-S 1168

VIMA: Aging and chronic diseases. Epidemiological and public health approaches.

16, avenue Paul Vaillant Couturier

94807 Villejuif

France

Phone: 33 (0) 145 59 50 60

Fax: 33 (0) 145 59 51 69

**Previously (2012-2014)**

Respiratory and Environmental Epidemiology Team

Inserm, UMRS Unit 1018

CESP, Centre for research in Epidemiology and Population Health

**Lebanon:**

Laboratory of Immunology, Faculty of Public Health - EDST E006.

Lebanese University, Fanar, Lebanon.

Phone: +96-1-686355

Fax: +961-1-686338

## *ABBREVIATIONS*

---

ACOS: Asthma-COPD Overlap Syndrome  
ACT: Asthma Control Test  
AHR: Airway Hyperresponsiveness  
ASF: Altered Structure and Function  
ATS: American Thoracic Society  
AUC: Area Under the Curve  
BAL: Broncho-Alveolar Lavage  
BED: Biological Effective Dose  
BMI: Body Mass Index  
CaCl<sub>2</sub>: Calcium Chloride  
CAT: COPD Assessment Test  
CD: Clinical Disease  
COPD: Chronic Obstructive Pulmonary Disease  
COX: Cyclooxygenase  
CRP: C - Reactive Protein  
CVD: Cardiovascular Disease  
DALYs: Disability-Adjusted Life Years  
DNA: Deoxyribonucleic acid  
DS-COPD: Diagnosis Score for COPD patients  
E: Exposure  
EBC: Exhaled Breath Condensate  
EBE: Early Biological Effects  
ECLIPSE: Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points  
ECRHS: European Community Respiratory Health Survey  
EGEA: Epidemiological study on the Genetics and Environment of Asthma  
EIA: Enzyme Immuno-Assay  
ELISA: Enzyme-Linked Immunosorbent Assay  
ERS: European respiratory Society  
FEV<sub>1</sub>: Forced Expiratory Volume in 1 second  
FVC: Forced Vital Capacity  
GERD: Gastro Esophageal Reflux Disease

GFR: Glomerular Filtration Rate  
GINA: Global INitiative for Asthma  
GM: Geometric Mean  
GOLD: Global Initiative for Chronic Obstructive Lung Disease  
H<sub>2</sub>O<sub>2</sub>: Hydrogen Peroxide  
H<sub>2</sub>SO<sub>4</sub>: Sulfuric Acid  
hs-CRP: high-sensitivity C-Reactive Protein  
ICS: Inhaled Corticosteroids  
ID: Internal Dose  
IgE: Immunoglobulin E  
IL: Interleukin  
IL-1Ra : Interleukin 1 receptor antagonist  
IMIM : Instituto Hospital del Mar de Investigaciones Médicas  
IsoPs : Isoprostanes  
IU: International Unit  
LABA: Long-Acting Beta<sub>2</sub>-Agonist  
LLN: Lower Limit of Normal  
mMRC: modified Medical Research Council Questionnaire  
NAS: National Academy of Sciences  
OR: Odds Ratio  
PAARC: “Pollution Atmosphérique et Affections Respiratoires Chroniques”  
PCA: Principal Component Analysis  
PG: Prostaglandin  
PS: Prognostic Significance  
RNA: RiboNucleic Acid  
ROC: Receiver-Operating Characteristic  
RV: Residual volume  
SABA: Short-Acting Beta<sub>2</sub>-Agonist  
SAS: Statistical Analytics Software  
SD: Standard Deviation  
SES: Socio-Economic Status  
SNP: Single Nucleotide Polymorphism  
SP-D: Surfactant Protein D  
SPSS: Statistical Package for the Social Sciences

SPT: Skin Prick Test

TH1: T-Helper type 1 lymphocytes

TH2: T-Helper type 2 lymphocytes

TLC: Total lung capacity

TNF- $\alpha$ : Tumor Necrosis factor - Alpha

UICTMR: "Union Internationale Contre la Tuberculose et les Maladies Respiratoires"

USA : United States of America.

## LIST OF TABLES

---

<b>Table 1.</b> Characteristics of controls, COPD and asthma patients.....	54
<b>Table 2.</b> Variations of SP-D, CRP and fibrinogen levels between patients and controls	55
<b>Table 3.</b> Associations between biological markers and lung function tests in COPD patients.....	55
<b>Table 4.</b> Associations between serum SP-D levels and COPD - Multivariate analyses.	57
<b>Table 5.</b> Coordinates of the ROC curve for all COPD patients and controls.....	59
<b>Table 6.</b> Coordinates of the ROC curve for ever-smoker COPD patients and controls	61
<b>Table 7.</b> Characteristics of the 558 adults with current asthma at the second survey (EGEA2).....	69
<b>Table 8.</b> Comparison of main characteristics between non-selected and selected participants.....	77
<b>Table 9.</b> Characteristics of the 252 adults with current asthma at the second survey (EGEA2).....	78
<b>Table 10.</b> Associations between serum hs-CRP levels and age, sex, BMI and smoking.....	79
<b>Table 11.</b> Cross-sectional associations between serum hs-CRP levels and asthma outcomes (EGEA2).....	81
<b>Table 12.</b> Longitudinal associations between serum hs-CRP levels and asthma outcomes (EGEA3).....	82
<b>Table 13.</b> Comparison of main characteristics between non-selected and selected participants.....	91
<b>Table 14.</b> Characteristics of the 283 adults with current asthma at the second survey (EGEA2).....	92
<b>Table 15.</b> Serum levels of the cytokines.....	93
<b>Table 16.</b> Pearson correlation coefficients for each pairwise correlation of cytokines	94
<b>Table 17.</b> Contribution of biological markers to each principal component.....	95
<b>Table 18.</b> Associations between PC scores and age, sex, BMI and smoking.....	97
<b>Table 19.</b> Cross-sectional associations between cytokines PC scores and asthma outcomes (EGEA2).....	98
<b>Table 20.</b> Cross-sectional associations between cytokines PC scores and neutrophil and eosinophil counts and IgE levels (at EGEA2).....	99
<b>Table 21.</b> Longitudinal associations between cytokines PC scores and asthma outcomes (EGEA3).....	100
<b>Table 22.</b> Longitudinal associations between individual cytokines and asthma outcomes (EGEA3).....	102
<b>Table 23.</b> Comparison of main characteristics between selected and non-selected participants and those having non-detectable 8-IsoPs levels.....	111
<b>Table 24.</b> Associations between EBC 8-IsoPs levels and age, sex, BMI and smoking.....	112
<b>Table 25.</b> Cross-sectional associations between EBC 8-IsoPs levels and asthma outcomes .....	113

## LIST OF FIGURES

---

<b>Figure 1.</b> Asthma phenotypes .....	36
<b>Figure 2.</b> Biologic marker components in sequential progression between exposure and disease.....	41
<b>Figure 3.</b> Participants of the study .....	50
<b>Figure 4.</b> ROC curve with all COPD patients and controls.....	58
<b>Figure 5.</b> ROC curve with ever-smoker COPD patients and controls.....	60
<b>Figure 6.</b> Participants and methodology of the EGEA study.....	68
<b>Figure 7.</b> Participants selected from the EGEA study.....	68
<b>Figure 8.</b> Flow chart of the participants – data on hs-CRP and current asthma.....	73
<b>Figure 9.</b> Cross-sectional associations between serum hs-CRP levels and asthma symptom control.....	80
<b>Figure 10.</b> Flow chart of the participants – data on cytokines and current asthma.....	87
<b>Figure 11.</b> Graphical representation of the scores in the space and box plots of the two principal components.....	95
<b>Figure 12:</b> Dendrogram of the seven cytokines obtained by using proc VARCLUS.....	96
<b>Figure 13.</b> Association between PC2 score at EGEA2 and subsequent risk of asthma symptom control.....	103
<b>Figure 14.</b> Flow chart of the participants – data on 8-IsoPs and current asthma.....	107
<b>Figure 15.</b> Histograms for 8-IsoPs levels before and after log10 transformation.....	109

## **1. Introduction**

Les maladies respiratoires chroniques représentent un large éventail de maladies graves. Elles prédisent fortement la santé globale, le vieillissement et la mortalité toutes causes, et sont une cause majeure de morbidité et de mortalité dans le monde. Les maladies respiratoires chroniques touchent des centaines de millions de personnes de tous âges et plus de 50% d'entre elles vivent dans les pays à revenu faible et moyen ou parmi des populations démunies. Elles représentent quatre millions de décès par an. Dans cette thèse, nous nous sommes intéressés à la broncho-pneumopathie chronique obstructive (BPCO) et à l'asthme, deux maladies respiratoires chroniques communes dont la charge mondiale semble être en croissance.

La BPCO est une maladie hétérogène avec différentes présentations cliniques, touchant environ 210 millions de personnes à travers le monde. Elle est caractérisée par une dyspnée, une production excessive de crachat, une toux chronique et une obstruction progressive des bronches qui n'est pas totalement réversible et qui est associée à une réponse inflammatoire chronique. Les principaux facteurs de risque sont le tabagisme, la pollution atmosphérique intérieure telle que les carburants de biomasse utilisés pour faire la cuisine et pour le chauffage, la pollution atmosphérique extérieure et l'exposition à des particules ou des gaz nocifs. La BPCO se développe surtout chez les adultes de plus de 40 ans, avec une augmentation considérable avec l'âge, en particulier chez les fumeurs. Le diagnostic de la BPCO est confirmé par la spirométrie, avec un rapport du volume expiratoire maximal seconde (VEMS) / capacité vitale forcée (CVF) après inhalation de bronchodilatateur inférieur à 70 %. Cette technique est cependant difficile pour certains patients et n'est pas toujours disponible. De plus, elle nécessite une certaine expertise pour son interprétation et est mal corrélée avec la sévérité de la maladie. Une alternative est donc nécessaire.

L'asthme est une maladie inflammatoire chronique des voies respiratoires, affectant environ 300 millions de personnes à travers le monde. L'asthme est une maladie complexe, avec une forte hétérogénéité clinique et une variabilité phénotypique dans le temps. La prévalence de l'asthme a presque doublé au cours des dernières décennies. L'asthme est caractérisé par des



symptômes respiratoires récurrents, tels que des sifflements, une oppression thoracique et une toux.

Il est maintenant reconnu que le vieillissement est largement déterminé au cours de la vie. Ainsi, afin d'assurer un meilleur vieillissement pour les sujets ayant des maladies respiratoires chroniques, nous avons besoin de mieux comprendre les mécanismes physiopathologiques de ces maladies afin de permettre un meilleur diagnostic, un meilleur traitement et une meilleure prévention. Dans la BPCO et l'asthme, l'étude des marqueurs biologiques est d'intérêt, et l'intégration de leurs mesures dans les études épidémiologiques peut être pertinente afin de mieux comprendre l'étiologie de ces maladies, et également d'affiner les phénotypes. Ces marqueurs peuvent permettre d'évaluer la gravité ou la progression de la maladie, ainsi que la réponse au traitement. Dans ces deux maladies, l'inflammation chronique est à la fois systémique et locale. Pour étudier l'inflammation systémique, l'étude des marqueurs biologiques dans le sang est des plus prometteuses, car le sang est plus facilement accessible que le lavage broncho-alvéolaire ou les expectorations induites, surtout dans le cadre de grandes études épidémiologiques ou en application clinique. Récemment, la recherche de marqueurs biologiques dans les maladies respiratoires a mis l'accent sur l'intérêt d'étudier plusieurs marqueurs simultanément plutôt qu'individuellement, et sur l'intérêt d'étudier des marqueurs biologiques au plus proche du poumon dans le condensat de l'air exhalé.

L'objectif général de cette thèse est donc d'étudier le rôle des marqueurs biologiques spécifiques dans la BPCO et dans l'asthme. Cette thèse comprend deux parties. La première partie a été réalisée dans le cadre d'une étude cas-témoin au Liban et visait à évaluer l'association entre un marqueur biologique spécifique du poumon et la BPCO. La deuxième partie a été réalisée dans le cadre de l'étude épidémiologique longitudinale Française des facteurs génétiques et environnementaux de l'asthme (EGEA) et visait à évaluer les associations entre des marqueurs biologiques de l'inflammation systémique et des marqueurs biologiques liés au stress oxydant dans le condensat de l'air exhalé et l'asthme.

## **2. Objectifs spécifiques du travail**

Le premier objectif spécifique a été réalisé parmi des patients Libanais consultant pour symptômes ou maladies respiratoires. Il visait à étudier l'association entre les niveaux d'un marqueur biologique spécifique du poumon, la protéine surfactant-D (SP-D) sérique, et la

BPCO. Un seuil optimal capable de discriminer les patients BPCO des témoins en utilisant les niveaux de la SP-D a été recherché. La validité de la SP-D a donc été comparée à celle de deux marqueurs sériques de l'inflammation systémique, qui sont la CRP et le fibrinogène. La CRP est un marqueur très sensible de l'inflammation systémique. Le fibrinogène est une protéine de phase aiguë et un facteur de coagulation du sang. Les taux de fibrinogène circulants ont été trouvés significativement plus élevés chez les patients atteints de BPCO par rapport aux témoins. Ces deux marqueurs ont été validés dans des essais cliniques de laboratoire, sont associés au degré de sévérité de l'inflammation des voies aériennes, mais ils ne sont pas spécifiques du poumon. Nous avons posé l'hypothèse que la SP-D, récemment associée avec la BPCO, pourrait être un marqueur biologique plus spécifique que la CRP ou le fibrinogène pour différencier des patients atteints de BPCO de personnes consultant pour d'autres maladies respiratoires y compris l'asthme ou ayant des symptômes respiratoires.

Le deuxième objectif spécifique a été réalisé dans le cadre de l'étude épidémiologique EGEA et visait à évaluer les associations entre les niveaux de marqueurs de l'inflammation (CRP ultra-sensible (hs-CRP) sérique, profils de cytokines sériques) ou de marqueurs du stress oxydant (8-isoprostanés (8-IsoPs) du condensat de l'air exhalé) d'une part et l'asthme d'autre part. Trois analyses différentes ont donc été effectuées dans l'étude EGEA. L'inflammation chronique dans l'asthme est orchestrée par de nombreux marqueurs grâce à des interactions complexes, mais peu d'études ont été réalisées à ce jour sur les associations entre les niveaux de hs-CRP sérique, ou les cytokines sériques et le contrôle de l'asthme, notamment sous une approche prospective. La hs-CRP est utile pour la détection de l'inflammation systémique de bas grade présente dans l'asthme et à notre connaissance, l'association avec le contrôle de l'asthme chez les adultes n'a été étudiée que dans deux études avec des résultats contradictoires. Les cytokines sont des protéines ayant un rôle dans l'inflammation chronique de l'asthme. Des thérapies ciblées par des anticorps anti-interleukine-(IL)-5 et IL-13 ont été récemment développées et ont abouti à des résultats encourageants chez certains patients, alors qu'elles sont demeurées inefficaces chez d'autres. Bien que ces cytokines soient utilisées comme cible thérapeutique pour améliorer le contrôle de l'asthme, l'association entre les niveaux de cytokines dans le sérum et contrôle de l'asthme a été peu étudiée. À notre connaissance, une seule étude cas-témoin a évalué les associations entre les profils de cytokines dans les crachats induits et le contrôle de l'asthme, et aucune étude n'a utilisé cette approche dans le sérum dans une étude prospective. Enfin, l'inflammation des voies respiratoires dans l'asthme pourrait être étudiée au plus proche du poumon à l'aide de

marqueurs biologiques mesurés dans le condensat de l'air exhalé. À notre connaissance, une seule étude a étudié les associations entre les niveaux de 8-IsoPs et le contrôle de l'asthme chez l'adulte et n'a rapporté aucune association.

### **3. Evaluation de la SP-D comme marqueur biologique de la BPCO**

Une étude cas-témoins incluant 90 patients BPCO (ayant un rapport VEMS / CVF après inhalation de bronchodilatateur inférieur à 70%) et 124 patients atteints d'asthme (diagnostic clinique confirmé par spirométrie) consultant dans les cliniques externes du département de pneumologie de l'hôpital universitaire et le centre médical Saint Georges à Beyrouth a été réalisée. Des témoins de la population générale (n = 92) ou venant pour des consultations non respiratoires dans le même hôpital (n = 88) ont également été recrutés. Tous les participants ont signé un consentement éclairé et ont rempli un questionnaire standardisé. Des prélèvements sanguins ont été effectués. Après centrifugation à 4°C, les échantillons de plasma / sérum ont été répartis en aliquots et conservés à -20°C jusqu'à leur analyse dans les 6 mois. Suite aux prélèvements sanguins, un test de spirométrie (avant et 30 minutes après administration de bronchodilatateur) a été effectué. La mesure de la SP-D a été effectuée par dosage immuno-enzymatique (ELISA). Les niveaux de CRP sérique et de fibrinogène plasmatique ont été mesurés en utilisant des kits ELISA.

Trois modèles de régressions logistiques ont été utilisés pour évaluer l'association entre la SP-D et de la BPCO. Dans la première et la deuxième régression, tous les patients BPCO et les témoins ont été inclus dans les analyses. Les variables indépendantes étaient la SP-D (dessus/dessous de la médiane), et les variables de confusion potentielles telles que l'âge, le sexe, le tabac et l'indice de masse corporelle (IMC), ainsi que toutes les caractéristiques sociodémographiques et les symptômes respiratoires ayant été trouvés associés dans les analyses univariées (valeur de  $P < 0.20$ ) ont été incluses dans le modèle. Dans la deuxième régression, seuls les patients BPCO et les témoins fumeurs actuels ou ex-fumeurs ont été également inclus. Une troisième régression a considéré les patients BPCO et les patients atteints d'asthme. Les odds-ratios ajustés (ORA) obtenus à partir de la première et de la deuxième régression ont ensuite été arrondis aux unités les plus proches et utilisés comme coefficients dans le calcul du score 1 (pour tous les patients BPCO et les témoins) et le score 2 (pour les patients BPCO et les témoins, fumeurs actuels ou ex-fumeurs). Des courbes ROC

ont ensuite été générées pour déterminer la capacité des scores 1 et 2 à discriminer les patients BPCO des témoins. Toutes les analyses ont été réalisées avec le logiciel SPSS version 17.0.

Les résultats ont montré que les niveaux de SP-D au-dessus de la valeur médiane étaient significativement et positivement associés à la BPCO (ORa = 3,86 et Intervalle de Confiance à 95% (IC 95%) [1,51-9,85], valeur de P=0,005 et 6,26 [1.81-21.65], valeur de P=0,004 pour tous les patients BPCO et pour ceux qui sont fumeurs actuels et ex-fumeurs respectivement). Ce résultat est concordant avec les résultats rapportés dans les études antérieures. Aucune association n'a été observée entre les taux de CRP, de fibrinogène et la BPCO. Aucune association n'a été trouvée entre la SP-D et l'asthme. Un premier score (score 1) a été identifié pour le diagnostic des patients BPCO. Le seuil optimal était de 15,5 et les sensibilité et spécificité étaient de 76,4% et de 89,3% respectivement. Chez les fumeurs actuels et les ex-fumeurs atteints de BPCO, un deuxième score (score 2) a été identifié. Le seuil optimal était de 18,5 et les sensibilité et spécificité étaient de 77,8% et 88,5% respectivement. Ces scores sont associés à d'excellentes valeurs discriminantes, avec la meilleure échelle pour le diagnostic de la BPCO obtenue en utilisant les niveaux de SP-D, les caractéristiques socio démographiques, et les symptômes respiratoires associés de façon significative avec la BPCO.

En conclusion, la SP-D semble pouvoir différencier les patients BPCO d'autres patients consultant pour des symptômes ou pour d'autres maladies respiratoires. Utilisée avec les caractéristiques sociodémographiques et les symptômes respiratoires associés à la BPCO, la SP-D est capable de discriminer les patients BPCO des témoins ainsi que des patients atteints d'asthme.

#### **4. Etude EGEA**

L'étude EGEA (<https://egeanet.vjf.inserm.fr/>) est une étude française longitudinale cas-témoin et familiale avec un premier groupe de cas asthmatiques et de leurs parents du premier degré, et des témoins (première enquête EGEA1, entre 1991 et 1995, n=2047). Après 12 ans, un suivi a été mené entre 2003 et 2007 (EGEA2). Parmi les participants en vie (n=2002), 92% (n=1845) ont rempli un auto-questionnaire court, et parmi eux 1601 (n=1570 adultes âgés de 18 ans et plus) ont eu un examen complet. Tous les sujets ont répondu à un questionnaire pour caractériser l'asthme et les symptômes respiratoires et allergiques associés, les traitements et les expositions environnementales. La troisième enquête (EGEA3) a été menée de 2011 à

2013 (suivi de 20 ans) en utilisant des auto-questionnaires et 1558 questionnaires ont été retournés (79% de suivi).

"L'asthme actuel" a été défini par la présence de symptômes respiratoires (sifflement, oppression thoracique nocturne, crise d'essoufflement suite à une activité intense, au repos ou pendant la nuit, et crise d'asthme) ou la prise de médicaments (inhalés et/ou oraux) en raison de problèmes respiratoires dans les douze derniers mois. "Le contrôle (des symptômes) de l'asthme" a été évalué à l'aide des réponses aux questionnaires afin d'être aussi proche que possible de la définition de Global Initiative for Asthma (GINA) 2015.

Les adultes ayant un asthme actuel à EGEA2 et revus à EGEA3 ont été sélectionnés. Ensuite, trois analyses ont été effectuées portant respectivement sur la hs-CRP sérique, des cytokines sériques et les 8-IsoPs du condensat de l'air exhalé. Trois groupes de sujets ont été sélectionnés en fonction de la disponibilité des données relatives à ces paramètres. Dans chaque analyse, les sujets ayant d'autres maladies qui peuvent influencer les niveaux de ces marqueurs biologiques ont été exclus.

#### **4.1. Hs-CRP et asthme actuel dans l'étude EGEA**

Les niveaux de hs-CRP (mg/mL) ont été dosés par le système Synchron d'un Beckman Coulter. Les valeurs de hs-CRP ont été transformées en log<sub>10</sub> en raison de leur distribution non-normale et exprimées en moyennes géométriques. Les niveaux de hs-CRP ont ensuite été étudiés en association avec les phénotypes de l'asthme, la fonction ventilatoire, la sensibilisation allergique, l'hyperréactivité bronchique (HRB), les niveaux d'immunoglobuline-(Ig)-E et les comptes de neutrophiles et d'éosinophiles sanguins à EGEA2 (analyses transversales), ainsi qu'avec les crises d'asthme, le contrôle de l'asthme, le score de symptômes d'asthme de Pekkanen à EGEA3 et l'évolution du score de symptômes d'asthme de Pekkanen entre EGEA2 et EGEA3 (analyses longitudinales). En raison de l'agrégation des données (étude familiale), les analyses multivariées (à l'exception des régressions logistiques multinomiales) ont été effectuées en utilisant des équations d'estimation généralisées. L'âge, le sexe, le tabac, l'IMC et le centre ont été inclus en tant que variables potentielles de confusion. Les analyses statistiques ont été effectuées à l'aide du logiciel SPSS, version 20.0. Le nombre de participants adultes inclus dans les analyses était de 252.

Dans les analyses transversales, les taux sériques de hs-CRP ont été associés significativement à l'asthme non contrôlé, une association qui était à la limite de la significativité après

l'ajustement sur l'âge, le sexe, le tabac et le centre, puis non significative après ajustement supplémentaire sur l'IMC.

À notre connaissance, seules trois études se sont intéressées aux associations entre les niveaux de hs-CRP et le contrôle de l'asthme chez l'adulte avec des résultats contradictoires qui peuvent en partie être expliqués par l'absence d'analyse multivariées, et des définitions variées du contrôle de l'asthme. Dans notre étude comme dans la littérature, les niveaux de hs-CRP augmentaient avec l'augmentation de l'IMC et l'IMC est associé à l'asthme. L'IMC pourrait donc être un facteur de confusion dans cette association. Dans notre étude, nous avons trouvé que les niveaux de hs-CRP étaient associés positivement avec le nombre de neutrophiles sanguins et avec l'HRB. Aucune autre association significative n'a été observée dans les analyses transversales et longitudinales.

En conclusion, aucune corrélation entre l'inflammation systémique estimée par les niveaux de hs-CRP et le contrôle de l'asthme classé selon GINA 2015 n'a été observée, probablement parce que l'IMC est un facteur de confusion dans l'association entre l'asthme et la hs-CRP.

#### **4.2. Cytokines et asthme actuel dans l'étude EGEA**

Les niveaux de 7 cytokines sériques comprenant le facteur de nécrose tumorale alpha (TNF- $\alpha$ ), et les interleukines *IL-1-Receptor antagonist*, (IL-1Ra), IL-5, IL-7, IL-8, IL-10 et IL-13 ont été dosés en utilisant la technique Luminex (xMAG®). Le taux de détection était de 100% pour l'IL-7 et l'IL-13. Moins de 3% des valeurs étaient au-dessus ou en dessous des limites de détection (LD) pour les autres cytokines. Afin de contrôler la variabilité inter-plaque, un même pool de sérum a été incorporé et mesuré en duplicata dans chaque plaque, et le rapport mesure du participant / mesure du pool a été inclus dans les analyses statistiques.

Une analyse en composantes principales (ACP) a été réalisée. Les composantes principales (CP) ont été exprimées en dessus/dessous de la valeur médiane. Les composantes ont été étudiées en association avec les phénotypes de l'asthme, la fonction ventilatoire, la sensibilisation allergique, les taux d'IgE et les neutrophiles et les éosinophiles sanguins à EGEA2 (analyses transversales), et avec les crises d'asthme, le contrôle de l'asthme, le score de symptômes d'asthme de Pekkanen à EGEA3 et l'évolution du score de symptômes d'asthme de Pekkanen) entre EGEA2 et EGEA3 (analyses longitudinales). A l'exception des régressions logistiques polytomiques, les analyses multivariées ont été effectuées en utilisant les équations d'estimation généralisées. L'âge, le sexe, le tabac, l'IMC et le centre ont été

inclus en tant que variables potentielles de confusion. Les analyses statistiques ont été effectuées à l'aide du logiciel SPSS, version 20.0 et du logiciel SAS, version 9.4.

Le nombre de participants adultes inclus dans les analyses était de 283. Le premier plan factoriel exprimait 82,5% de la variabilité. La première composante principale (CP1) regroupait cinq cytokines qui contribuent positivement à l'activation des cellules immunitaires (TNF- $\alpha$  et IL-8 sont pro-inflammatoires, IL-7 est impliqué dans l'hématopoïèse et IL-5 et IL-13 qui sont caractéristiques d'une réponse TH2) et la deuxième composante (CP2) deux cytokines anti-inflammatoires (IL-1Ra et IL-10). CP1 était significativement associée à un niveau plus élevé de neutrophiles sanguins (valeur de  $P=0,02$ ) à EGEA2, et avec un risque ultérieur accru de crises d'asthme et un mauvais contrôle de l'asthme à la limite de la significativité (valeur de  $P<0,10$ ) 7 ans plus tard (à EGEA3). CP2 était significativement associée à un niveau plus faible d'IgE totales et à moins d'HRB à EGEA2, et à un plus faible risque ultérieur de crises d'asthme et de mauvais contrôle de l'asthme à EGEA3 (ORa et IC 95% de 0,56 (0,31-1,00) pour les crises d'asthme, et de 0,48 (0,23-1,02) et 0,12 (0,03-0,45) pour l'asthme partiellement contrôlé et l'asthme non contrôlé respectivement).

Ce travail a identifié pour la première fois que le profil de cytokines sériques avec des niveaux élevés d'IL-1Ra et d'IL-10 était associé avec moins de crises d'asthme et moins de risque de mauvais de contrôle de l'asthme chez les adultes dans une étude longitudinale. Ces résultats mettent en évidence l'importance de l'inclusion d'IL-1Ra et d'IL-10, et éventuellement d'autres cytokines anti-inflammatoires pour étudier les associations entre des profils de cytokines et le contrôle de l'asthme avec la possibilité de suivre l'évolution de la maladie chez les patients non contrôlés.

En conclusion, ce travail montre l'intérêt de l'étude des profils de cytokines dans les associations avec le contrôle de l'asthme en complément de l'étude des niveaux de cytokines individuelles. Ces profils pourraient également permettre de raffiner les phénotypes de certains patients, et pourraient aider à cibler les patients souffrant d'asthme non contrôlé pour la thérapie.

Les résultats préliminaires des analyses sur les associations entre les niveaux de 8-IsoPs mesurés dans le condensat de l'air exhalé et l'asthme sont également présentés. Aucune association significative n'a été observée entre les niveaux des 8-IsoPs et les phénotypes de l'asthme. Des résultats complémentaires sont en cours afin de confirmer ces résultats préliminaires.

## 5. Conclusion

Dans la première partie de cette thèse, la SP-D a été trouvée associée significativement avec la BPCO, mais pas avec l'asthme. De plus, un score pour le diagnostic de la BPCO avec d'excellentes valeurs discriminantes a été identifié en utilisant les niveaux de SP-D sériques. Le diagnostic précoce de la BPCO reste toujours une tâche difficile pour le clinicien, et des efforts ont été faits pour trouver des marqueurs spécifiques pour le diagnostic et le pronostic de cette maladie. Dans l'étude réalisée au Liban, la BPCO a été définie comme un rapport VEMS / CVF après inhalation de bronchodilatateur inférieur à 70%. Cette définition semble être la plus utilisée dans la littérature, mais elle a l'inconvénient d'être peu spécifique chez les patients âgés. La limite inférieure à la normale (LLN) basée sur la stratification selon l'âge du rapport d'après inhalation de bronchodilatateur du VEMS /CVF a été proposée comme une alternative. Cependant, cette méthode a l'inconvénient d'être peu sensible. Les marqueurs biologiques spécifiques du poumon, tels que la SP-D, ou les scores générés à partir de ces marqueurs biologiques peuvent donc être utilisés pour identifier les patients BPCO.

Parallèlement, dans l'asthme, il est pertinent de trouver des marqueurs biologiques capables de raffiner certains phénotypes de l'asthme, d'optimiser le diagnostic et d'orienter le traitement. Dans la deuxième partie de cette thèse, nous avons trouvé que des profils de cytokines ont été associés différemment avec les phénotypes de l'asthme. Différents marqueurs biologiques de l'asthme sont déjà disponibles à partir du lavage broncho-alvéolaire ou de la biopsie bronchique ou des expectorations induites qui sont des méthodes invasives. Une procédure moins invasive peut être utilisée qui est la collecte de sang périphérique, pratique à utiliser dans les grandes études épidémiologiques. L'utilisation des méthodes statistiques telles que l'analyse en composante principale, qui permet l'évaluation de plusieurs marqueurs biologiques simultanément, pourrait capturer des profils de marqueurs à partir d'un panel de mesures de nombreux marqueurs biologiques, et ainsi aider au développement de traitements ciblés de l'asthme. L'asthme étant une maladie inflammatoire chronique et une maladie liée au stress oxydant, la recherche de marqueurs biologiques dans ces deux voies est peut-être une solution à envisager. Enfin, que ce soit dans le sang ou dans le condensat de l'air exhalé, la nécessité est de trouver des profils de marqueurs qui prédisent le mieux la réponse au traitement chez les patients atteints d'asthme.



Certains des résultats obtenus pourraient avoir des implications de santé publique. Concernant la BPCO, son fardeau semble augmenter, en particulier dans les pays en développement principalement en raison de l'augmentation de l'usage du tabac. Dans ce contexte, trouver un moyen pratique pour diagnostiquer la BPCO, en particulier dans les régions où la spirométrie n'est pas toujours disponible, pourra cibler les patients BPCO nécessitant une thérapie afin de leur fournir une meilleure qualité de vie. En outre, le score créé en utilisant les niveaux de SP-D pourrait être utilisé dans les établissements de soins primaires afin d'assurer une transition de la recherche à la pratique. Concernant l'asthme, sa prévalence est en augmentation dans les communautés qui s'urbanisent. Il semble donc primordial de viser à maintenir un contrôle optimal de l'asthme. Dans ce contexte, la poursuite des recherches sur les profils de cytokines semble nécessaire car les profils pourraient peut-être aider à cibler les patients à la thérapie et à réduire les risques futurs d'asthme non contrôlé.

Plusieurs perspectives peuvent être envisagées à partir des travaux de cette thèse et certaines sont citées ci-après. Puisque la SP-D est un marqueur spécifique du poumon, sa valeur prédictive devrait être maintenant évaluée dans le cadre d'une étude à grande échelle avec un suivi prospectif, et nous espérons être en mesure de finaliser ce travail dans un futur proche au Liban. Les études des associations entre les profils des cytokines sériques, en incluant un grand nombre de cytokines inflammatoires et anti-inflammatoires, et l'asthme doivent maintenant être poursuivies dans un cadre prospectif. Ces travaux futurs pourraient améliorer notre compréhension de la physiopathologie de l'asthme en ouvrant de nouvelles possibilités pour la gestion de la maladie, d'autant plus que les cytokines sont actuellement déjà mises en œuvre comme thérapies ciblées pour les patients asthmatiques. Le passage de la recherche à des pratiques de santé publique est une des clés pour réduire le fardeau de la BPCO et de l'asthme dans le monde dans les années à venir.

## Contents

FOREWORD.....	28
1. GENERAL INTRODUCTION.....	31
1.1. COPD.....	31
1.1.1. COPD definition and overview.....	31
1.1.2. Epidemiological aspects .....	32
1.2. Asthma .....	34
1.2.1. Asthma definition and overview .....	34
1.2.2. Epidemiological aspects .....	37
1.3. COPD and asthma comorbidities and differential diagnosis .....	40
1.4. Biological markers in epidemiological studies .....	40
1.5. Biological markers in COPD and asthma .....	42
1.5.1. C-reactive protein (CRP) and high-sensitivity C-reactive protein (hs-CRP).....	42
1.5.2. Fibrinogen.....	43
1.5.3. Surfactant protein-D (SP-D).....	44
1.5.4. Cytokines.....	45
1.5.5. 8-isoprostanes .....	46
1.6. Objectives and flow of the thesis .....	46
2. EVALUATION OF SP-D AS A BIOMARKER FOR COPD.....	49
2.1. Material and methods .....	49
2.1.1. Study design and participants .....	49
2.1.2. Questionnaire.....	50
2.1.3. Blood collection and pulmonary function tests.....	50
2.1.4. Measurement of SP-D, CRP and fibrinogen.....	51
2.1.5. Statistical analyses .....	51
2.2. Results .....	53
2.2.1. Participants' characteristics .....	53
2.2.2. Associations between biological markers and COPD and asthma – Univariate analyses ..	54
2.2.3. Associations between SP-D and COPD - Multivariate analyses .....	56
2.2.4. Construction, properties and thresholds of the scores for COPD diagnosis .....	58
2.3. Discussion.....	62
2.3.1. Summary of findings.....	62
2.3.2. Comparisons with other studies and interpretation .....	62
2.3.3. Strengths and limitations .....	64
2.3.4. Conclusion.....	65
3. BIOLOGICAL MARKERS AND ASTHMA CONTROL OUTCOMES.....	66

3.1.	EGEA: Study design and follow-up.....	66
3.2.	Participants selection.....	68
3.3.	Characteristics of the participants with current asthma from the EGEA study .....	69
3.4.	Respiratory phenotyping .....	70
3.5.	Blood tests and EBC collection .....	71
3.6.	First analysis: Cross-sectional and longitudinal associations between hs-CRP and current asthma	72
3.6.1.	Material and methods .....	72
3.6.1.1.	<i>Participants' selection</i> .....	72
3.6.1.2.	<i>Biological phenotypes</i> .....	73
3.6.1.3.	<i>Statistical analyses</i> .....	74
3.6.2.	Results .....	76
3.6.2.1.	<i>Study participants</i> .....	76
3.6.2.2.	<i>Association between serum hs-CRP levels and potential confounding variables</i> .....	79
3.6.2.3.	<i>Cross-sectional associations between serum hs-CRP levels and asthma symptom control at EGEA2</i> .....	80
3.6.2.4.	<i>Cross-sectional associations between serum hs-CRP levels and asthma outcomes at EGEA2</i>	80
3.6.2.5.	<i>Longitudinal associations between serum hs-CRP levels and asthma outcomes at EGEA3</i>	81
3.6.2.6.	<i>Sensitivity analyses</i> .....	82
3.6.3.	Discussion.....	82
3.6.3.1.	<i>Summary of findings</i> .....	82
3.6.3.2.	<i>Comparison with other studies and interpretation</i> .....	83
3.6.3.3.	<i>Strengths and limitations</i> .....	85
3.6.3.4.	<i>Conclusion</i> .....	85
3.7.	Second analysis: cross-sectional and longitudinal associations between cytokine profiles and current asthma .....	86
3.7.1.	Materials and methods.....	86
3.7.1.1.	<i>Participants' selection</i> .....	86
3.7.1.2.	<i>Cytokine measurements</i> .....	87
3.7.1.3.	<i>Statistical analyses</i> .....	88
3.7.2.	Results .....	90
3.7.2.1.	<i>Study participants</i> .....	90
3.7.2.2.	<i>Profiles of serum cytokines</i> .....	93
3.7.2.3.	<i>Cross-sectional associations between PC scores and asthma outcomes at EGEA2</i> .....	97
3.7.2.4.	<i>Longitudinal associations between PC scores and asthma outcomes at EGEA3</i> .....	100

3.7.2.5.	<i>Associations between individual cytokines and asthma outcomes</i> .....	101
3.7.2.6.	<i>Sensitivity analyses</i> .....	103
3.7.3.	<i>Discussion</i> .....	104
3.7.3.1.	<i>Summary of findings</i> .....	104
3.7.3.2.	<i>Comparisons with other studies and interpretation</i> .....	104
3.7.3.3.	<i>Strengths and limitations</i> .....	106
3.7.3.4.	<i>Conclusion</i> .....	106
3.8.	<i>Third analysis: Preliminary findings for the association between 8-isoprostanes and current asthma</i> .....	107
3.8.1.	<i>Materials and methods</i> .....	107
3.8.1.1.	<i>Participants' selection</i> .....	107
3.8.1.2.	<i>8-isoprostanes measurements</i> .....	108
3.8.1.3.	<i>Statistical analyses</i> .....	108
3.8.2.	<i>Preliminary results</i> .....	110
3.8.2.1.	<i>Study participants</i> .....	110
3.8.2.2.	<i>Association between EBC 8-IsoPs levels and potential confounding variables</i> .....	112
3.8.2.3.	<i>Cross-sectional associations between EBC 8-IsoPs and asthma outcomes at EGEA2</i> .....	113
3.8.3.	<i>Conclusion</i> .....	113
4.	<i>GENERAL DISCUSSION</i> .....	115
4.1.	<i>Summary of findings and interpretation</i> .....	115
4.2.	<i>Common strengths and limitations</i> .....	117
4.3.	<i>Implications for public health</i> .....	118
4.4.	<i>Recommendations and perspectives</i> .....	119
5.	<i>REFERENCES</i> .....	121

## FOREWORD

Chronic respiratory diseases are chronic diseases of the airways and other structures of the lungs that represent a wide array of serious diseases (1). They strongly predict the overall health, ageing and all-cause mortality (2), and they are a major cause of global morbidity and mortality (3). Chronic respiratory diseases affect hundreds of millions of people of all ages (more than 50% of them live in low and middle income countries or are deprived populations) and account for four million deaths annually (1,4). The fraction of disability-adjusted life years (DALYs) from all chronic respiratory diseases was 44.9% among 306 diseases from 188 countries measured from 1990 till 2013 (5). The most common chronic respiratory diseases include chronic obstructive pulmonary disease (COPD), asthma, allergic rhinitis, occupational lung diseases, sleep apnea syndrome and pulmonary hypertension (6). In this thesis, we will focus on COPD and asthma, the two diseases from which the global burden appears to be growing (2).

COPD is a heterogeneous disease with various clinical presentations, affecting around 210 million individuals around the world (7). COPD is characterized by persistent airflow limitation that is usually progressive and is the fourth leading cause of death worldwide (8). COPD prevalence is growing especially in adults over 40 years of age, with a considerable increase with age, particularly among smokers (1). Asthma is a chronic inflammatory disease of the airways, affecting around 300 million individuals around the world (9). It is a complex disease, with strong clinical heterogeneity and phenotypic variability over time (10). The prevalence of asthma has nearly doubled over the last decades, more particularly in westernized countries (11). Even though COPD and asthma are two distinct disease entities, they often present overlapping conditions of clinical features particularly among smokers and older adults. This is called the Asthma-COPD Overlap Syndrome (ACOS) (12).

It is now widely recognized that ageing is largely determined throughout the life course, including the pre-birth period (13). Thus, in order to ensure a healthy ageing for subjects having chronic respiratory diseases, we need to better understand the pathophysiologic mechanisms of these diseases to allow a better diagnosis, treatment and prevention (3). In both COPD and asthma, the study of biological markers is of interest since they are likely to play a key role in the persistent inflammation and in the pathogenesis of these two diseases (14,15). In COPD and asthma, the inflammation is both local to the lungs and systemic (16).

To assess systemic inflammation, biological markers from the blood are the most promising ones and are easily procured using standard venipuncture techniques (17). Moreover, and in order to assess local inflammation in asthma, the exhaled breath condensate (EBC), which is obtained by non-invasive sample collection technique, was brought to the attention of researchers in the last decade. It is a useful tool to study the inflammatory molecules in the airway lining fluid (18).

The general aim of this thesis is to study the role of biological markers in COPD and asthma. The thesis includes two parts. The first part was performed in a study conducted in Lebanon and aimed at assessing the association between a lung specific biological marker and COPD, in comparison with two systemic markers of inflammation validated in the clinical laboratory assays. The second part was performed on French data as part of the Epidemiological study on the Genetics and Environment of Asthma (EGEA) and aimed at evaluating the cross-sectional and longitudinal associations between inflammatory biological markers from the blood and biological markers related to oxidative stress from EBC, on one hand and asthma outcomes on the other.

Two guidelines have oriented my thesis:

- 1- Research has recently focused on the assessment of lung specific biological markers in the diagnosis and prognosis of COPD. Thus, the first part of the thesis was focused on the role of a lung specific biological marker in COPD, an association that has not yet been evaluated in Lebanese patients.
- 2- The integration of the study of biological markers in respiratory epidemiology, especially in asthma, in view of improving the respiratory health of these patients by opening new possibilities for the disease management, such as the development of new therapies, is currently a hot topic. Furthermore, and since achieving controlled asthma is an important goal in asthma management, the second part of the thesis focused on the cross-sectional and longitudinal associations between biological markers and asthma control outcomes.

The thesis is divided in four parts:

The first part is a general introduction in which general aspects regarding the epidemiology and the clinical features of COPD and asthma are presented. Since discussing all cells and cellular elements implicated in the inflammatory process associated with COPD or asthma is

beyond the scope of this thesis, selected biological markers used to diagnose COPD or asthma or to predict asthma outcomes are illustrated, and the general and specific objectives are presented.

The second part presents the study conducted in Lebanon about the association between a lung specific biomarker and COPD. This part includes the methods used, the results obtained, the clinical implications of the main results, the comparison with other studies and the strengths and main limitations of the performed analyses.

The third part presents the analyses of the data selected from the EGEA study. This part includes first a description of the EGEA study. Then, the three different analyses that were carried out are presented, the association between asthma outcomes on one hand and inflammatory biological markers as well as biological markers related to oxidative stress on the other. In each analysis, the methods used, the results obtained, the clinical implications of the main results, the comparison with other studies and the strengths and main limitations of the performed analyses are presented.

The fourth and last part is a general conclusion that includes a summary of the main results from both parts of the thesis, the common strengths and limitations, the implications regarding public health, and finally the recommendations and perspectives from the thesis.

## **1. GENERAL INTRODUCTION**

### **1.1. COPD**

In this part, the general characteristics of COPD are presented regarding clinical features and epidemiological aspects.

#### **1.1.1. COPD definition and overview**

COPD is a heterogeneous disease with various clinical presentations. It is characterized by persistent airflow limitation that is usually progressive, not fully reversible and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases (8). The characteristic symptoms of COPD are chronic and progressive dyspnea, cough and sputum production that can vary from day to day (19).

A clinical diagnosis of COPD is considered in any patient who has dyspnea, a chronic cough or sputum production, and a history of exposure to the disease risk factors (tobacco smoke, smoke from home cooking and heating fuels, occupational dusts and chemicals). Spirometry is required to confirm a COPD diagnosis. The presence of a post bronchodilator ratio of the forced expiratory volume at one second ( $FEV_1$ )/forced vital capacity (FVC) being less than 70% confirms the presence of persistent airflow limitation, thus of COPD (8,20). In order to determine COPD severity, COPD patients are classified into four categories (A, B, C and D) based on the symptomatic assessment according to the modified Medical Research Council Questionnaire (mMRC), on breathlessness according to the COPD assessment test (CAT), on the patient's spirometric classification according to post-bronchodilator  $FEV_1$  and/or on the risk of exacerbations (8). Diagnosing COPD accurately has been a priority for many researchers. We can cite the Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE) study, which is a large 3-year observational controlled multicenter international study aimed at defining clinically relevant subtypes of COPD and identifying novel biological markers and genetic factors. It has produced more than 50 original publications and 75 communications to international meetings so far (21).



The goals of COPD management are to determine the severity of the disease, and to improve the patient's functional status and quality of life by preserving optimal lung function, improving symptoms, and preventing the risk of future events such as the recurrence of exacerbations, the hospital admissions or death, in order to guide therapy (22). The identification and reduction of risk factors, mainly cigarette smoke and regular intake of medications are important components in COPD management. Medications include short-acting anticholinergic or short-acting beta<sub>2</sub>-agonist (SABA) for patients in group A and long-acting anticholinergic or long-acting beta<sub>2</sub>-agonist (LABA) for patients in group B associated with inhaled corticosteroids (ICS) for patients in group C. ICS along with LABA and/or long-acting anticholinergic are recommended for group D patients (8).

### **1.1.2. Epidemiological aspects**

#### ***Prevalence***

There are at least 210 million persons in the world with COPD (7). Most data on COPD prevalence came from high income countries. COPD prevalence greatly underestimates the total burden of COPD because the disease is usually not diagnosed until it is clinically apparent and moderately advanced. Moreover, COPD prevalence presents remarkable variation due to the differences in COPD definitions between studies (23). It was estimated to range from 4% to 20% in adults over 40 years of age, with a considerable increase by age, particularly among ever-smokers and men. COPD can occur as well in subjects aged 20-44 years (1). COPD prevalence was reported to be 6% in France (24) and 9.7% in Lebanon (25). COPD has been classified as the 5<sup>th</sup> (out of 306 diseases and injuries) cause of DALYs worldwide (5). COPD is the 3<sup>rd</sup> leading cause of death in the world causing 3 million deaths per year (7). Deaths attributable to COPD have increased sharply in countries where data is available. The burden of COPD is expected to increase mainly in Asia and Africa in the coming decades, due to increased tobacco use in those regions (2).

#### ***Social and economic impact***

The social and economic cost of COPD is considerable both in terms of direct costs (drugs, medical visits, hospitalizations) and indirect ones (days of absences, premature death). In Europe, the total direct costs of respiratory diseases are estimated to be about 6% of the total

health care budget, with COPD accounting for 56% of this cost of the respiratory disease, thus €38.6 billion per year. COPD exacerbations account for the greatest proportion of the total COPD burden on the health care system (8).

### *Etiological aspects*

Although 90% of COPD is caused by long term cigarette smoking, only 25% of chronic tobacco smokers develop COPD (26). COPD tends to occur more frequently in smokers with a family history of obstructive airways disorders including asthma and COPD. Besides smoking, there are underlying genetic factors in the development of COPD. Variants of some genes have been investigated and identified to have close associations with COPD (27,28). COPD is thus a multifactorial disease resulting from complex interactions between genetic, environmental, lifestyle and social factors.

### *Genetic factors*

Although it is relevant to some populations, the hereditary deficiency in alpha-1 antitrypsin is a genetic risk factor for COPD that shows the interaction between genes and environmental exposures leading to COPD (29). Other genes have been related to a decline in lung function parameters (30).

### *Exposure to particles*

Any type of tobacco (cigarette, waterpipe, cigar, environmental tobacco smoke...) (31,32), as well as occupational exposures (organic and inorganic dusts, chemical agents and fumes) (33) and indoor pollution (biomass cooking, heating in poorly ventilated areas...) (34) are all risk factors for COPD. Outdoor air pollution is a potential risk factor for COPD, but more research is required to clarify its role in the initiation of COPD (35).

### *Age and sex*

Age is known to be a risk factor for COPD, but it is unclear if this is the result of the cumulative exposures throughout life. Males have a higher COPD prevalence than females. However, COPD prevalence in females has recently been reported to be equal to that in males, probably due to changes in tobacco smoking habits (36).

### *Lung growth and infections*

Factors that affect lung growth during gestation and childhood (low birth weight, lung infections...) have the potential of increasing an individual's risk of developing COPD (37,38). Moreover, it has been demonstrated that childhood asthma and childhood respiratory infections have much the same impact on adult lung function and the risk of COPD as heavy smoking (38), suggesting that COPD can be considered as a disease of childhood that manifests in adults (39,40).

### *Other respiratory diseases*

Asthma and chronic bronchitis might be risk factors for COPD development. After adjusting for smoking, adults with asthma had a higher risk of having COPD than those without asthma (41). Another study has reported that chronic bronchitis in younger adult smokers is associated with a higher risk of having COPD (42).

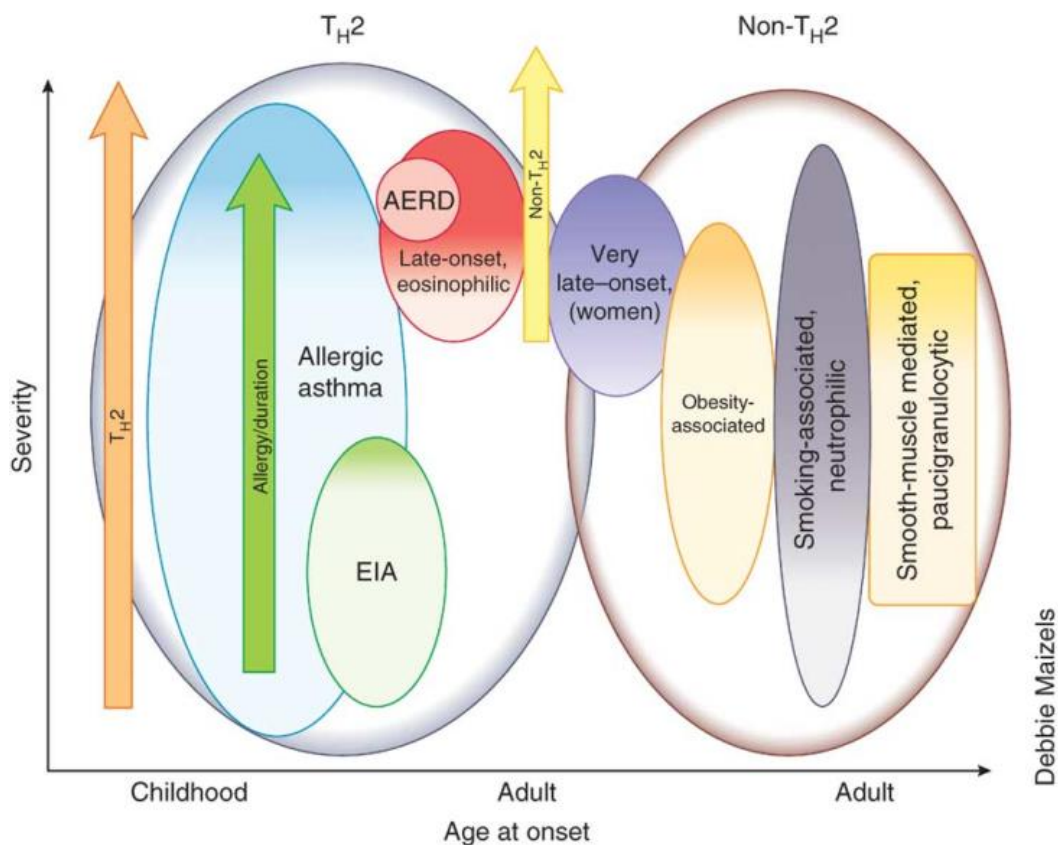
## **1.2. Asthma**

In this section, general characteristics of asthma are presented regarding the clinical features and the epidemiological aspects.

### **1.2.1. Asthma definition and overview**

Asthma is a common and potentially serious chronic inflammatory disease of the airways, clinically characterized by recurring respiratory symptoms, such as wheezing, shortness of breath, chest tightness and cough. The symptoms are associated with variable expiratory airflow (e.g., difficulty breathing air out of the lungs due to bronchoconstriction (airway narrowing), airway wall thickening, and increased mucus) caused by airway inflammation. The symptoms may be triggered or worsened by factors such as allergens, tobacco smoke, viral infections, exercise and stress. Moreover, variable airflow obstruction that is reversible and may resolve spontaneously or in response to medication, and airway hyperresponsiveness (AHR) to allergen, pharmacologic or physiological stimuli are as well asthma characteristics (43). Furthermore, an important pathogenic feature of asthma is the imbalance of oxidative stress and antioxidant capacities (44,45).

In asthma, strong clinical heterogeneity and phenotypic variability are observed (Figure 1) (10). Its clinical expression varies over time (during the same day, month, depending on seasons or years) in its occurrence, frequency and intensity. Asthma patients may experience periods of remission, sometimes followed by a relapse of symptoms (46). Asthma usually occurs during childhood but can also occur at any age. During childhood, boys has a higher risk of developing asthma than girls whereas after puberty, the risk in men is lower (47). For many years, asthma had been considered as a single disease, but now it is well-known that asthma consists of multiple phenotypes. Recent studies have focused on asthma heterogeneity, which has promoted the concept that asthma consists of multiple phenotypes or of consistent groupings of characteristics. Asthma heterogeneity can be observed at different levels: clinical (e.g. control of the disease, evolution of the disease), biological (e.g. allergic/non-allergic, eosinophilic/non-eosinophilic inflammation), or etiological (10). Many years ago, asthma was considered to be an allergic, eosinophilic and TH2-(T-helper type 2 lymphocytes) mediated disease, but epidemiological studies have shown that atopic asthma is observed in less than half of the cases (48). Other phenotypes include late-onset asthma, and asthma with obesity or with fixed airflow limitation (43,49). Epidemiological research performed over the past decade has identified novel phenotypes able to simultaneously integrate multiple disease features (50,51). Examples of these phenotypes include early-onset mild allergic asthma, severe non-atopic asthma with frequent exacerbations...(52–55), but understanding poorly controlled asthma phenotype remains the great unmet clinical need (10).



**Figure 1:** Asthma phenotypes (Wenzel, Nature Medicine 2012; 18 (5): 716-725).

Asthma diagnosis is based on a history of characteristic symptom patterns and an evidence of variable airflow limitation, from bronchodilator reversibility testing or other tests (43). Asthma control, which reflects the activity of the disease over a short period of time, and asthma severity, which reflects the intrinsic severity of the disease, are important in evaluating patients and their response to treatment (9). The control of asthma has incorporated both symptom control over the past 4 weeks (assessed with day and night symptoms, use of reliever therapies, and activity limitations) and the risk factors for poor asthma outcomes (risk factors at diagnosis, particularly for patients having exacerbations, low lung function, risk factors for developing fixed airflow limitation and for medication side effects) (43,56).

Over the recent decades, the public health priority has been to improve the assessment and management of asthma, thus to achieve and maintain optimal asthma control by reducing airway inflammation and preventing fixed airway obstruction (43,57). The fine-tuning and regular intake of medications even in the absence of symptoms, the identification of triggers, the use of a written action plan and the facilitation of self-management education may help to

achieve asthma control. Reliever medications such as SABA, associated with anticholinergic drugs and oral corticosteroids or not, are used to reverse bronchoconstriction occurring during exacerbations. Maintenance therapies are based on ICS, associated or not with LABA or other medications according to the control of the disease (43). LABA might increase severe attacks and mortality in some subpopulations of subjects with asthma (58).

### **1.2.2. Epidemiological aspects**

#### ***Prevalence***

Around 300 million individuals are affected by asthma worldwide (9). The prevalence of asthma or respiratory symptoms associated with asthma is higher in children than in adults (59). It has nearly doubled over the past decades, more particularly in westernized countries (11). It is also higher in industrialized countries and is associated with the adoption of an urban lifestyle in developing countries (60,61). It is estimated that an additional 100 million people will have asthma by 2025 (2). A large study conducted among 178,215 adults from 70 countries in 2002-2003 estimated the prevalence of physician-diagnosed asthma at 4.3% worldwide, ranging from 0.2% (in China) to 21.0% (in Australia) (62). However, while the prevalence of asthma is still increasing in some countries, it has been suggested that the increase in the asthma epidemic is coming to an end in some countries with a high prevalence of the disease (61,63). In 2006, more than 10% of the whole population in France had ever had asthma and the prevalence of current asthma, defined by the presence of asthma symptoms or the use of asthma medication in the past 12 months, was of 7% (64). In 2009, in Lebanon, the prevalence of ever having asthma was 8.3% among 13 to 14 year-old students (65), and the prevalence of physician diagnosed asthma was estimated to be 4.8% among 5 to 12 year-old students (66). Asthma has been classified as the 28<sup>th</sup> (out of 291 diseases) cause of DALYs worldwide (67). It is estimated that around 200,000 people die prematurely each year as a result of asthma, with over 80% of these deaths occurring in low- and middle-income countries (68,69). Uncontrolled asthma, defined according to the recommendations of the Global INitiative for Asthma (GINA), affects one third of the subjects with asthma in Europe, with significant geographical variability (20% to 67% depending on the country, about one third in France) (70).

### ***Economic and social impact***

The social and economic cost of asthma is considerable both in terms of direct costs (drugs, medical visits, hospitalizations) and indirect ones (disease stops, days of absences, premature death). The total cost of asthma is approximately €17.7 billion per year in Europe (71), and €1.5 billion per year in France (72). Patients with severe or poorly controlled asthma are the ones that concentrate the largest expenditures (73,74).

### ***Etiological aspects***

Asthma is a multifactorial disease resulting from complex interactions between genetic, environmental, lifestyle and social factors (11). In a recent international workshop on asthma and allergies, Bousquet *et al.* confirmed the importance of gene–environment interactions as well as early-life risk factors (75). However, the causation of asthma remains largely unknown.

#### ***Genetic factors***

Asthma has an important genetic component. As of 2011, 173 genes have been associated with asthma or clinical conditions related to it (76). Interactions between genes and environmental factors were reported; therefore, research is now focusing on the development of gene–gene and gene–environment interaction studies, including epigenetic alterations (77,78).

#### ***Environmental factors***

Exposure to indoor environment (allergens such as dust mites, pets (mainly cats and dogs), cockroaches, mice and fungi) is an important risk factor for the development and exacerbations of allergic asthma (79). Contact with livestock in the farming environment during childhood, causing optimal immunological maturation may provide a healthy lifestyle for those at risk of developing allergic asthma (80,81). Exposure to air pollution, which causes inflammation, can increase asthma outcomes such as exacerbations, emergency visits and increased medication intake (79). Asthma is now the first occupational respiratory disease in adults, and more than 400 agents have been specifically identified as disease-related (82). The role of exposure to cleaning products is reported to explain part of the increase in asthma prevalence observed in most developed countries (43,83).

### *Smoking*

Tobacco smoke contains several chemicals, which promote oxidative stress leading to inflammation, thus to asthma (79). Maternal smoking during pregnancy and exposure to tobacco smoke during childhood increase the risk for the child to have asthma during adolescence (57). In contrast, the effects of both passive and active smoking on asthma in adults are more unclear. Tobacco exposure has an effect on asthma control; smoking asthma patients have more symptoms, exacerbations and higher risk of hospitalizations than non-smoking asthma patients (84).

### *Diet, obesity and physical activity*

The dietary regimes (less fruit/vegetables, more ready to eat meals), the obesity epidemic, and the decrease in physical activity, have been suggested to play a role in the increase of asthma worldwide (85). It is still discussed whether the decrease in physical activity relates more to the evolution than the development of asthma (86).

### *Socio-economic factors*

The role of socio-economic status (SES) in asthma has been rarely discussed in the literature even though it is related to air pollution, smoking, nutrition and occupation (11). Measuring SES is difficult in epidemiological studies since variables can be taken into account either at individual level or at area level. At the individual level, lower SES was reported to be associated with more severe/symptomatic asthma or poorly controlled asthma, and poor adherence to ICS was reported to be associated with lower income and lower educational level (79).

### *Lung growth and infections*

Factors that affect lung growth during gestation or childhood such as pre-term birth (<37 weeks), low birth weight (<2.5 kg) and high infant weight gain (>600 g/month) are associated with subsequent risk of asthma in childhood (87). In adults, the risk of asthma is increased with self-reported recent lower or upper respiratory tract infection or atopy (88).



### **1.3. COPD and asthma comorbidities and differential diagnosis**

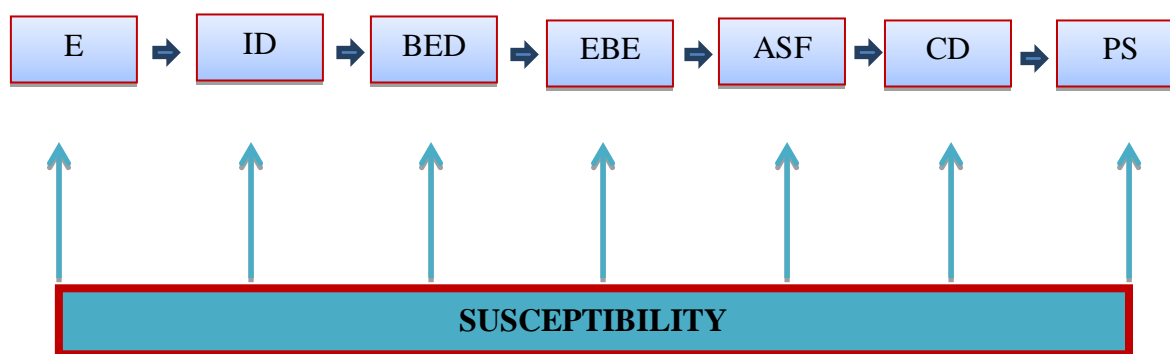
Several comorbidities, such as cardiovascular diseases (CVD), osteoporosis, depression and lung cancer are commonly present in patients with COPD (8). Asthma as well may often coexist with other diseases, such as obesity, gastro esophageal reflux disease (GERD), anxiety and depression, food allergy and anaphylaxis, rhinitis, sinusitis and nasal polyps (43).

Even though some features may help to diagnose COPD (onset in mid-life, slow progression of symptoms, history of tobacco smoking or exposure to other types of smoke) and asthma (early onset in life, variation of symptoms from day to day and getting worse at night or in the early morning, presence of allergy, rhinitis and/or eczema and a family history of asthma), in some patients (particularly smokers and older adults), a clear distinction between both diseases is not possible (8,43). COPD and asthma often present overlapping conditions of clinical features called the ACOS. The ACOS is characterized by persistent airflow limitation with several features associated on the one hand with asthma, and on the other with COPD and is therefore identified in clinical practice (12,89). A systematic literature review reported that the prevalence of asthma–COPD overlap was approximately 20% in patients with obstructive airway disease (asthma or COPD) and was consistent across study designs and varying definitions (90).

### **1.4. Biological markers in epidemiological studies**

The biological markers are in the continuum between the exposure from the environment and the disease (91). Integrating their measurement in epidemiological studies of complex diseases may be pertinent in order to better understand the etiology of these diseases, and therefore, to refine the phenotypes. The types of biological markers were defined in 1987 by the National Academy of Sciences (NAS) (92) upon the work of Perera and Weinstein (93) and were categorized as follows: biological markers of exposure, of effect and of susceptibility. These categories have been extensively discussed mainly with regard to pulmonary diseases. A biological marker of exposure is an exogenous substance or the product of an interaction between a xenobiotic agent and a molecule measured in an organism. A biological marker of effect is a measurable biochemical, physiologic, or other alteration within an organism that could be a potential health impairment or disease. A biological

marker of susceptibility is an indicator of an acquired limitation of an organism's ability to respond to the challenge of exposure to a specific xenobiotic substance. Then, the continuum between exposure (E) and the development of clinical disease (CD) has been elaborated to include the following classes of biological markers: internal dose (ID), biological effective dose (BED), early biological effects (EBE), altered structure and function (ASF), and biological markers of prognostic significance (PS) (94) (Figure 2). In this thesis, we will focus on CD, ASF and PS biological markers.



**Figure 2:** Biologic marker components in sequential progression between exposure and disease (From Schulte PA, Environmental Research, 1989; 48: 129-144).

Abbreviations: E, exposure; ID, internal dose ; BED, biological effective dose; EBE, early biological effects; ASF, altered structure and function; CD, clinical disease; PS, prognostic significance.

The considerable progress made in biology in the past 50 years has generated more than 30,000 reports on biological markers, but less than one thousand (3%) have emerged as clinically useful (Diagnostic and Prognostic biomarkers SciClips database). In the future, advanced bioinformatics will help the use of new biological markers in clinical practice by improving the quality and standardization of input data in the databases, refining the definition of prognostic or predictive markers, and establishing links between existing databases (95). Biological markers included in epidemiological studies need to be valid, reliable and practical (96). The best biological markers for the future should be indicators of a normal or pathological biological process, of the presence of pathogens, or of the response to a therapeutic intervention.

## **1.5. Biological markers in COPD and asthma**

In order to assess COPD or asthma presence, severity or state such as progression or response to treatment, clinically assessable biological markers are needed and have become a hot topic. Inflammation, which is associated with both COPD and asthma, is a highly complex, interrelated network of cells and cellular elements (14,15) and could be assessed through the biological markers measurements. Moreover, biological markers research in asthma has recently focused on the multidimensional approach, thus the combination of various markers instead of the assessment of the value of biological markers studied separately (97), as well as biomarkers obtained using non-invasive sampling techniques (98).

It is clearly beyond the scope of this thesis to discuss all cells and cellular elements (such as cytokines, proteins....) implicated in the inflammatory or pathogenic processes associated with COPD or asthma, which is a large body of literature. Thus, selective biological markers investigated in the study performed in Lebanon as clinical biomarkers for COPD or asthma (serum C-reactive protein (CRP) and surfactant protein D (SP-D) and plasma fibrinogen) or in the French EGEA study as predictors of asthma control (serum high-sensitivity CRP (hs-CRP), serum cytokines and EBC 8-IsoProstanes (8-IsoPs)) will be illustrated.

### **1.5.1. C-reactive protein (CRP) and high-sensitivity C-reactive protein (hs-CRP)**

CRP is the first acute-phase protein to be described and is a highly sensitive systemic marker of inflammation, infection and tissue damage. CRP is predominantly produced and secreted by hepatocytes although other cells including alveolar macrophages may also synthesize it under the control of pro-inflammatory cytokines, mainly Interleukin-6 (IL-6) (99). In healthy subjects, CRP is increased with increasing age and body mass index (BMI), in women and in heavy smokers (100,101). In addition, CRP is probably the most non-specific marker of inflammation and is not particularly a good marker of respiratory health outcomes or rate of decline in lung function in COPD patients because the predominant source of circulating CRP is the liver and not the lungs (102,103). There has been no clear mechanism to implicate CRP in the pathophysiology of COPD, and it does not reflect known genetic polymorphism, although CRP levels are genetically determined, suggesting it is merely a general marker of the underlying inflammatory process associated with COPD (14). High CRP levels were

associated with increased hospitalizations although probably reflecting disease severity (104). CRP levels are higher during exacerbations, most probably when the cause is bacterial (105). Thus, they can be used to distinguish between exacerbations with or without bacterial infections (106). Regarding the association with lung function tests, results have been inconsistent, with reports of weak to moderate unadjusted correlations between FEV<sub>1</sub> and CRP (107–109). CRP has shown high variability over time when measured at baseline and 3 months later (110). It seems, according to a review conducted in 2014, that CRP causal role in COPD is controversial and that CRP may not be a suitable biological marker for COPD due to the conflicting reports regarding its value to predict mortality and hospitalizations, its low specificity and high variability (111). Regarding CRP and asthma, it has been reported that the mean CRP levels of patients with asthma and rhinitis were not significantly different from those of healthy control groups (112). Another study reported that CRP levels did not discriminate between COPD and asthma patients (113). The hs-CRP test is able to detect small amounts of the protein in the serum, 0.5–10 mg/L *versus* 10-1000 mg/L for regular CRP test. Serum hs-CRP test was reported to be suitable for the detection of systemic inflammation in asthma (99,100,114) as CRP levels were significantly elevated in neutrophilic asthma, which is more severe and represents an increased systemic inflammation (115). Moreover, serum hs-CRP levels correlated with asthma characteristics such as BHR (100), FEV<sub>1</sub> (116), and sputum cytology (117). However, other studies have reported contradictory results regarding the above mentioned associations (118,119). Overall, hs-CRP appears to be clinically more reliable as a marker of asthma when compared to ordinary CRP.

### **1.5.2. Fibrinogen**

Fibrinogen is an acute phase protein and a blood clotting factor which is synthesized by hepatocytes and released in large amounts into the circulation primarily in response to IL-6 stimulation (120,121). In healthy subjects, positive associations were reported between fibrinogen and age and female gender (122). It is possible that fibrinogen could be used as a non-invasive measurement of ongoing airway inflammation and lung tissue destruction. Circulating fibrinogen levels were shown to be significantly higher in COPD patients compared to controls (123,124). Plasma fibrinogen levels are higher in COPD patients with frequent exacerbations and are associated with hospitalizations (124,125). Conflicting results were reported regarding the association between fibrinogen and lung function parameters in COPD patients (126,127). No genetic polymorphisms of fibrinogen levels have been

identified in COPD although circulating fibrinogen levels are genetically determined, indicating that fibrinogen is also more likely to be a reflective feature of COPD rather than a causal factor (125). Even though studies indicate that therapy can reduce fibrinogen levels, it remains unknown whether this affects progression or other outcomes (125).

### **1.5.3. Surfactant protein-D (SP-D)**

To capture respiratory health events, lung-specific biological markers are the most advantageous (128). Recent studies have described associations between a pulmonary-specific marker, SP-D, and COPD (129–131). SP-D protein is a member of a family of collagenous carbohydrate-binding proteins, known as collectins, which is a subgroup of C-type (calcium dependant) lectins (132). SP-D is produced in the lungs by alveolar type II cells and non-ciliated Clara cells as well as by bronchial and tracheal gland cells. It is secreted into the airspaces, where it forms a large, oligomeric complex. Outside the lungs, SP-D is also synthesized by epithelial cells of many human tissues, including the salivary, sweat, and lachrymal glands, pancreas and bile ducts, renal tubules as well as gastrointestinal and genital tract (133). However, it is assumed that SP-D in serum mainly originates from the respiratory tract (134). SP-D facilitates the resolution of lung inflammation (133) and has the advantage to be a stable molecule over a period of 6 months that can be reliably measured in serum (135). Serum SP-D seems to rise as a consequence of leakage from the lung due to increased vascular permeability associated with lung inflammation (131,132). In healthy subjects, serum SP-D is increased in men, in smokers, and with increasing age and BMI (131,136–138). Serum SP-D was reported to be significantly higher in COPD patients compared to controls (130,131,139) and during exacerbation of COPD compared to stable COPD (139,140). Among smokers, most studies have reported reduced levels of SP-D in broncho-alveolar lavage (BAL) (130,141) and increased circulating levels (130,131,142). Conflicting results were reported regarding the association between SP-D and lung function tests in COPD patients (130,131,143,144). Several single nucleotide polymorphisms (SNPs) are associated with serum SP-D levels in COPD patients (111). Furthermore, there is no clear evidence regarding the associations between SP-D levels and asthma. Some have reported an increase in serum SP-D levels in asthma patients (145) while others have reported no significant difference in serum SP-D levels between controls and asthma patients (142).

#### 1.5.4. Cytokines

Cytokines are small proteins (sometimes classified as peptides and can also be glycosylated) that are secreted by a variety of cellular sources acting either on the cells producing them or on the surrounding cells. Some of them can also act on distant cells. They play an important role in cell signaling. Cytokines have been named based on their numerical order of discovery (especially, in the interleukin family, currently up to IL-38) or on a given functional activity (e.g., tumor necrosis factor (TNF) family). During a specific immune response, the main producers of cytokines are TH cells. Among these TH1 cells secrete INF- $\gamma$  and IL-2 and enhance cellular immune responses, while TH2 cells secrete IL-4, IL-5, IL-6, IL-13 and favor antibody responses (146). Over 50 cytokines have now been identified in asthma, but their role in the pathophysiology of this complex airway disease is often unclear (147). They play a role in the chronic inflammation in asthma through complex interactions (148). Among key cytokines, IL-5, IL-7 and IL-13 are implicated in eosinophilic asthma triggered by the TH2 pathway, IL-8 is crucial in neutrophil recruitment (10,149,150), and tumor necrosis factor-alpha (TNF- $\alpha$ ) has a central role in systemic inflammation (151). Additionally, Interleukin 1 receptor antagonist (IL-1Ra), an antagonist of IL-1, has anti-inflammatory activity (152), and IL-10 is able to inhibit pro-inflammatory cytokine synthesis (153,154). Cytokine levels have been often measured after direct and invasive sampling procedures i.e. BAL or induced sputum to better understand the inflammatory mechanisms in relation with asthma control outcomes (147,155–157). The measurement of cytokine levels in serum may be a valuable alternative, as standardized collection of peripheral blood is practical among participants of all ages for large epidemiological studies and for further routine clinical practice (151,158). Furthermore, this approach seems to be more powerful since it allows the identification of pools of cytokines with diagnostic performance superior to single cytokines in terms of sensitivity, specificity and robustness (97). However, it has been reported that recent studies utilizing multiplex analysis for the parallel analysis of multiple cytokines within one serum/plasma sample have neglected to include anti-inflammatory cytokines (such as receptor antagonists) (97).

### **1.5.5. 8-isoprostanes**

The exhaled breath condensate (EBC) was introduced in the mid-1990s as a new technique for measuring airway oxidative stress in lung diseases. It is a non-invasive method that can be carried out on mechanically ventilated patients as well as on children. Oxidative stress, which is characterized by an imbalance between increased exposure to free radicals, mainly derived from oxygen, and antioxidant defenses, is involved in the pathophysiology of asthma. Lipids are a major target of free radical attacks, which induce lipid peroxidation. One of the major biological markers for the successful measurement of oxidative stress in EBC is the 8-IsoPs (159). The IsoPs are prostaglandin (PG)-like substances that are produced in vivo independently of cyclooxygenase (COX) enzymes, primarily by free radical-induced lipid peroxidation of arachidonic acid (18). They are chemically stable, formed in vivo and are the most specific markers of lipid peroxidation. Elevated IsoPs levels have been associated with inflammatory pulmonary diseases. 8-IsoPs levels were found to be elevated in the EBC of asthma patients compared to healthy controls, but this increase was related to the disease severity (98). Moreover, it has been reported that 8-IsoPs levels were not affected by ICS therapy in children having asthma (98,160).

## **1.6. Objectives and flow of the thesis**

As previously mentioned, the general objective of the thesis is to study the role of biological markers in COPD and asthma. The thesis includes two specific objectives:

- The first specific objective was to assess the association between serum SP-D levels and COPD, an association that has not yet been evaluated in Lebanese patients, in comparison with serum CRP and plasma fibrinogen levels among subjects consulting for respiratory diseases or symptoms and to search for the optimal cut-off point able to discriminate COPD patients from controls using SP-D levels.

In reply to the first specific objective, a case-control study was performed in Lebanon. As COPD diagnosis is confirmed by spirometry (8), and since spirometry is a difficult technique, that is not always available, requires a certain expertise for its interpretation and is poorly

correlated with disease severity (161,162), an alternative is needed. Research has recently focused on the measurement of a variety of biological markers that appear to exhibit a utility in the diagnosis and prognosis of COPD. Among these biological markers, the circulating levels of systemic markers of inflammation, such as C-reactive protein (CRP) and fibrinogen, are validated in clinical laboratory assays (21). They have been shown to reflect the degree of severity of airway inflammation (99,124), but they are not lung-specific (125,163,164). Therefore, associations between a pulmonary-specific marker, SP-D and COPD were investigated (130,131). In this study, it was hypothesized that SP-D is a more specific biological marker than CRP or fibrinogen for the differentiation of COPD patients among individuals consulting for respiratory symptoms or diseases, including those with asthma. Therefore, the associations of SP-D, CRP and fibrinogen with patients with COPD or asthma were investigated. The optimal cut-off point able to discriminate COPD patients from controls using serum SP-D levels was also sought.

- The second specific objective was to assess the cross-sectional and longitudinal associations between asthma outcomes and serum CRP levels, serum cytokine profiles as well as EBC 8-IsoPs levels respectively.

In reply to the second specific objective, data from the EGEA study was used and three different analyses were performed including systemic as well as EBC biological markers of damage due to oxidative stress.

As the chronic inflammation in asthma is orchestrated by multiple cells and biological markers through complex interactions (148), and as little is known about the associations of these biological markers with asthma control outcomes, especially in a prospective design, the association between systemic markers, such as CRP levels (first analysis) and cytokine profiles (second analysis) and asthma outcomes were evaluated. Hs-CRP levels were chosen since hs-CRP is useful for the detection of low grade systemic inflammation present in asthma and to our knowledge, its association with asthma control in adults was previously investigated in two studies using cross-sectional designs and conflicting results were reported (165,166). Then, the associations between serum cytokines profiles and asthma outcomes were performed because recently, target-directed therapies with anti-Immunoglobulin (Ig) E or anti-interleukin (IL)-5 and anti-IL-13 antibodies were developed. However, these therapies have shown encouraging results in some patients, while they remained ineffective in others (167,168). Although cytokines are used as target therapy to improve asthma control (43,169),



little is known about their association with asthma control outcomes. To our knowledge, only one case-control study has evaluated associations between cytokine profiles in sputum and asthma control outcomes (156), and no study has used this approach in serum within a prospective design.

Moreover, biological markers potentially related to asthma could also be measured in EBC. Thus, the associations between EBC 8-IsoPs and asthma outcomes were evaluated (third analysis). EBC has raised the hope of finding an easy, non-invasive and reliable method to measure biological markers in asthma. As far as we know, a single study has assessed the relation between 8-IsoPs and asthma control using asthma control test (ACT) in 25 asthma patients and by performing univariate analyses and reported no correlation (170).

In the three above mentioned analyses, it was hypothesized that biological markers could predict asthma outcomes, and therefore longitudinal associations were also sought.

## **2. EVALUATION OF SP-D AS A BIOMARKER FOR COPD**

This part is devoted to assess the association between serum SP-D, a lung specific biological marker, and COPD, an association that has not yet been evaluated in Lebanese patients, and to find a cut-off point able to discriminate COPD patients from controls using serum SP-D levels.

### **2.1. Material and methods**

#### **2.1.1. Study design and participants**

A case-control study was conducted and the data collection, entry and statistical analyses took place in Lebanon between June 2011 and April 2013. All COPD patients consulting at the outpatients' clinics of the Pulmonology department of Saint George Hospital University and Medical Center in Beirut during the above mentioned period were recruited. COPD diagnosis was defined as a post-bronchodilator ratio of  $FEV_1/FVC$  of  $< 70\%$  (171). Ninety COPD patients were classified into categories based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines 2013 (171). There were 35 patients in group A, 38 in group B, 3 in group C, and 14 in group D. None of the patients had an exacerbation one month prior to the inclusion. With regard to medication, COPD patients were classified as treated when they were receiving beta2-agonists or anti-cholinergic agents (whether short acting or long acting) combined or not with ICS, as suggested previously (172). A total of 46 COPD patients were found to be on regular medication. Patients with asthma (n=124) were defined according to GINA guidelines 2013 (173). A clinical diagnosis based on symptoms (breathlessness, wheezing, cough and chest tightness), family history of asthma, worsening symptoms when exposed to various risk factors was performed and lung function measurements were assessed. Having another respiratory disease was the exclusion criterion for COPD or asthma patients. Healthy individuals from the general population (n=92) and outpatients consulting for a variety of non-respiratory problems in the same hospital (n=88) were recruited. Controls had normal lung function tests as defined by GOLD guidelines (171). The exclusion criteria were previous or current diagnosis of any respiratory disease such as asthma, COPD, chronic bronchitis, fibrosis, tuberculosis or lung cancer. Finally, genotyping of three coding SNPs of the SP-D gene was performed on 115 healthy controls from the

general population, 62 COPD and 97 asthma patients since previous studies have reported a genetic influence on SP-D serum levels (132,174). I performed the statistical analyses for the genotype association with clinical outcomes. The results are not reported in this thesis since they are part of an immunology thesis for another PhD student. Ethical approval was obtained from the ethical committee at the Lebanese university. Written informed consent was provided by all participants. The study participants are presented in Figure 3.

COPD patients	Asthma patients	Controls
<ul style="list-style-type: none"> <li>• <b>n = 90</b></li> <li>• GOLD classification: post-bronchodilator ratio FEV<sub>1</sub>/FVC &lt;70%</li> </ul>	<ul style="list-style-type: none"> <li>• <b>n = 124</b></li> <li>• A clinical diagnosis (symptoms, family history, worsening symptoms when exposed to risk factors) and lung function measurements.</li> </ul>	<ul style="list-style-type: none"> <li>• <b>n = 180</b></li> <li>• 92 from the general population</li> <li>• 88 outpatients consulting for non-respiratory diseases</li> </ul>

**Figure 3.** Participants of the study

Abbreviations: COPD, chronic obstructive pulmonary disease; GOLD, global initiative for chronic obstructive lung disease; FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity.

### 2.1.2. Questionnaire

A standardized questionnaire, adapted to the local Arabic language, was filled out by all participants with the assistance of trained interviewers. The questionnaire included detailed information on the socio-demographic characteristics, exposure to the disease risk factors, clinical assessment of the disease and its symptoms using the American Thoracic Society (ATS) questionnaire (175), the “MRC” breathlessness scale (176), and smoking history.

### 2.1.3. Blood collection and pulmonary function tests

Before pulmonary function tests, venous blood samples with or without anticoagulant were withdrawn from all participants, immediately transported at 4°C to the Immunology Laboratory of the Lebanese University and centrifuged at 4°C within 12 hours after

withdrawal. Plasma and serum were then aliquoted and stored frozen at -20°C until their analysis within 6 months.

All participants underwent baseline spirometry by a trained physician. Reversibility assessments (post-bronchodilator spirometry) were performed following the inhalation of two puffs of Ventoline (albuterol) with 30 minutes after baseline spirometry.

#### **2.1.4. Measurement of SP-D, CRP and fibrinogen**

Quantification of SP-D was performed by a five-layered enzyme-linked immunosorbent assay (ELISA) as previously described by Leth-Larsen *et al.* (177). Briefly, microtiter plates were coated with F(ab')<sub>2</sub> anti-human SP-D antibody (cat.no., K477; dilution, 1:1500; Grith Sorensen's laboratory, Odense, Denmark) in sodium carbonate buffer (pH 9.6). Following overnight incubation at 4°C, the plates were washed and left in contact with washing buffer (Tris-buffered saline, 0.05% Tween20, 5mM CaCl<sub>2</sub>) for 1 hour at room temperature. Calibrator, controls and samples were added at a dilution of 1:10 and incubated overnight at 4°C. This was followed by successive incubations with biotinylated monoclonal anti-human SP-D antibody (dilution 1:2,000; Grith Sorensen's laboratory), horseradish peroxidase-conjugated streptavidin (cat.no., 43-4323; dilution 1:20,000; Zymed; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and *o*-phenylenediamine (Zymed; Thermo Fisher Scientific, Inc.) in citrate-phosphate buffer pH 5, containing 0.014% H<sub>2</sub>O<sub>2</sub>. Plates were read at 492 nm in a multichannel spectrophotometer (Bio-Tek ELx800 (BioTek instruments, Inc., Vermont, USA)) after adding H<sub>2</sub>SO<sub>4</sub>. Serum CRP and plasma fibrinogen levels were measured using double antibody sandwich ELISA kits (Immunology Consultants Laboratory, Inc., Portland, OR, USA) according to the manufacturer's protocol. All samples were tested in duplicate. The coefficient of variation was 4.8% for SP-D, 2.7% for CRP, and 2% for fibrinogen.

#### **2.1.5. Statistical analyses**

Fibrinogen was normally distributed, whereas SP-D and CRP levels were not even after log<sub>10</sub> transformation. Results are expressed as median (interquartile ranges). Differences between groups were tested using the T-test or Chi-squared when appropriate (non-parametric tests gave same results). Associations between biological markers and lung function parameters in

COPD patients were estimated using generalized linear models with adjustment for age (continuous), sex, smoking (ever-smokers *versus* non-smokers) and BMI classes.

Three logistic regressions models were used to evaluate the association between SP-D and COPD. In the first and second regressions, COPD patients *versus* controls were used as dependent variable. The independent variables were SP-D expressed as above/below the median value, and all potential confounding variables such as age, sex, smoking and BMI classes, and all the remaining socio-demographic characteristics and respiratory symptoms (cough, wheezing and expectoration) having P-values < 0.20 in the univariate analyses. In the first regression, smoking was forced into the model since serum SP-D levels were previously reported to be higher in smoker subjects (131). As a sensitivity analysis, the second regression was performed in ever-smokers (smokers and ex-smokers) only. Then, in order to confirm the ability of SP-D to differentiate patients with COPD from those with asthma, a third regression including COPD *versus* asthma patients as the dependent variable was performed.

The adjusted odds ratios (aORs) obtained from the first and second regressions respectively were then rounded to the nearest units and used as coefficients in the calculation of score 1 (for all COPD patients and controls) and score 2 (for ever-smoker COPD patients and controls) with the purpose of predicting COPD diagnosis. Receiver-operating characteristic (ROC) curves were then generated in order to determine the ability of the scores 1 and 2 to discriminate between patients with COPD and controls. The ROC curve is a graphical technique widely accepted as a method for selecting an optimal cut-off point for a test able to discriminate between subjects with and those without disease and at which optimal sensitivity and specificity are achieved. Moreover, it allows the assessment of the diagnostic accuracy of a test. The curve is obtained by calculating the sensitivity and specificity of the test at every possible cut-off point, and plotting sensitivity (on the  $y$  axis) against 1-specificity (on the  $x$  axis). The shape of the curve reflects the overall strength of association between the underlying test and the disease status. The best cut-off point for balancing the sensitivity and specificity of a test is the point on the curve closest to the (0, 1) point. Optimal sensitivity and specificity are defined as those yielding the minimal value for  $(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2$ . The cut-off point corresponding to these sensitivity and specificity values is the one closest to the (0, 1) point and is considered to be the best point that differentiates between subjects with and those without disease. The total area below the ROC curve is known as the area under the curve (AUC) (178). An AUC > 0.8 is considered to reflect a good

accuracy of the diagnostic test (179). P-values  $< 0.05$  were considered to indicate a statistically significant difference. All analyses were performed using SPSS software, version 17.0 (SPSS, INc., Chicago, IL, USA).

### *Sample size calculation*

According to a previous study on SP-D in COPD (180), the smallest difference that could exist between healthy individuals and patients with COPD was 56.6 ng/mL, while the standard deviation (SD) was 80.2 ng/mL. With an alpha error of 5%, a power of 80%, a minimum of 32 patients and 64 controls was required for the study (181).

## **2.2. Results**

### **2.2.1. Participants' characteristics**

Characteristics of COPD and asthma patients and controls are described in Table 1. The three groups were significantly different regarding age, sex, smoking, marital status and education. Compared with controls, patients with COPD were more frequently men, ever-smokers and unmarried (all P-values  $\leq 0.003$ ); while patients with asthma were younger, more often unmarried, non-smokers and were more likely to have a university degree (all P-values  $\leq 0.02$ ). Compared to asthma patients, COPD patients were older, more frequently men, ever-smokers and were less likely to have a university degree (all P-values  $\leq 0.001$ ). No other significant associations were found.

**Table 1.** Characteristics of controls, COPD and asthma patients.

	<b>Controls (N=180)</b>	<b>COPD patients (N=90)</b>	<b>Asthma patients (N=124)</b>
<b>Age, Median (Q1;Q3)</b>	55 (51;64)	62 (50;71)	46 (32;61)
<b>Women, n (%)</b>	113 (63.5)	38 (42.2)	82 (67.8)
<b>Body Mass Index (BMI), n (%)</b>			
[20 – 25[ kg/m <sup>2</sup>	52 (29.9)	33 (37.1)	46 (37.4)
[25 – 30[ kg/m <sup>2</sup>	82 (47.1)	33 (37.1)	44 (35.8)
>=30 kg/m <sup>2</sup>	40 (23.0)	23 (25.8)	33 (26.8)
<b>Married, n (%)</b>	134 (84.3)	61 (71.8)	70 (60.9)
<b>Education, (University) n (%)</b>	28 (16.4)	12 (13.8)	43 (35.0)
<b>Work, (yes %)</b>	69 (39.9)	35 (41.7)	55 (45.8)
<b>Ever-smokers, n (%)</b>	112 (62.2)	68 (75.6)	60 (48.4)

Abbreviations: COPD, chronic obstructive pulmonary disease; %, percentage; (Q1; Q3): first and third quartiles.

Ever-smokers: Current and ex-smokers.

### **2.2.2. Associations between biological markers and COPD and asthma – Univariate analyses**

The three groups were significantly different regarding SP-D levels only. Serum SP-D levels were significantly increased in COPD patients as compared with controls and with asthma patients. There were no significant differences in SP-D levels between asthma patients and controls. No other significant associations were observed (Table 2).

**Table 2.** Variations of SP-D, CRP and fibrinogen levels between patients and controls

	<b>Controls</b> (N=180)	<b>COPD</b> <b>patients</b> (N=90)	<b>P-value*</b>	<b>Asthma</b> <b>patients</b> (N=124)	<b>P-value#</b>	<b>P-value§</b>
<b>SP-D (ng/mL)</b>	1269 (664;1884)	1510 (986;2174)	<b>0.02</b>	1130 (676;1852)	0.7	<b>0.02</b>
<b>CRP (ng/mL)</b>	9.72 (4.37;15.5)	8.41 (3.48;14.3)	0.5	8.35 (3.35;15.8)	0.4	0.3
<b>Fibrinogen (µg/mL)</b>	3135 (2730;3597)	2992 (2550;3965)	0.5	3358 (2575;4079)	0.09	0.06

Results expressed as Median (Q1; Q3). \*COPD patients compared to controls; #Asthma patients compared to controls; §COPD patients compared to asthma patients; **Bolded results** are statistically significant at P-values < 0.05.

Abbreviations: COPD, chronic obstructive pulmonary disease; SP-D, surfactant protein-D; CRP, C-reactive protein; (Q1; Q3), first and third quartiles.

SP-D levels were lower in the COPD patients who were receiving inhaled therapy (ICS and/or bronchodilators) compared with the COPD patients who were not, but the difference did not reach the level of significance [n=46 *versus* 42, Median (Q1;Q3): 1298 (892;2132) *versus* 1681 (1182;2469) ng/mL respectively, P-value=0.10].

No significant associations were identified between biological markers and FEV<sub>1</sub>% predicted or FEV<sub>1</sub>/FVC following the administration of a bronchodilator in COPD patients (Table 3).

**Table 3.** Associations between biological markers and lung function tests in COPD patients

	<b>FEV<sub>1</sub>%predicted*</b> <b>β (95 % CI)</b>	<b>P-value</b>	<b>FEV<sub>1</sub>/FVC*</b> <b>β (95 % CI)</b>	<b>P-value</b>
<b>SP-D</b>	-0.51 (-9.50;8.49)	0.9	-8.55 (-31.6;14.5)	0.5
<b>CRP</b>	-0.02 (-0.18;0.13)	0.8	-0.06 (-0.45;0.33)	0.8
<b>Fibrinogen</b>	5.60 (-2.46;13.7)	0.2	5.95 (-15.0;26.9)	0.6

Generalized linear models were performed; β (95 % CI) were adjusted for age, sex, smoking and BMI.

\*After administration of a bronchodilator.



Abbreviations: FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity; SP-D, surfactant protein-D; CRP, C-reactive protein; CI: confidence interval.

### **2.2.3. Associations between SP-D and COPD - Multivariate analyses**

Multivariate analyses performed on all COPD patients and controls showed that having SP-D levels above the median value, being a man, being unmarried and having symptoms such as a morning cough, cough during the day and wheezing during the day were significantly and positively associated with COPD (Table 4a). The same associations were observed in ever-smoker COPD patients and controls (Table 4b).

In the third regression (Table 4c) with COPD patients *versus* asthma patients as the dependent variable, SP-D levels above the median value, older age, being a man and having a morning cough were significantly and positively associated with COPD.

**Table 4.** Associations between serum SP-D levels and COPD - Multivariate analyses

<i>(a) - First regression – N =221 – COPD patients versus controls as dependent variable</i>			
	<b>aOR</b>	<b>95% CI</b>	<b>P-value</b>
SP-D (above the median)	3.86	1.51; 9.85	0.005
Men	2.92	1.23; 6.93	0.02
Not Married	3.07	1.19; 7.96	0.02
Cough in the morning	39.2	10.1; 153	<0.001
Cough during the day	10.9	3.25; 36.6	<0.001
Wheezing during the day	64.9	11.8; 356	<0.001
Ever-smokers	1.31	0.54; 3.18	0.5
<i>Nagelkerke R<sup>2</sup> = 60%; Hosmer-Lemeshow goodness-of-fit, P-value = 0.541; 86.4% of the subjects correctly classified.</i>			
<i>(b) - Second regression – N =141 – COPD patients versus controls as dependent variable for ever-smokers</i>			
SP-D (above the median)	6.26	1.81; 21.65	0.004
Men	4.22	1.43; 12.5	0.009
Not Married	3.38	1.04; 10.9	0.04
Cough in the morning	53.8	10.7; 272	<0.001
Cough during the day	8.66	1.80; 41.8	0.007
Wheezing during the day	35.8	4.82; 267	<0.001
<i>Nagelkerke R<sup>2</sup> = 62%; Hosmer-Lemeshow goodness-of-fit, P-value = 0.926; 84.4% of the subjects correctly classified.</i>			
<i>(c)- Third regression- N =201 – COPD patients versus asthma patients as dependent variable</i>			
SP-D (above the median)	2.53	1.29; 4.96	0.007
Age	1.04	1.02; 1.07	<0.001
Men	2.84	1.44; 5.62	0.003
Cough in the morning	8.70	3.29; 23.0	<0.001
<i>Nagelkerke R<sup>2</sup> = 39%; Hosmer-Lemeshow goodness-of-fit, P-value = 0.867; 76.1% of the subjects correctly classified.</i>			

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; SP-D, surfactant protein-D; COPD, chronic obstructive pulmonary disease; Ever-smokers: Current & ex-smokers.

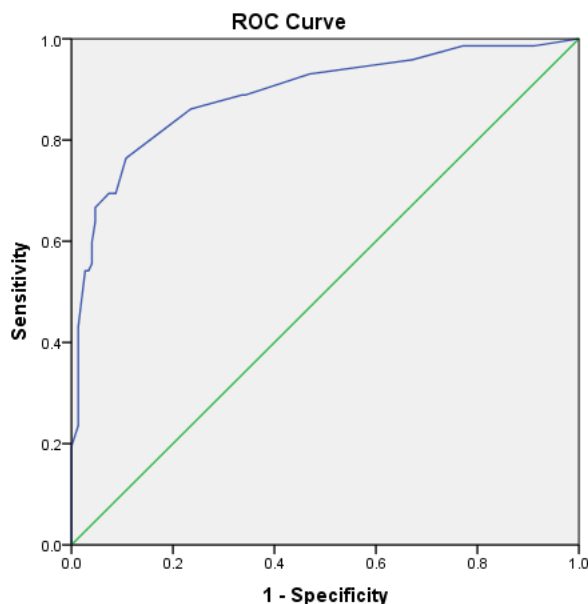
#### 2.2.4. Construction, properties and thresholds of the scores for COPD diagnosis

Taking into account the aORs from the first regression (from Table 4a) and rounding to the nearest unit, a first score (score 1) for COPD diagnosis was computed as follows:

Score 1 = (SP-D above/below the median x 4) + (sex x 3) + (marital status x 3) + (cough in the morning x 39) + (cough during the day x 11) + (wheezing during the day x 65) + (smoking x 1).

In COPD patients, score 1 has a minimum of 6 and a maximum of 129. The mean was 47.2, the median was 49 and the SD was 31.9. In controls, the minimum was 6, the maximum was 75, with a mean of 12.6, a median of 10 and a SD of 10.1.

The ROC curve generated for score 1 is shown in Figure 4 for the comparison of COPD patients with controls. The AUC was at 0.890 (0.841-0.940; P-value < 0.001). The most optimal cut-off point was calculated to be 15.5 (Table 5) at which point sensitivity, specificity, positive predictive value and negative predictive value were 76.4%, 89.3%, 81% and 74% respectively.



**Figure 4.** ROC curve with all COPD patients and controls.

**Table 5.** Coordinates of the ROC curve for all COPD patients and controls

<b>COPD positive if <math>\geq</math> (a)</b>	<b>Sensitivity</b>	<b>1 – Specificity</b>
5.0000	1.000	1.000
6.5000	0.986	0.913
8.0000	0.986	0.772
9.5000	0.958	0.671
10.5000	0.931	0.470
11.5000	0.889	0.342
12.5000	0.889	0.336
13.5000	0.861	0.235
<b>15.5000</b>	<b>0.764</b>	<b>0.107</b>
17.5000	0.694	0.087
19.0000	0.694	0.074
20.5000	0.681	0.060
22.5000	0.667	0.047
24.5000	0.639	0.047
26.5000	0.597	0.040
36.5000	0.569	0.040
45.5000	0.556	0.040
47.0000	0.542	0.034
48.5000	0.542	0.027
49.5000	0.486	0.020
51.5000	0.431	0.013
56.5000	0.403	0.013
62.0000	0.306	0.013
67.5000	0.278	0.013
71.5000	0.264	0.013
73.0000	0.250	0.013
74.5000	0.236	0.013
75.5000	0.194	0.000
77.0000	0.181	0.000
78.5000	0.153	0.000
80.0000	0.125	0.000
81.5000	0.111	0.000
83.5000	0.097	0.000
85.5000	0.083	0.000
87.5000	0.069	0.000
101.5000	0.056	0.000
119.5000	0.042	0.000
127.0000	0.028	0.000
130.0000	0.000	0.000

Abbreviation: COPD, chronic obstructive pulmonary disease.

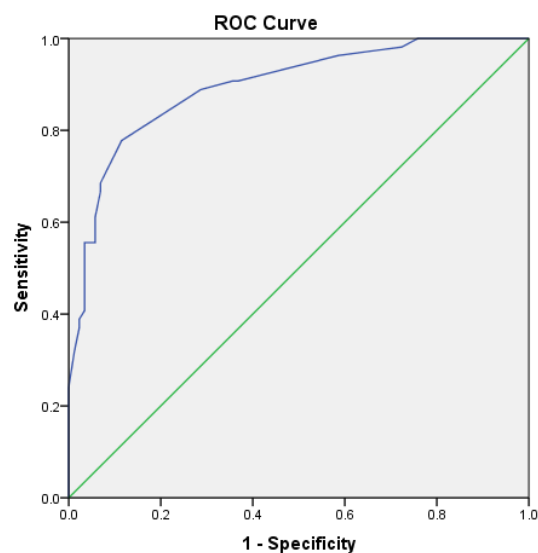
The value in bold is the optimum cut-off point.

A second score (score 2) was calculated from the second regression (from Table 4b) for ever-smoker COPD patients and controls as follows:

Score 2 = (SP-D above/below the median x 6) + (sex x 4) + (marital status x 3) + (cough in the morning x 54) + (cough during the day x 9) + (wheezing during the day x 36).

In ever-smoker COPD patients, score 2 has a minimum of 10 and a maximum of 116. The mean was 47.7, the median was 51 and the SD was 28.5. In ever-smoker controls, the minimum was 7, the maximum was 67, with a mean of 15, a median of 13 and a SD of 11.4.

The ROC curve generated from score 2 is shown in Figure 5. The AUC was at 0.895 (0.841-0.950; P-value < 0.001). The most optimal cut-off point was 18.5 at which point sensitivity, specificity, positive predictive value and negative predictive value were 77.8%, 88.5%, 70% and 82% respectively (Table 6).



**Figure 5.** ROC curve with ever-smoker COPD patients and controls.

**Table 6.** Coordinates of the ROC curve for ever-smoker COPD patients and controls.

<b>COPD positive if <math>\geq</math> (a)</b>	<b>Sensitivity</b>	<b>1 – Specificity</b>
6.0000	1.000	1.000
8.5000	1.000	0.759
10.5000	0.981	0.724
12.0000	0.963	0.586
13.5000	0.907	0.368
15.0000	0.907	0.356
16.5000	0.889	0.287
<b>18.5000</b>	<b>0.778</b>	<b>0.115</b>
21.5000	0.685	0.069
24.5000	0.667	0.069
27.5000	0.611	0.057
36.0000	0.574	0.057
44.5000	0.556	0.057
46.5000	0.556	0.034
48.0000	0.537	0.034
49.5000	0.519	0.034
51.0000	0.500	0.034
52.5000	0.481	0.034
54.5000	0.463	0.034
57.5000	0.426	0.034
60.0000	0.407	0.034
62.5000	0.389	0.023
64.5000	0.370	0.023
66.0000	0.315	0.011
69.0000	0.241	0.000
72.5000	0.204	0.000
77.0000	0.093	0.000
95.0000	0.056	0.000
113.0000	0.037	0.000
117.0000	0.000	0.000

Abbreviation: COPD, chronic obstructive pulmonary disease.

The value in bold is the optimum cut-off point.

## **2.3. Discussion**

### **2.3.1. Summary of findings**

In this study, we investigated the association of COPD with serum SP-D levels among subjects consulting for respiratory diseases or symptoms, and in comparison with serum CRP and plasma fibrinogen levels. SP-D levels were found to be significantly and positively associated with COPD whereas serum CRP and plasma fibrinogen levels were not. Furthermore, a score for COPD diagnosis with excellent discriminant values was identified, with the best scale for the diagnosis of COPD being obtained using SP-D levels, socio-demographic characteristics, and respiratory symptoms significantly associated with COPD.

### **2.3.2. Comparisons with other studies and interpretation**

The present study found that SP-D levels were significantly and positively associated with COPD as compared with controls, a result in line with previous studies (130,139,182). To the best of our knowledge, the present study is the first to confirm such a result through multivariate analyses. The association between SP-D and COPD has previously been investigated by bivariate analyses (130,139,142). Ou *et al.* did not find an association between SP-D and COPD in bivariate and multivariate analyses (183) and Ilumets *et al.* adjusted for a single confounding factor, which is age (180). Others have performed multivariate analyses concerning associations of SP-D, but considering COPD exacerbation rather than diagnosis (140,183). Since smoking is known to highly affect SP-D levels, the present study considered ever-smokers only, and it was found that SP-D remained significantly and positively associated with COPD, a result concordant with previous literature (130,138). No association of CRP or fibrinogen with COPD was found in the present study. Previous studies have reported higher CRP or fibrinogen levels in stable COPD patients compared with controls (123,184). However, the use of CRP and fibrinogen as biological markers for COPD is limited by their low specificity and low predictive value for COPD (172) and any inflammatory or infectious condition, even if not related to lung inflammation, can modify CRP levels (185).

In the present study, SP-D levels were also significantly elevated in COPD patients as compared with asthma patients, a result in agreement with a previous study by Mutti *et al.* (142). We observed no significant differences in SP-D levels between asthma patients and controls. To the best of our knowledge, there is no clear evidence regarding the association between SP-D and asthma. One study has reported no association of SP-D levels with asthma (142), while some small clinical/case studies have indicated that raised serum SP-D levels are associated with allergy (186,187). Finally, SP-D appears to be a clinical biological marker for COPD, able to differentiate COPD patients among individuals consulting for respiratory diseases or symptoms including those with asthma, while serum CRP and plasma fibrinogen levels are not.

The present study did not find an association between SP-D and FEV<sub>1</sub>% predicted or FEV<sub>1</sub>/FVC in COPD patients. Previous studies failed to provide a clear evidence of an association between biological marker levels and lung function parameters. Some studies have reported similar results, whether in bivariate analyses (129,188) or in multivariate analysis (143). However, others have described significant negative correlations between SP-D levels and lung function parameters in univariate analyses with a borderline P-value for the association between SP-D and FEV<sub>1</sub>% predicted (189) or a weak but significant association with FEV<sub>1</sub>/FVC in only 20 COPD smokers (130). Others have found an association with FEV<sub>1</sub> through multivariate analyses in only 23 patients with advanced COPD (128), in stable COPD patients (139) or in smokers only (190). Regarding CRP and fibrinogen, the results of the present study are concordant with two previous studies that found no association with FEV<sub>1</sub>% in COPD patients (110,128). By contrast, others have found an inverse correlation between CRP or fibrinogen and lung function test results (143,191). Differences between the studies may be explained in part by differences in the sample size, inclusion or exclusion of non-smoker COPD patients and the severity of the disease. In the present study, 81% of the COPD patients were classified in groups A and B and 19% in groups C and D, and smoker, ex-smoker and non-smoker COPD patients were included.

To the best of our knowledge, the present study is the first to suggest a score for COPD diagnosis with excellent discriminant values and validity results (AUC, sensitivity, specificity, positive predictive value and negative predictive value). There are validated diagnosis scales that could be used in primary care settings without the need for blood marker measurements such as DS-COPD (diagnosis score for COPD patients) (192). A previous study has created



ROC curves based on 44 stable COPD patients to evaluate the diagnostic accuracy of SP-D, and found a total AUC of 0.734 (180). In the present study, to obtain more accurate results, ROC curves were generated for two scores calculated from the variables identified to be associated with COPD in the logistic regression models for all COPD patients and controls and for ever-smoker COPD patients and controls, respectively. In addition to SP-D, the formula for calculating the score included socio-demographic characteristics and respiratory symptoms associated with COPD. The AUCs were 0.890 and 0.894 for all subjects and ever smokers, respectively, which may be considered as an improvement compared with the results reported in previous literature.

### **2.3.3. Strengths and limitations**

The particular selection of patients and controls allowed us to support our hypothesis that SP-D is able to differentiate COPD patients among individuals consulting for respiratory diseases or symptoms, including those with asthma. COPD and asthma patients, healthy controls and outpatients consulting for non-respiratory diseases were recruited. One of the study limitations was the low percentage of COPD patients presenting severe and very severe stages of the disease that required treatment with ICS. Therefore, we assessed the effect of medical treatment as a whole, regrouping patients receiving  $\beta$ 2-agonists (or anticholinergics) combined or not with ICS. SP-D levels were lower in the group of COPD patients who were receiving treatment compared with those that were not receiving treatment, but the association did not reach the level of significance reached by previous studies (182,193,194). The present study may have a selection bias (75.6% of COPD patients were ever smokers *versus* 62.2% of controls) but this did not affect the analytical results. Recall bias might be possible because information on previous smoking history was based on self-reports. The information bias introduced by underreporting is probable, as smoking behaviors are sensitive issues, particularly among patients that do not want to stop smoking. However, as the patients knew that the study outcome may be beneficial in their medical follow up, it was assumed that the information bias was minimized.

#### **2.3.4. Conclusion**

In conclusion, SP-D appears to be able to differentiate COPD patients from patients consulting for other respiratory symptoms or diseases. Used along with socio-demographic characteristics and respiratory symptoms associated with COPD, SP-D is able to discriminate COPD patients from controls, particularly among smokers. The predictive value of SP-D as a biological marker of COPD was planned to be evaluated in Lebanese patients within a large scale study with prospective follow-up but due to financial issues, it was interrupted. However, we hope we will be able to finalize this work in the future.

*As a result of the work performed in this part of the thesis, an article entitled “SP-D as a clinical biomarker for COPD with excellent discriminant values” was published in Experimental and Therapeutic Medicine Journal. Another article on SP-D genotyping entitled “Surfactant protein D (SP-D) levels, polymorphisms and multimerization in healthy and inflammatory disease state” is in the process of being finalized and will include my name as a co-author.*

### 3. BIOLOGICAL MARKERS AND ASTHMA CONTROL OUTCOMES

This part describes the three analyses aiming to find the association between inflammatory biological markers (serum hs-CRP and serum cytokine profiles) and a marker of damage due to oxidative stress (8-IsoPs) on one hand and asthma outcomes on another in a longitudinal design using data from the French EGEA study.

#### 3.1. EGEA: Study design and follow-up

The EGEA study (<https://egeanet.vjf.inserm.fr/index.php/fr/>) is a French multicenter longitudinal study. It combines a case-control and a family based study with an initial group of asthma cases and their first-degree relatives and a group of controls, followed-up for over 20 years. It was conducted in five French cities (Grenoble, Lyon, Marseille, Montpellier and Paris) (56,195–197). The general objective of the EGEA study is to assess genetic and environmental risk factors and their interactions with asthma and the associated phenotypes of bronchial hyperresponsiveness and allergic sensitization. This study is important in order to develop preventive measures and new asthma management. It provides public health information with the goal of improving the respiratory health of the population.

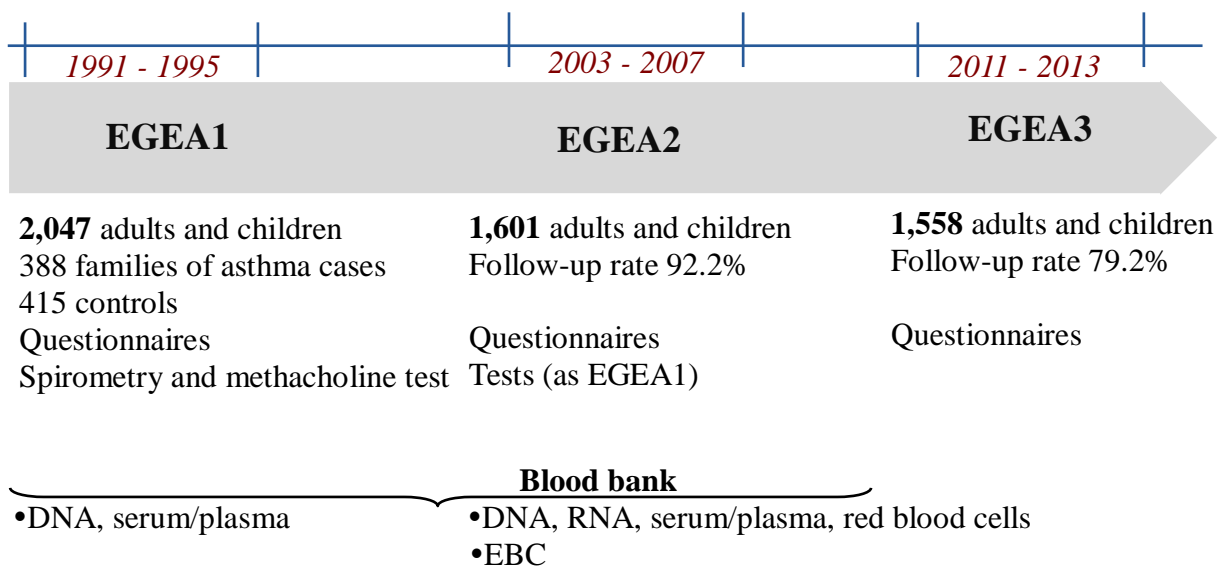
The first cross-sectional survey (EGEA1) took place between 1991 and 1995. The questionnaire used in the study included parts of standardized questionnaires, such as the questionnaire of the European Community Respiratory Health Survey (ECRHS) (198), of the International Union against Tuberculosis and Lung diseases / “Union Internationale Contre la Tuberculose et les Maladies Respiratoires” (UICTMR) study (199), of the Tucson study (200), of the British Medical Research Council / European Coal and Steel Community (201), of the “Pollution Atmosphérique et Affections Respiratoires Chroniques” (PAARC) (202), and of the ATS (175) with additional questions when needed. Questionnaires were distributed. After the collection of self-administered questionnaires, the eligible applicants as well as first degree relatives of potential cases were invited to participate in the study. Overall, 2,047 participants aged from 7 to 70 years were enrolled, including 388 families of asthma cases (children <16 years and adults) and 415 controls. Asthma cases were recruited from 7 chest clinics. Two thirds of adult control subjects were population-based (electoral rolls), and one-third from surgery hospitals or check-up centers. Children control participants were recruited

in surgery hospitals. All in all, half of the controls were population-based. Control subjects were matched for age, sex, month of exam and city. This first data collection allowed a good characterization of the population in terms of health, environmental exposures and the creation of a bio-bank, including genetics.

Between 2003 and 2007, this population was contacted for the second survey. In total, 2002 individuals were still alive. Among them, 1845 participants (92.2%) in addition to 73 new family members, who were not examined at the first survey or were born later, completed a short self-administered questionnaire. Responders to the self-completed questionnaire were invited for a complete examination, and detailed information was available for 1601 subjects (77.1% of the alive cohort + 58 new family members). The examination included detailed questionnaires to assess respiratory and allergic symptoms, personal and familial history of respiratory and allergic diseases, domestic and professional environmental factors from early childhood, lung function tests (spirometry and methacholine challenge test), a blood sampling, an EBC collection and a skin prick test (SPT). This second data collection aimed at improving the asthma phenotypic characterization since it included very detailed information on health, and the environment as well as a thorough program of collection of biological material.

As a follow-up study of EGEA2, the third survey (EGEA3) was initiated from 2011 to 2013 using a self-completed questionnaire. Among the 1996 subjects invited to respond to the questionnaire, 1558 filled it out (79.2% follow-up rate for more than 20 years).

Ethical approval was obtained from the relevant institutional review board committees (Cochin Port-Royal Hospital and Necker-Enfants Malades Hospital, Paris). All participants signed written informed consent. The EGEA study participants and follow-up from EGEA1 till EGEA3 are summarized in Figure 6.



**Figure 6.** Participants and methodology of the EGEA study

EGEA collection is certified ISO 9001 since 2006 (AFNOR certificate N° 2006/26271.7)

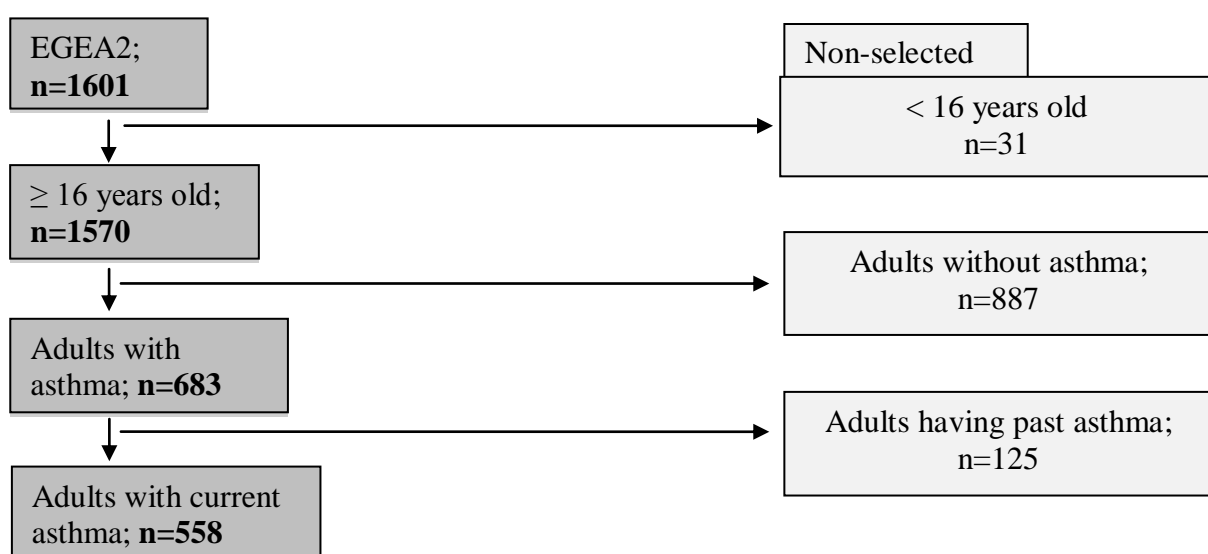
Abbreviations: EGEA, epidemiological study on the genetics and environment of asthma;

DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EBC, exhaled breath condensate.

### 3.2. Participants selection

We performed three analyses on adults with current asthma from EGEA2 (Figure 7).

Subsequent selections were done for each analysis.



**Figure 7.** Participants selected from the EGEA study.

Abbreviations: EGEA, epidemiological study on the genetics and environment of asthma.

### 3.3. Characteristics of the participants with current asthma from the EGEA study

At EGEA2, the mean age of the 558 adults with current asthma was 39 years, half of them were women, and 48% were non-smokers. Almost half of them reported asthma attacks in the past year and 52% were classified as controlled according to GINA 2015. More than 80% had an allergic sensitization (Table 7).

**Table 7.** Characteristics of the 558 adults with current asthma at the second survey (EGEA2)

	<b>At EGEA2</b>
<b>Age, years, Mean ± SD, min, max</b>	39.2 ± 16.6, 16.9, 79.5
<b>Women, n (%)</b>	270 (48.4)
<b>Body mass index (BMI), n (%)</b>	
<20 kg/m <sup>2</sup>	63 (12.5)
[20 – 25[ kg/m <sup>2</sup>	257 (50.9)
[25 – 30[ kg/m <sup>2</sup>	127 (25.1)
>=30 kg/m <sup>2</sup>	58 (11.5)
<b>Smoking, n (%)</b>	
Non-smokers	267 (47.9)
Ex-smokers	139 (25.0)
Smokers	151 (27.1)
<b>Age at asthma onset, n (%)</b>	
[0-4] years	167 (31.8)
]4-16] years	179 (34.1)
>16 years	179 (34.1)
<b>Exacerbations, past 12 months, n (%)</b>	77 (16.8)
<b>Asthma attacks, past 12 months, n (%)</b>	251 (45.1)
<b>Asthma symptom control, n (%)</b>	
Controlled	268 (52.4)
Partly controlled	178 (34.8)
Uncontrolled	65 (12.7)
<b>Asthma symptom score (Pekkanen), n (%)</b>	
0	55 (10.0)
1	125 (22.7)
≥2	370 (67.3)
<b>ICS use, past 12 months, n (%)</b>	268 (48.8)
<b>FEV<sub>1</sub> % predicted, Mean ± SD</b>	95.3 ± 18.8
<b>Methacholine test*, PD20 ≤ 4 mg, n (%)</b>	206 (70.5)
<b>SPT+, n (%)</b>	382 (81.1)
<b>Allergic rhinitis, n (%)</b>	404 (74.0)
<b>IgE, IU/mL, GM (Q1;Q3)</b>	154 (66.0; 378)
<b>Eosinophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	213 (140; 320)
<b>Neutrophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	3856 (3015; 4825)

Abbreviations: ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV in 1 second from baseline; \*Methacholine challenge test was performed if baseline FEV<sub>1</sub> ≥ 80% predicted (n=292). SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; GM, geometric mean; (Q1;Q3), first and third quartiles.

### 3.4. Respiratory phenotyping

Respiratory phenotypes used in the three different analyses performed using data from the EGEA study are described below:

Inclusion criteria used to define “*ever asthma*” in cases were based on self-reported answers to the four questions: “*Have you ever had attacks of breathlessness at rest with wheezing?*”, “*Have you ever had asthma attacks?*”, “*Was this diagnosis confirmed by a physician?*”, and “*Have you had an asthma attack in the past 12 months?*”, or a positive response to at least two questions and a positive review of their medical record. Asthma in relatives of cases was defined as a positive answer to at least one of the two first questions. Among subjects with asthma, “*current asthma*” was defined by a report of respiratory symptoms (wheezing, nocturnal chest tightness, attacks of breathlessness following strenuous activity, at rest or at night time, and asthma attacks) or use of inhaled and/or oral medicines because of breathing problems in the past twelve months.

ICS medications were defined among subjects who reported to have used ICS in the past 12 months prior to inclusion.

A composite score proposed by Pekkanen (203,204), labeled here as the “*asthma symptom score*” was calculated both at EGEA2 and EGEA3. The score varies from 0 to 5 and is based on the number of asthma symptoms reported in the past 12 months (wheezing and breathlessness, woken up with chest tightness, woken up by an attack of shortness of breath, attack of shortness of breath at rest, attack of shortness of breath after exercise). In the analyses, the score is presented as 0, 1, and  $\geq 2$ . We assessed the change of asthma symptoms from EGEA2 to EGEA3 using the difference of asthma symptom scores (Pekkanen) as follows and defined three groups: i) the “better” group: EGEA3 score - EGEA2 score  $< 0$ , ii) the “stable” group: EGEA3 score - EGEA2 score = 0, iii) the “worse” group: EGEA3 score - EGEA2 score  $> 0$ . In the analyses we put the stable group and the better group together.

“*Asthma symptom control*” at EGEA2 and EGEA3 was evaluated using answers to survey questions to approximate as closely as possible the GINA 2015 definition. Subjects were defined with controlled, partly-controlled and uncontrolled asthma if they fit none, 1-2 or 3-4 of the following criteria, respectively: frequent daytime symptoms (defined by at least 1 asthma attack or 1 or more episode of shortness of breath per week in the past 3 months), any nighttime symptom (defined by woken up because of asthma or by an attack of shortness of

breath in the past 3 months), frequent use of reliever medications (defined by an average use of short acting bronchodilators twice per week in the past 3 months), and any activity limitation (defined by the following answer “totally limited”, “extremely limited”, “very limited”, “moderately limited” or “somewhat limited” to the question “Overall, in all the activities that you have done during the past two weeks, how limited have you been by your asthma? ”).

Asthma exacerbation was defined by hospital or emergency admissions because of respiratory problems or the use of oral steroids for breathing difficulties in the past year.

Allergic sensitization was defined by an SPT+ with a mean wheal diameter  $\geq 3$ mm than the negative control for at least one of 12 aeroallergens (indoor: cat, *Dermatophagoides pteronyssinus*, *Blattella germanica*, outdoor: olive, birch, *Parietaria judaica*, timothy grass, *Cupressus* and ragweed pollen, and molds: *Aspergillus*, *Cladosporium herbarum*, *Alternaria tenuis*).

Pulmonary function test was performed using a standardized protocol with similar equipment across centers according to the ATS / ERS (European Respiratory Society) guidelines (205). FEV<sub>1</sub> % predicted value was based on Quanjer *et al.* reference equations (206). It is defined as FEV<sub>1</sub> divided by the average FEV<sub>1</sub> in the population for any person of similar age, sex and body composition. For subjects with an FEV<sub>1</sub> of  $\geq 80\%$  of the predicted value, a methacholine bronchial challenge test was performed. The patients inhaled at most 4 mg of methacholine which provokes a constriction of the airways. The dose of methacholine causing a 20% fall in FEV in 1 second from baseline (PD20) was recorded. The degree of narrowing was then quantified by spirometry.

### **3.5. Blood tests and EBC collection**

Venous blood samples were drawn from all participants with or without anti-coagulant. Tubes without anti-coagulant were centrifuged immediately after collection at 4°C and serum was separated in aliquots and stored at -80°C from 6.6 to 9.6 years until analysis, according to standardized procedures.

Total Immunoglobulin E (IgE) levels were assessed by UniCAP system (Pharmacia®) in serum in a centralized laboratory, and expressed in international units (IU) per milliliter.



Eosinophil and neutrophil cell counts were obtained from white blood cell counts from tubes with anti-coagulant.

EBC samples were collected with RTubes TM (Respiratory Research Inc., Charlottesville, VA, USA) according to a standardized method. The RTubes were rinsed with deionized water and dried thoroughly. Participants breathed orally at tidal volumes into a mouthpiece attached to a cold condenser (-20°C). They were seated comfortably with a headrest. All headrests and back seats were tilted slightly to avoid any saliva contamination during breathing maneuvers. Breathing was quiet and regular. After 15 minutes, EBC collection was immediately separated in aliquots and stored at -80°C from 5 to 9 years according to standardized procedures. Maintenance of the cold chain was guaranteed in every case for blood and EBC samples.

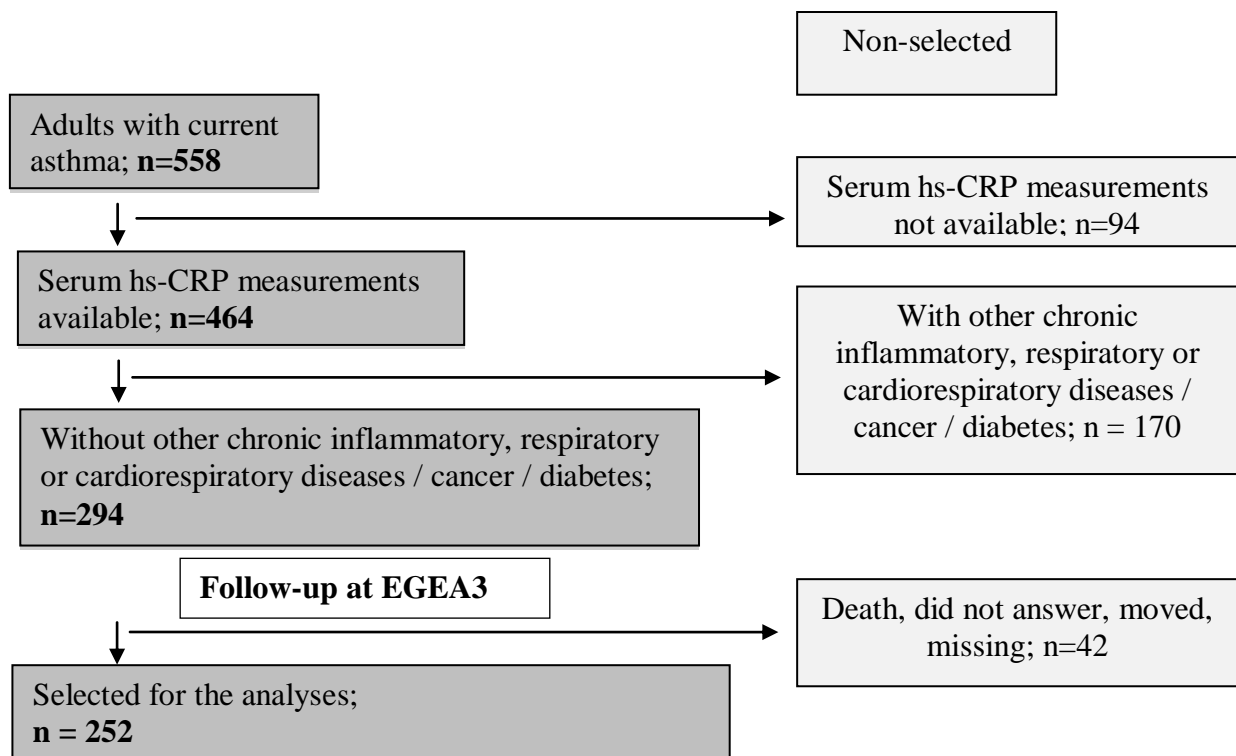
### **3.6. First analysis: Cross-sectional and longitudinal associations between hs-CRP and current asthma**

This analysis attempts to characterize the cross-sectional and longitudinal associations between serum hs-CRP levels and asthma outcomes in a selected population from the French EGEA study of adult patients with current asthma.

#### **3.6.1. Material and methods**

##### ***3.6.1.1. Participants' selection***

Among the 558 participants with current asthma who were adults at EGEA2 ( $\geq 16$  years old), the present analysis included 252 participants who were followed-up at EGEA3, excluding those who had no available serum hs-CRP measurements (n=94), those who reported other chronic inflammatory diseases, respiratory or cardiorespiratory diseases, cancer or diabetes (n=170) since these diseases may affect CRP levels as reported by previous literature (Figure 8).



**Figure 8.** Flow chart of the participants – data on hs-CRP and current asthma

Abbreviations: EGEA, epidemiological study on the genetics and environment of asthma; hs-CRP, high sensitivity C-reactive protein.

### 3.6.1.2. *Biological phenotypes*

#### *Hs-CRP measurements*

Hs-CRP tests were performed at the laboratory of Alfred Bernard, at the Catholic University of Louvain, Belgium, in January 2011, using the Synchron system of a Beckman Coulter purchased from the firm Analys. Hs-CRP measurement was based on the formation of turbidity following the reaction of the antigen with an antibody coated on a particle. This kinetic turbidimetric reaction was measured at 940 nm. The standard curve was adjusted every day of analysis by adding a calibrator. The value of 3 mg/L was accepted as the normal limit by Beckman or the Catholic University of Louvain laboratories.

### *The glomerular filtration rate*

The glomerular filtration rate (GFR), measured in mL/1.73m<sup>2</sup>min is associated with kidney function (207). In the EGEA study, the GFR was not measured directly but was calculated from the serum level of creatinine (mg/dL), which was assessed via the following formula (208):

$$\text{GFR} = 141 \cdot \min(\text{Scr}/k, 1)^\alpha \cdot \max(\text{Scr}/k, 1)^{-1.209} \cdot 0.993^{\text{Age}} \cdot 1.018[\text{if woman}] \cdot 1.159[\text{if black}]$$

where *Scr* is serum creatinine (mg/dL), *K* is 0.7 for women and 0.9 for men and  $\alpha$  equals 0.329 for women and 0.411 for men.

GFR was measured in order to test if there is any association between hs-CRP levels and kidney diseases.

#### **3.6.1.3. Statistical analyses**

Hs-CRP values were log10 transformed due to their skewed distribution and expressed as geometric means (GMs) (interquartile range). The GMs are more robust comparing variables with different numeric ranges (209). They apply only to strictly positive numbers. The GM  $\mu_g$  is calculated via:

$$\mu_g = \left( \prod_{i=1}^n x_i \right)^{\frac{1}{n}} = \exp \left( \frac{1}{n} \sum_{i=1}^n \ln x_i \right)$$

Associations between hs-CRP levels and storage time, GFR, hour of blood test and smoking in the 24 hours prior to the blood sampling and with age (continuous), sex, smoking (never, ex-, current smokers), BMI (4 classes), and ICS use in the past 12 months were assessed using standard statistical tests such as student T-test or ANOVA test and Pearson correlation coefficient or its non-parametric Spearman correlation coefficient.

Due to the familial aggregation of the data, multivariate analyses (except multinomial logistic regressions) were performed using generalized estimating equations (GEE) with an exchangeable working correlation to account for the potential clustering within the families, thus, the dependence between observations. Hs-CRP levels were studied in association with asthma outcomes (exacerbations, attacks, asthma symptom control, asthma symptom score (Pekkanen)), lung function, allergic sensitization, BHR (PD20), IgE levels and blood cell counts at EGEA2 (cross sectional analyses), and with asthma attacks, asthma symptom control, asthma symptom score (Pekkanen) at EGEA3 and evolution of asthma symptom

score (Pekkanen) between EGEA2 and EGEA3 (longitudinal analyses). Age, sex, smoking, BMI and center at EGEA2 were included as covariates.

As the presence of respiratory infections in the past four weeks may affect blood neutrophil counts, we further adjusted the association between hs-CRP levels and neutrophil counts on respiratory infections as a sensitivity analysis. P-values < 0.05 were considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS software, version 20.0.

## **3.6.2. Results**

### ***3.6.2.1. Study participants***

#### *Participants selected for the hs-CRP analysis versus those non-selected*

Participants selected for this analysis (n=252) included more subjects with controlled asthma and had more positive methacholine challenge test than those not selected (n=306). The two groups were similar for age, sex, BMI, smoking status, allergic sensitization, exacerbations, asthma attacks, asthma symptom score, ICS use, FEV1 % predicted, and blood cell counts (Table 8).

**Table 8.** Comparison of main characteristics between non-selected and selected participants

	<b>Non-selected N=306</b>	<b>Selected N=252</b>	<b>P-value</b>
<b>Age, years, Mean ± SD</b>	39.8 ± 17.4	38.5 ± 15.5	0.4
<b>Women, n (%)</b>	141 (46.1)	129 (51.2)	0.2
<b>Body mass index (BMI), n (%)</b>			0.4
<20 kg/m <sup>2</sup>	27 (10.5)	36 (14.5)	
[20 – 25[ kg/m <sup>2</sup>	130 (50.6)	127 (51.2)	
[25 – 30[kg/m <sup>2</sup>	67 (26.1)	60 (24.2)	
>=30 kg/m <sup>2</sup>	33 (12.8)	25 (10.1)	
<b>Smoking, n (%)</b>			0.6
Non-smokers	141 (46.2)	126 (50.0)	
Ex-smokers	76 (24.9)	63 (25.0)	
Smokers	88 (28.9)	63 (25.0)	
<b>Exacerbations, past 12 months, n (%)</b>	39 (17.0)	38 (16.5)	0.9
<b>Asthma attacks, past 12 months, n (%)</b>	138 (45.4)	113 (44.8)	0.9
<b>Asthma symptom control, n (%)</b>			<b>0.02</b>
Controlled	132 (48.7)	136 (56.7)	
Partly controlled	94 (34.7)	84 (35.0)	
Uncontrolled	45 (16.6)	20 (8.30)	
<b>Asthma symptom score, n (%)</b>			0.7
0	29 (9.60)	26 (10.4)	
1	65 (21.6)	60 (24.1)	
≥2	207 (68.8)	163 (65.5)	
<b>ICS use, past 12 months, n (%)</b>	156 (52.2)	112 (44.8)	0.09
<b>FEV<sub>1</sub>% predicted, Mean ± SD</b>	94.1 ± 20.8	96.6 ± 16.4	0.1
<b>Methacholine test*, PD20 ≤ 4 mg, n (%)</b>	85 (63.9)	121 (76.1)	<b>0.02</b>
<b>SPT+, n (%)</b>	190 (79.2)	192 (83.1)	0.3
<b>Eosinophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	215 (133;328)	211 (140;310)	0.8
<b>Neutrophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	3947 (3000;4918)	3766 (3040;4800)	0.07

Abbreviations: ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV in 1 second from baseline; \*Methacholine challenge test was performed if baseline FEV<sub>1</sub> ≥ 80% predicted; SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; GM, geometric mean; (Q1;Q3), first and third quartiles.

#### *Participants' characteristics*

At EGEA2, the mean age of the selected 252 adults with current asthma was 39 years, half of them were women, and 50% were non-smokers. Almost half of them reported asthma attacks in the past year and 57% were classified as controlled according to GINA 2015. More than 80% had an allergic sensitization. At EGEA3, 26% of them had a worsening of asthma symptom between EGEA2 and EGEA3 (Table 9).

**Table 9.** Characteristics of the 252 adults with current asthma at the second survey (EGEA2)

	<b>At EGEA2</b>	<b>At EGEA3</b>
<b>Age, years, Mean ± SD</b>	38.5 ± 15.5	45.4 ± 15.6
<b>Women, n (%)</b>	129 (51.2)	129 (51.2)
<b>Body mass index (BMI), n (%)</b>		
<20 kg/m <sup>2</sup>	36 (14.5)	17 (8.70)
[20 – 25[ kg/m <sup>2</sup>	127 (51.2)	99 (50.8)
[25 – 30[ kg/m <sup>2</sup>	60 (24.2)	52 (26.7)
≥30 kg/m <sup>2</sup>	25 (10.1)	27 (13.8)
<b>Smoking, n (%)</b>		
Non-smokers	126 (50.0)	115 (46.0)
Ex-smokers	63 (25.0)	85 (34.0)
Smokers	63 (25.0)	50 (20.0)
<b>Age at asthma onset, n (%)</b>		
[0-4] years	77 (32.1)	77 (32.1)
]4-16] years	79 (32.9)	79 (32.9)
>16 years	84 (35.0)	84 (35.0)
<b>Exacerbations, past 12 months, n (%)</b>	38 (16.5)	NA
<b>Asthma attacks, past 12 months, n (%)</b>	113 (44.8)	100 (43.9)
<b>Asthma symptom control, n (%)</b>		
Controlled	136 (56.7)	84 (53.2)
Partly controlled	84 (35.0)	60 (38.0)
Uncontrolled	20 (8.30)	14 (8.90)
<b>Asthma symptom score (Pekkanen), n (%)</b>		
0	26 (10.4)	55 (23.7)
1	60 (24.1)	46 (19.8)
≥2	163 (65.5)	131 (56.5)
<b>Asthma symptom evolution (Pekkanen), n (%)</b>		
Better	-	96 (41.9)
Stable	-	74 (32.3)
Worse	-	59 (25.8)
<b>ICS use, past 12 months, n (%)</b>	112 (44.8)	100 (52.4)
<b>FEV<sub>1</sub> % predicted, Mean ± SD</b>	96.6 ± 16.4	NA
<b>Methacholine test*, PD20 ≤ 4 mg, n (%)</b>	121 (76.1)	NA
<b>SPT+, n (%)</b>	192 (83.1)	NA
<b>IgE, IU/mL, GM (Q1;Q3)</b>	162 (71.8; 403)	NA
<b>Eosinophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	211 (140; 310)	NA
<b>Neutrophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	3766 (3040; 4800)	NA

Abbreviations: ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, Dose of methacholine causing a 20% fall in FEV<sub>1</sub> in 1 second from baseline; \*Methacholine challenge test was performed if baseline FEV<sub>1</sub> ≥ 80% predicted (n=159). SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; IgE, immunoglobulin E; GM, geometric mean; (Q1;Q3), first and third quartiles; NA, not available; EGEA: epidemiological study on the genetics and environment of asthma.

**3.6.2.2. Association between serum hs-CRP levels and potential confounding variables**

No significant associations were observed between serum hs-CRP levels and hour of blood test (P-value = 0.6), storage time (P-value = 0.5), GFR (P-value = 0.06), and smoking in the 24 hours prior to blood sampling (P-value = 0.6). Hs-CRP levels were positively associated with age. Significantly, higher hs-CRP levels were observed in women, in overweight and obese participants. No associations were observed with smoking status or between those who used ICS in the past 12 months *versus* not (Table 10).

**Table 10.** Associations between serum hs-CRP levels and age, sex, BMI and smoking

	<b>Hs-CRP n; GM (Q1;Q3)*</b>	<b>P-value</b>
<b>Age, (years)</b>	252; 0.13	<b>0.04</b>
<b>Age classes</b>		<b>0.03</b>
[16-25[	76; 0.91 (0.41;1.92)	
[25-35[	52; 1.52 (0.67;2.92)	
[35-45[	34; 1.59 (0.79;2.95)	
[45-55[	44; 1.45 (0.69;2.67)	
≥ 55	46; 1.36 (0.54;3.36)	
<b>Sex</b>		<b>0.003</b>
Women	129; 1.55 (0.72;2.93)	
Men	123; 1.04 (0.47;2.10)	
<b>Body mass index (BMI)</b>		<b>&lt;0.001</b>
<20 kg/m <sup>2</sup>	36; 1.07 (0.41;2.24)	
[20 – 25[ kg/m <sup>2</sup>	127; 0.96 (0.46;1.78)	
[25 – 30[ kg/m <sup>2</sup>	60; 1.73 (0.80;3.06)	
>=30 kg/m <sup>2</sup>	25; 2.99 (1.46;6.73)	
<b>Smoking</b>		0.6
Non-smokers	126; 1.20 (0.53;1.95)	
Ex-smokers	63; 1.41 (0.60;3.04)	
Smokers	63; 1.29 (0.52;2.74)	
<b>ICS use, past 12 months</b>		0.2
No	138;1.19 (0.57;2.24)	
Yes	112;1.41 (0.55;3.11)	

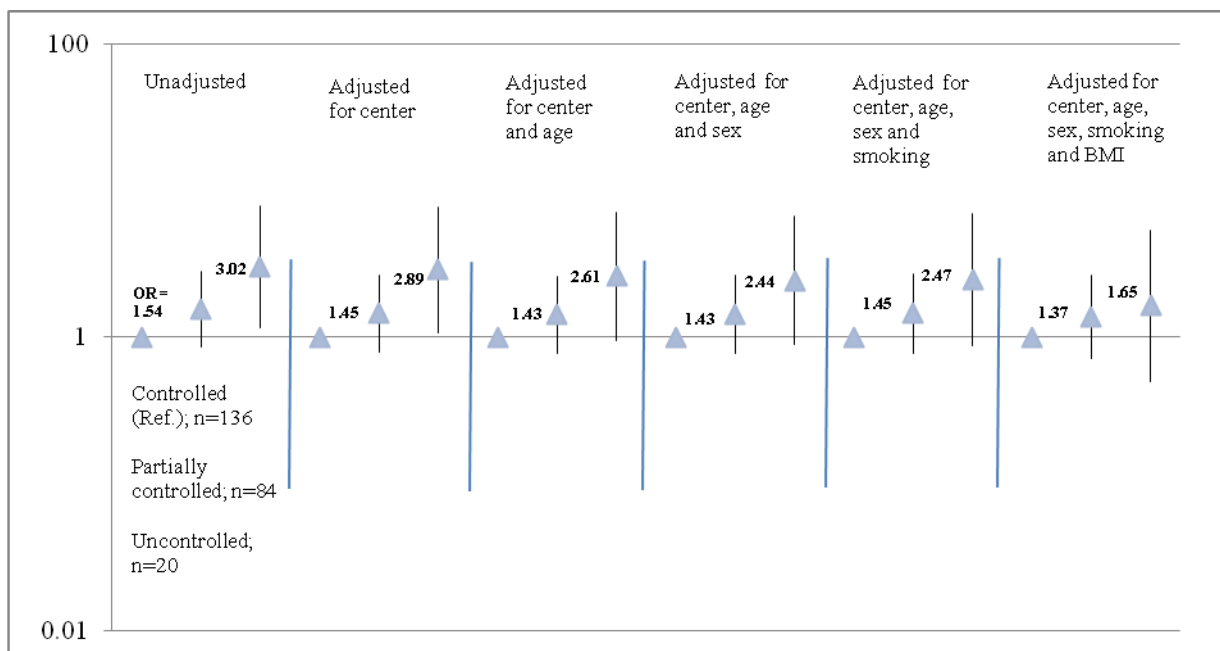
\*All values are expressed as n; GM (Q1; Q3) except for age that is expressed as n; correlation coefficient. **Bolded results** are statistically significant at P-values <0.05.

Abbreviations: GM, geometric mean; (Q1; Q3), first and third quartiles.



### 3.6.2.3. Cross-sectional associations between serum hs-CRP levels and asthma symptom control at EGEA2

Serum hs-CRP levels were significantly and positively associated with uncontrolled *versus* controlled asthma. This association was borderline significant after adjustment for center, age, sex and smoking and non-significant after additional adjustment for BMI (Figure 9).



**Figure 9.** Cross-sectional associations between serum hs-CRP levels and asthma symptom control.

Subjects were classified as controlled, partly controlled and uncontrolled according to asthma symptom control as per GINA 2015.

Abbreviations: BMI, body mass index; OR, odds ratio.

### 3.6.2.4. Cross-sectional associations between serum hs-CRP levels and asthma outcomes at EGEA2

No associations were found between hs-CRP levels and exacerbations or asthma attacks in the past 12 months, asthma symptom score (Pekkanen), FEV1% predicted, allergic sensitization,

eosinophil counts and IgE levels. Hs-CRP levels were positively associated with a higher risk of BHR and higher neutrophil counts (Table 11).

**Table 11.** Cross-sectional associations between serum hs-CRP levels and asthma outcomes (EGEA2)

	<b>Hs-CRP</b>	
	<b>Unadjusted</b>	<b>Adjusted*</b>
	<b>OR (95% CI)</b>	<b>OR (95% CI)</b>
<b>Exacerbations</b> (past 12 months)	1.52 (0.78;2.95)	1.12 (0.52;2.41)
<b>Asthma attacks</b> (past 12 months)	1.32 (0.77;2.25)	1.23 (0.67;2.26)
<b>Asthma symptom score (Pekkanen)</b>		
1 <i>versus</i> 0	1.76 (0.62;4.96)	1.29 (0.37;4.55)
$\geq 2$ <i>versus</i> 0	1.75 (0.68;4.51)	1.08 (0.34;3.41)
<b>Methacholine test, PD20 <math>\leq</math> 4 mg</b>	<b>2.53 (1.13;5.70)</b>	<b>3.80 (1.39;10.4)</b>
<b>SPT+</b>	0.87 (0.42;1.77)	1.35 (0.55;3.32)
	<b><math>\beta</math> (95% CI)</b>	<b><math>\beta</math> (95% CI)</b>
<b>FEV<sub>1</sub> % predicted</b>	-0.02 (-0.06;0.03)	-0.01 (-0.06;0.04)
<b>Neutrophils, cells/mm<sup>3</sup></b>	<b>0.06 (0.02;0.09)</b>	<b>0.04 (0.001;0.08)</b>
<b>Eosinophils, cells/mm<sup>3</sup></b>	0.03 (-0.05;0.11)	0.04 (-0.05;0.13)
<b>IgE, IU/ml</b>	0.03 (-0.14;0.19)	0.14 (-0.04;0.32)

\*OR or  $\beta$  (95% CI) were adjusted for age, sex, smoking, BMI and center; GEE regression methods were performed, except for asthma symptom score where multinomial logistic regressions were applied. **Bolded results** are statistically significant at P-values < 0.05.

Abbreviations: FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV in 1 second from baseline; SPT+, a mean wheal diameter  $\geq$ 3mm than the negative control for at least one of 12 aeroallergens; hs-CRP, high sensitivity C-reactive protein; IgE, immunoglobulin E; OR: odds ratio; CI: confidence Interval.

### 3.6.2.5. Longitudinal associations between serum hs-CRP levels and asthma outcomes at EGEA3

Serum hs-CRP levels at EGEA2 were unrelated to asthma attacks in the past 12 months, asthma symptom control or asthma symptom score (Pekkanen) at EGEA3 and change in asthma symptom score (Pekkanen) between EGEA2 and EGEA3 (Table 12).

**Table 12.** Longitudinal associations between serum hs-CRP levels and asthma outcomes (EGEA3)

	<b>Unadjusted</b> <i>OR (95% CI)</i>	<b>Adjusted*</b> <i>OR (95% CI)</i>
<b>Asthma attacks</b> (past 12 months)	1.12 (0.65;1.93)	0.98 (0.49;1.97)
<b>Asthma symptom control</b>		
Partially controlled <i>versus</i> controlled	1.44 (0.67;3.11)	1.20 (0.51;2.84)
Uncontrolled <i>versus</i> controlled	2.09 (0.58;7.48)	1.04 (0.21;5.03)
<b>Asthma symptom score (Pekkanen)</b>		
1 <i>versus</i> 0	0.62 (0.25;1.51)	0.61 (0.23;1.64)
$\geq 2$ <i>versus</i> 0	1.07 (0.54;2.12)	0.73 (0.34;1.57)
<b>Change in the asthma symptom score between EGEA2 and EGEA3,</b>		
Better/stable <i>versus</i> worse	0.85 (0.47;1.54)	1.29 (0.62;2.70)

\*OR (95% CI) were adjusted for age, sex, smoking status, BMI and center; GEE regression methods were performed, except for asthma symptom control and asthma symptom score where multinomial logistic regressions were applied.

Abbreviations: EGEA, epidemiological study on the genetics and environment of asthma; OR, odds ratio; CI, confidence interval.

### 3.6.2.6. Sensitivity analyses

The association between hs-CRP levels at EGEA2 and neutrophil counts remained significant after further adjustment for respiratory infections in the past four weeks [ $\beta$  (95%CI): 0.04 (0.001; 0.08), P-value=0.045].

## 3.6.3. Discussion

### 3.6.3.1. Summary of findings

In the cross-sectional analyses, serum hs-CRP levels were found to be significantly associated with uncontrolled asthma, an association that became borderline significant after adjustment for age, sex, smoking and center and non-significant after additional adjustment for BMI.

Moreover, serum hs-CRP levels were positively associated with blood neutrophil counts and a higher risk of BHR at baseline. No associations were found with asthma attacks or exacerbations in the past 12 months, with asthma symptom score, lung function, allergic sensitization, eosinophil counts and IgE levels at baseline. No associations were found with asthma attacks, asthma symptom control, asthma symptom score (Pekkanen), and change of asthma symptom score (Pekkanen) between EGEA2 and EGEA3 seven years later.

### **3.6.3.2. Comparison with other studies and interpretation**

A positive correlation was found between CRP and age and is confirmed by previous literature (101,210). The relation between CRP and sex is less clear. Women had higher CRP levels in line with a previous study (211); however, another did not find a relation between CRP and sex (101). Moreover, positive associations between smoking and CRP levels were previously reported (101,212) in contrast with our results, yet Wood *et al.* in 2012 stated that there is no association between hs-CRP levels and smoking (115). The positive association with overweight or obesity is well established (213–215). Since adipose tissue has an active role in CRP metabolism (216), this association was expected and has been described several times.

Even though the association did not reach the level of significance, serum hs-CRP levels were increased in asthma patients who were receiving ICS therapy compared to those who were not. Previously, steroid untreated asthma patients were reported to have significantly higher levels of CRP compared to the treated ones (117,217). The difference in findings might be due to the fact that in previous studies, asthma patients included in the analyses used ICS medication currently, whereas the definition of ICS treatment in the EGEA2 study included treatment over the past 12 months. Hence, the group of asthma patients receiving ICS therapy might include subjects with former treatment. They might increase the mean CRP values in this group since the anti-inflammatory properties of ICS are limited to the time of their intake. Furthermore, we can state that our study population is larger (n=252) compared to the study by Takemura *et al.* (n=59) or Hoshino *et al.* (n=99) which might be the reason for the different findings. Further, Takemura *et al.* excluded smoker subjects and those with CRP levels > 10 mg/L which might be severe cases needing asthma treatment. The exclusion of

these subjects might have led to an underestimation of mean CRP levels in the asthma treated group.

In the cross-sectional analyses, hs-CRP levels were significantly higher in patients having uncontrolled asthma. This association was borderline significant after adjustment for the potential confounding variables age, sex, smoking and center and non-significant after additional adjustment for BMI. According to the previous literature, CRP levels increase with increasing BMI and BMI may confound the association between CRP and asthma (218). Thus, BMI might be a confounding factor for the association between hs-CRP and asthma control. To our knowledge, there are three previous studies which reported conflicting results in the association of hs-CRP levels and asthma control assessed by univariate analyses. Some have reported no association with asthma control assessed through GINA, ACT or asthma control questionnaire (115,165), and others have reported significant negative correlation with asthma control assessed through ACT (166). Since the definition of asthma control per ACT includes four symptoms/reliever questions plus a patient's self-assessed level of control in the past 4 weeks (219), while the definition per asthma symptom control includes daytime symptoms, night waking, and need for reliever and activity limitation in the past 4 weeks as well (43), we can suppose that assessing asthma control by using asthma symptom control is more objective and may lead to more accurate results.

Moreover, hs-CRP levels were unrelated to allergic sensitization which is consistent with previous literature (101,166). No associations were found with IgE levels. Previous reports showed absence or weak positive correlation with IgE levels (101,220). Positive associations with bronchial hyperresponsiveness were observed in contrast with previous literature (101,221). Our results might be more accurate since we explored this association using multivariate analysis while previous studies have used univariate analyses. No association with eosinophil counts was found. Previously, no associations were reported with blood eosinophil counts (220), but positive correlations were observed with sputum eosinophils in 45 asthma patients assessed through multivariate analysis (221) or in 99 asthma patients assessed through univariate analysis (217). Our study population included more subjects (n=252) and blood eosinophils were measured instead of sputum eosinophils.

Furthermore, positive associations with neutrophil counts were found, which is concordant with previous results reporting this association in sputum neutrophils (115,221). No

association was found with FEV<sub>1</sub>%. There is no clear evidence regarding the association with lung function parameters. Some have reported a negative correlation in the univariate analysis for 45 patients with mild asthma (221), or in the multivariate analysis for 99 asthma patients. Others have reported negative significant associations in asthma patients who were not receiving ICS treatment and no associations in asthma patients who were receiving ICS treatment (117). However, other studies have reported no associations with FEV<sub>1</sub>% whether in the univariate or multivariate analyses (115,118,165,166). No associations were found between hs-CRP levels and asthma attacks, exacerbations or asthma symptom score. As far as we know, these associations were not previously explored in the literature.

In the longitudinal analyses, no associations were found with asthma attacks, asthma symptom control, and asthma symptom score or change of asthma symptom score between EGEA2 and EGEA3 seven years later. These associations were not previously investigated in a prospective design, thus comparisons were not possible.

#### ***3.6.3.3. Strengths and limitations***

Most participants with asthma included in this study were recruited in chest clinics as asthma cases, with a careful procedure set up to include true asthmatics. Some were also recruited as relatives of asthma cases, based on answers to questions on asthma diagnosis. This leads to a group of asthma patients with a wide range of severity and response to methacholine. Asthma symptom control was defined according to GINA 2015. No correlations were found between hs-CRP levels measured in the EGEA2 study and variables like storage time and hour of blood test which is consistent with a high quality of the EGEA data collection. However, the selection of the participants with asthma included in the present analyses was driven first by an adult status with current asthma and the availability of hs-CRP measurements at the EGEA2 survey, and secondly by a follow-up at the EGEA3 survey.

#### ***3.6.3.4. Conclusion***

In conclusion, no correlation between the degrees of systemic inflammation estimated by hs-CRP levels and asthma symptom control classified as per GINA 2015 was observed since BMI might be a confounding factor in this association.

Compared to the study performed in the first part of the thesis in Lebanon where no associations were found between CRP and asthma, hs-CRP levels from the EGEA study were shown to be significantly increased in asthma patients compared to controls (preliminary analyses performed before excluding the non-asthmatics from the database). Thus, hs-CRP tests are more useful to study the associations with asthma and asthma outcomes than the ordinary CRP test.

*It was hypothesized that hs-CRP levels are associated with asthma control but this association was not found nor any other innovative result. Thus, we decided not to write an article in regard to this part of the thesis.*

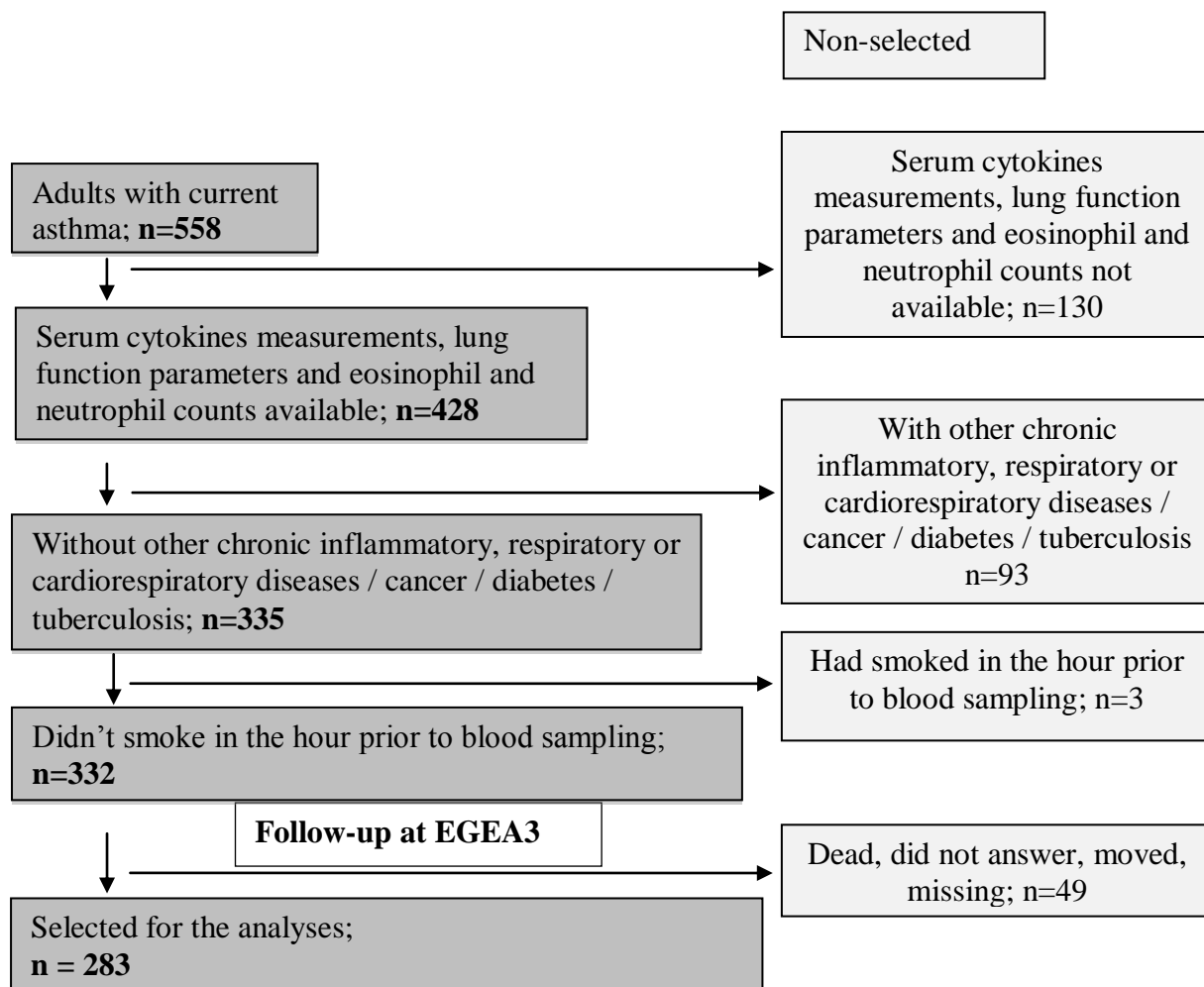
### **3.7. Second analysis: cross-sectional and longitudinal associations between cytokine profiles and current asthma**

This analysis is devoted to assess the cross-sectional and longitudinal associations between serum cytokine profiles and asthma outcomes using data from the EGEA study.

#### **3.7.1. Materials and methods**

##### ***3.7.1.1. Participants' selection***

Among the 558 participants with current asthma who were adult at EGEA2 ( $\geq 16$  years old), the present analysis included 283 participants with available data for serum cytokines measurements, lung function parameters and eosinophil and neutrophil counts and who were followed-up at EGEA3, excluding those who reported respiratory, cardiorespiratory diseases, cancer, diabetes, other chronic inflammatory diseases, or tuberculosis (n=93) or who had smoked one hour prior to blood sampling (n=3) (Figure 10).



**Figure 10.** Flow chart of the participants – data on cytokines and current asthma  
Abbreviations: EGEA, epidemiological study on the genetics and environment of asthma.

### 3.7.1.2. Cytokine measurements

Determination of serum cytokine levels was carried out at the IMIM (*Instituto Hospital del Mar de Investigaciones Médicas*) in Barcelona, Spain in July 2013. IL-1Ra, IL-5, IL-7, IL-8, IL-10, IL-13 and TNF- $\alpha$  levels were tested using the Luminex xMAG<sup>®</sup> technique in a BioPlex system (Bio-Rad, Hercules, California, United States). No value above or below the limit of detection (LD) was observed for IL-7 and IL-13 measurements. Less than 3% of the values were above or below the LD for other cytokines. Values below the lower limit of detection (LLD) were assigned the LLD/2 and values above the upper limit of detection (ULD) were assigned the ULD+1. An aliquot of the same human serum pool was run by duplicate in each plate. In order to minimize inter-plate variability, each serum cytokine value was divided by



the pool result obtained from the same plate and expressed as ratio. These ratios were included in the statistical analyses.

### **3.7.1.3. Statistical analyses**

The Pearson correlation coefficient was used to estimate correlations between cytokine levels. To reduce the dimension of the cytokines data set (n=7) to a few uncorrelated components that account for a meaningful amount of the variance contained in the original set of variables, principal component analysis (PCA) was performed by using the PRINCOMP procedure in the SAS software.

PCA does not rely on a prior knowledge of the relationship among the variables of interest. It identifies uncorrelated linear combinations of observed variables, called principal components (PC), which explain the greatest degree of variance among a set of observed variables, within a specified study population (222). The PCs are organized in order of descending independent contribution to discriminate the variance in the data. Each PC produced from PCA corresponds to a different linear combination of the measured quantities. The Kaiser criterion is used for significant PC inclusion, thus only PCs that have eigenvalues greater than 1 will be retained (223). These PC can be used simultaneously in statistical analyses, and may be thought of as independent constructs represented by the markers that mainly explain that component. Interpretability of the final solution is a major determinant of the minimum number of components to be used in the analyses. In this analysis, no rotations were made on the data. Components were used to calculate PC scores for each subject. PC1 and PC2 scores obtained were expressed as above/below the median (high/low PC score) as a result of their skewed distribution, and for clinical meaningfulness.

To rigorously confirm the partitioning of the data set, the VARCLUS procedure was also performed (<http://www.math.wpi.edu/saspdf/stat/chap68.pdf>). This procedure is closely related to PCA and is used as an alternative method for eliminating redundant dimensions. It finds clusters of variables that are as correlated as possible among themselves and as uncorrelated as possible with variables in other clusters.

Standard statistical tests (chi<sup>2</sup> or Fisher exact test when appropriate, and student T-test or ANOVA) were performed to test the univariate associations between the PC scores and the

socio-demographic characteristics. Then and except for polytomous logistic regression, multivariate analyses were conducted using GEE regression methods to take into account the familial aggregation of the data (GENMOD and MIXED procedures in SAS). PC1 and PC2 scores were studied in association with asthma outcomes, lung function, allergic sensitization, IgE levels and blood cell counts at EGEA2 (cross sectional analyses), and with asthma attacks, asthma symptom control, asthma symptom score (Pekkanen) at EGEA3 and evolution of asthma symptom score (Pekkanen) between EGEA2 and EGEA3 (longitudinal analyses). Age (continuous), sex, smoking status (never, ex-, current smokers), BMI status (4 classes), and center at EGEA2 were included as covariates. Associations between individual cytokines and asthma outcomes at EGEA3 were as well investigated.

As sensitivity analyses, the association between PC1 and neutrophil counts was further adjusted on respiratory infection, which may affect blood neutrophil counts. As use of ICS in the past 12 months could reflect asthma severity and therefore lead to an issue of over-adjustment, we further adjusted for ICS in the past 12 months in the association between PC2 at EGEA2 and subsequent risk of asthma control at EGEA3 after main analyses. As lung function and the level of asthma symptom control at EGEA2 may also reflect asthma severity, we further adjusted for FEV<sub>1</sub> and asthma control at EGEA2 in the association between PC2 at EGEA2 and subsequent risk of asthma control at EGEA3. Finally, both PC1 and PC2 were used in the same model.

P-values < 0.05 were considered to indicate a statistically significant difference. Statistical analyses were done using SPSS software version 20.0 then repeated using the SAS software, version 9.3 (SAS Institute, Inc., Cary, North Carolina, USA). Similar results were obtained and the results presented are those of SAS software.

## **3.7.2. Results**

### ***3.7.2.1. Study participants***

#### *Participants selected for the analyses versus those non-selected*

Participants selected for the analysis were younger, more often non-smokers, had lower BMI and a better lung function than those not selected (n=145). The two groups were similar for sex, allergic sensitization, asthma-related phenotypes and blood cell counts (Table 13).

**Table 13.** Comparison of main characteristics between non-selected and selected participants

	<b>Non-selected N=145</b>	<b>Selected N=283</b>	<b>P-value</b>
<b>Age, years, Mean ± SD</b>	44.5 ± 17.7	36.6 ± 15.4	<b>&lt;0.001</b>
<b>Women, n (%)</b>	62 (42.8)	140 (49.5)	0.2
<b>Body mass index (BMI), n (%)</b>			<b>&lt;0.001</b>
<20 kg/m <sup>2</sup>	13 (9.00)	40 (14.1)	
[20 – 25[ kg/m <sup>2</sup>	62 (42.8)	157 (55.5)	
[25 – 30[ kg/m <sup>2</sup>	43 (29.7)	66 (23.3)	
>=30 kg/m <sup>2</sup>	27 (18.6)	20 (7.10)	
<b>Smoking, n (%)</b>			<b>0.02</b>
Non-smokers	63 (43.4)	151 (53.4)	
Ex-smokers	48 (33.1)	60 (21.2)	
Smokers	34 (23.4)	72 (25.4)	
<b>Exacerbations, past 12 months, n (%)</b>	23 (17.6)	47 (18.0)	0.9
<b>Asthma attacks, past 12 months, n (%)</b>	5 (44.8)	132 (46.8)	0.7
<b>Asthma symptom control, n (%)</b>			0.08
Controlled	68 (50.4)	150 (54.9)	
Partly controlled	43 (31.9)	96 (35.2)	
Uncontrolled	24 (17.8)	27 (9.90)	
<b>Asthma symptom score (Pekkanen), n (%)</b>			0.8
0	13 (9.00)	31 (11.0)	
1	30 (20.7)	60 (21.2)	
≥2	102 (70.3)	192 (67.8)	
<b>ICS use, past 12 months, n (%)</b>	78 (54.2)	142 (50.7)	0.5
<b>FEV<sub>1</sub> % predicted, Mean ± SD</b>	91.1±22.8	97.0±16.8	<b>0.007</b>
<b>Methacholine test*, PD20 ≤ 4 mg, n (%)</b>	41 (63.1)	138 (73.0)	0.1
<b>SPT+, n (%)</b>	110 (79.7)	228 (84.1)	0.3
<b>Eosinophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	211 (130;305)	215 (140;320)	0.6
<b>Neutrophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	3999 (2965;4890)	3745 (3000;4690)	0.05

Abbreviations: ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV in 1 second from baseline; \*Methacholine challenge test was performed if baseline FEV<sub>1</sub> ≥ 80% predicted; SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; GM, geometric mean; (Q1;Q3): first and third quartiles.

#### *Participants' characteristics*

At EGEA2, the mean age of the selected 283 adults with current asthma was 37 years, half of them were women, and 53% were non-smokers. Almost half of them reported asthma attacks in the past year, and 55% were classified as having controlled asthma, according to GINA 2015. More than 80% had an allergic sensitization. At EGEA3, 50% of them were classified as having controlled asthma and 27% of them had a worsening of asthma symptoms, as compared to EGEA2 (Table 14).

**Table 14.** Characteristics of the 283 adults with current asthma at the second survey (EGEA2)

	<b>At EGEA2</b>	<b>At EGEA3</b>
<b>Age, year, Mean ± SD</b>	36.6 ± 15.4	43.5 ± 15.5
<b>Sex, women, n (%)</b>	140 (49.5)	140 (49.5)
<b>Body mass index (BMI), n (%)</b>		
<20 kg/m <sup>2</sup>	40 (14.1)	26 (11.7)
[20 – 25[ kg/m <sup>2</sup>	157 (55.5)	114 (51.1)
[25 – 30[ kg/m <sup>2</sup>	66 (23.3)	54 (24.2)
≥30 kg/m <sup>2</sup>	20 (7.10)	29 (13.0)
<b>Smoking, n (%)</b>		
Non-smokers	151 (53.4)	136 (48.6)
Ex-smokers	60 (21.2)	86 (30.7)
Smokers	72 (25.4)	58 (20.7)
<b>Age at asthma onset, n (%)</b>		
[0-4] years	93 (34.3)	93 (34.3)
]4-16] years	99 (36.5)	99 (36.5)
>16 years	79 (29.2)	79 (29.2)
<b>Exacerbations, past 12 months, n (%)</b>	47 (18.0)	NA
<b>Asthma attacks, past 12 months, n (%)</b>	132 (46.8)	128 (49.0)
<b>Asthma symptom control, n (%)</b>		
Controlled	150 (54.9)	94 (49.7)
Partly controlled	96 (35.2)	73 (38.7)
Uncontrolled	27 (9.90)	22 (11.6)
<b>Asthma symptom score (Pekkanen), n (%)</b>		
0	31 (11.0)	59 (22.6)
1	60 (21.2)	51 (19.5)
≥2	192 (67.8)	151 (57.9)
<b>Asthma symptom evolution (Pekkanen), n (%)</b>		
Better	-	105 (40.2)
Stable	-	85 (32.6)
Worse	-	71 (27.2)
<b>ICS use, past 12 months, n (%)</b>	142 (50.7)	125 (56.1)
<b>FEV<sub>1</sub> % predicted, Mean ± SD</b>	97.0 ± 16.8	NA
<b>Methacholine test*, PD20 ≤ 4 mg, n (%)</b>	138 (73.0)	NA
<b>SPT+, n (%)</b>	228 (84.1)	NA
<b>IgE, IU/mL, GM (Q1;Q3)</b>	157 (69.2; 396)	NA
<b>Eosinophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	215 (140; 320)	NA
<b>Neutrophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	3745 (3000; 4690)	NA

Abbreviations: ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV<sub>1</sub> in 1 second from baseline; \*Methacholine challenge test was performed if baseline FEV<sub>1</sub> ≥ 80% predicted (n=189). SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; IgE, immunoglobulin E; GM, geometric mean; (Q1;Q3), first and third quartiles; NA, not available; EGEA, epidemiological study on the genetics and environment of asthma.

### 3.7.2.2. Profiles of serum cytokines

The mean, minimum and maximum serum levels of the seven studied cytokines, along with their ratio values in order to minimize the inter-plate variability are summarized in Table 15.

**Table 15.** Serum levels of cytokines

<b>Adults with current asthma, N=283</b>			
	<b>Mean ± SD</b>	<b>Minimum</b>	<b>Maximum</b>
<b>IL-1Ra (pg / mL)</b>	19801 ± 29281	58.9	176381
<b>Ratio IL-1Ra /pool</b>	0.36 ± 0.69	0.002	6.99
<b>IL-5 (pg / mL)</b>	69.1 ± 57.2	4.92	403.6
<b>Ratio IL-5/pool</b>	0.42 ± 0.37	0.03	3.94
<b>IL-7 (pg / mL)</b>	245 ± 170	40.0	1168
<b>Ratio IL-7/pool</b>	0.58 ± 0.37	0.11	2.09
<b>IL-8 (pg / mL)</b>	620 ± 523	10.9	3283
<b>Ratio IL-8/pool</b>	0.40 ± 0.32	0.007	2.00
<b>IL-10 (pg / mL)</b>	167 ± 265	9.67	3291
<b>Ratio IL-10/pool</b>	0.46 ± 0.77	0.03	8.98
<b>IL-13 (pg / mL)</b>	275 ± 221	22.0	1410
<b>Ratio IL-13/pool</b>	0.49 ± 0.37	0.04	2.35
<b>TNF-α (pg / mL)</b>	613 ± 567	11.4	3552
<b>Ratio TNF-α/pool</b>	0.37 ± 0.33	0.01	2.17

Abbreviations: IL, interleukin; TNF-α, tumor necrosis factor-alpha; SD, standard deviation.

As shown in Table 16a, IL-1Ra was more strongly correlated with IL-10 ( $r=0.61$ ) than with other cytokines ( $r=0.29-0.50$ ), whereas IL-5, IL-7, IL-8, IL-13 and TNF-α were highly correlated together ( $r=0.65-0.90$ ). Correlations were similar using raw cytokine levels (Table 16.b).

**Table 16.** Pearson correlation coefficients for each pairwise correlation of cytokines.

**a.** By taking the variability into account

<b>IL-1Ra/pool</b>						
0.29	<b>IL-5/pool</b>					
0.42	0.76	<b>IL-7/pool</b>				
0.34	0.76	0.89	<b>IL-8/pool</b>			
0.61	0.29	0.44	0.32	<b>IL-10/pool</b>		
0.38	0.72	0.90	0.77	0.42	<b>IL-13/pool</b>	
0.50	0.65	0.79	0.75	0.55	0.71	<b>TNF-<math>\alpha</math>/pool</b>

All P-values are  $< 0.001$ .

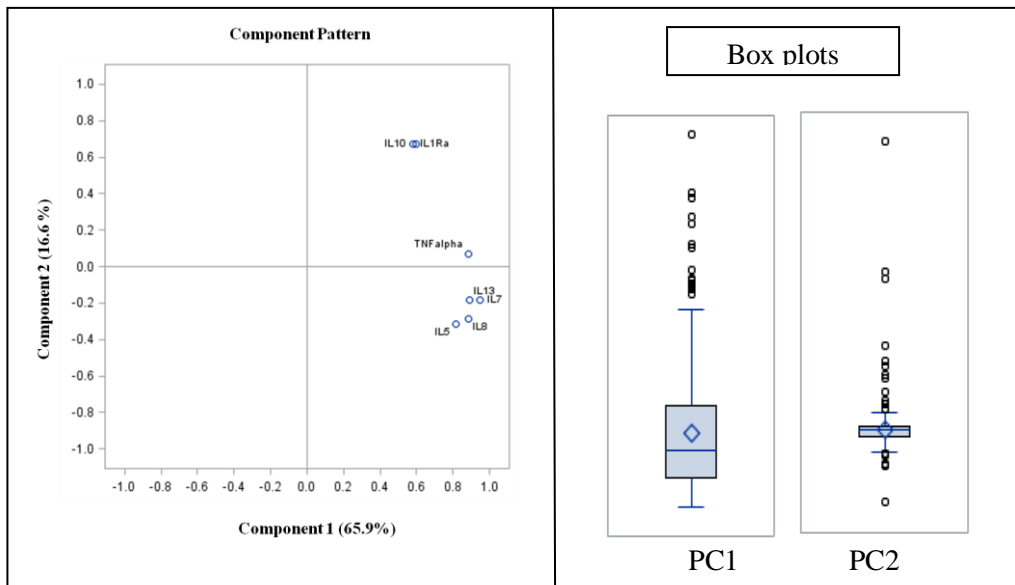
**b.** By taking raw cytokine levels into account

<b>IL-1Ra</b>						
0.51	<b>IL-5</b>					
0.59	0.86	<b>IL-7</b>				
0.57	0.86	0.93	<b>IL-8</b>			
0.63	0.38	0.46	0.40	<b>IL-10</b>		
0.53	0.80	0.90	0.81	0.44	<b>IL-13</b>	
0.60	0.75	0.80	0.78	0.53	0.72	<b>TNF-<math>\alpha</math></b>

All P-values are  $< 0.001$ .

Abbreviations: IL, interleukin; TNF- $\alpha$ , tumor necrosis factor-alpha.

Using PCA, we retained the first two PCs, which accounted for 82.5% of the variability. The correlations between cytokines and PC are presented in Figure 11 and Table 17. PC1 was mainly explained by the inflammatory cytokines IL-5, IL-7, IL-8, IL-13 and TNF- $\alpha$  (0.81 to 0.95), while PC2 was mainly explained by the anti-inflammatory cytokines IL-1Ra and IL-10 (0.67). Participants with high scores on one PC have therefore high levels for the cytokines that mainly explain the PC. The dendrogram obtained with the VARCLUS procedure confirmed the partition in two clusters: cluster 1 {IL-5, IL-7, IL-8, IL-13 and TNF- $\alpha$ } and cluster 2 {IL-1Ra and IL-10} (Figure 12).



**Figure 11.** Graphical representation of the scores in the space and box plots of the two principal components.

The first principal component (PC1) included IL-5, IL-7, IL-8, IL-13, and TNF- $\alpha$ . The second principal component (PC2) included IL-1Ra and IL-10. PC1 and PC2 have skewed distributions.

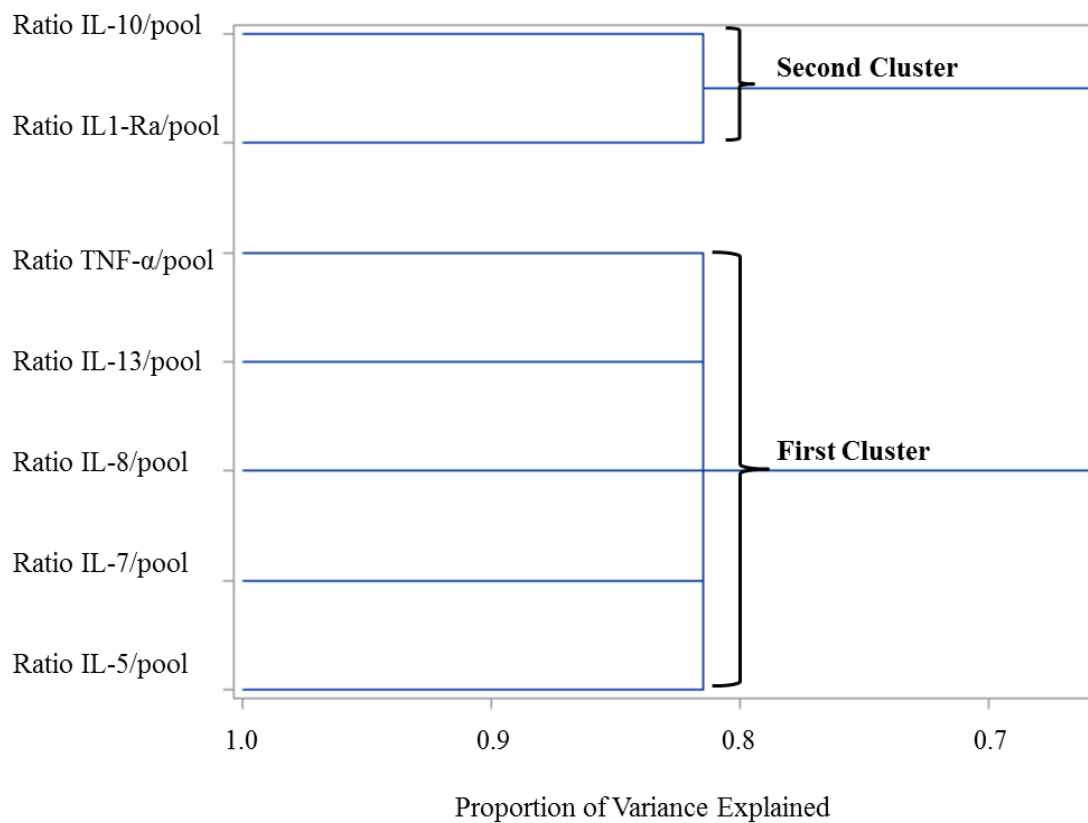
Abbreviations: IL, interleukin; TNF- $\alpha$ , tumor necrosis factor-alpha.

**Table 17.** Contribution of biological markers to each principal component

Marker	PC1	PC2
Ratio IL-7/ pool	<b>0.95</b>	-0.18
Ratio IL-13/ pool	<b>0.90</b>	-0.18
Ratio IL-8/ pool	<b>0.89</b>	-0.29
Ratio TNF- $\alpha$ / pool	<b>0.88</b>	0.07
Ratio IL-5/ pool	<b>0.81</b>	-0.32
Ratio IL-1Ra/ pool	0.58	<b>0.67</b>
Ratio IL-10/ pool	0.60	<b>0.67</b>

Abbreviations: IL, interleukin; TNF- $\alpha$ , tumor necrosis factor-alpha





**Figure 12:** Dendrogram of the seven cytokines obtained by using proc VARCLUS. Abbreviations: IL, interleukin; TNF- $\alpha$ , tumor necrosis factor-alpha.

At EGEA2, a higher PC2 score was associated with overweight or obesity. No association was observed between PC1 and BMI. No other significant associations of PC1 or PC2 scores with age, sex, or smoking were observed (Table 18).

**Table 18.** Associations between PC scores and age, sex, BMI and smoking

	PC1 above the median	P-value	PC2 above the median	P-value
<b>Age</b> , years, Mean $\pm$ SD	36.7 $\pm$ 16.1	0.9	35.5 $\pm$ 14.4	0.2
<b>Women</b> , n(%)	65 (45.8)	0.2	74 (52.1)	0.4
<b>Body Mass Index (BMI)</b> , n (%)		0.1		<b>0.004</b>
<20 kg/m <sup>2</sup>	24 (16.9)		17 (12.0)	
[20 – 25[ kg/m <sup>2</sup>	83 (58.5)		69 (48.6)	
[25 – 30[ kg/m <sup>2</sup>	28 (19.7)		40 (28.2)	
$\geq$ 30 kg/m <sup>2</sup>	7 (4.90)		16 (11.3)	
<b>Smoking</b> , n(%)		0.6		0.7
Non-smokers	73 (51.4)		77 (54.2)	
Ex-smokers	29 (20.4)		32 (22.5)	
Smokers	40 (28.2)		33 (23.2)	

Abbreviations: PC, principal component; SD, standard deviation.

### 3.7.2.3. *Cross-sectional associations between PC scores and asthma outcomes at EGEA2*

Both PC1 and PC2 scores were unrelated to exacerbations, asthma attacks, asthma symptom control, asthma symptom score (Pekkanen), lung function, and allergic sensitization. Participants with higher PC2 score had a lower risk of BHR (Table 19).

**Table 19.** Cross-sectional associations between cytokines PC scores and asthma outcomes (EGEA2)

	PC1 score above the median		PC2 score above the median	
	Unadjusted <i>OR (95% CI)</i>	Adjusted* <i>OR (95% CI)</i>	Unadjusted <i>OR (95% CI)</i>	Adjusted* <i>OR (95% CI)</i>
<b>Exacerbations,</b> (past 12 months)	0.79 (0.42;1.49)	0.72 (0.33;1.53)	0.68 (0.36;1.28)	0.65 (0.29;1.47)
<b>Asthma attacks,</b> (past 12 months)	1.17 (0.73;1.85)	1.09 (0.65;1.82)	0.74 (0.46;1.18)	0.68 (0.40;1.16)
<b>Asthma symptom control, (controlled reference)</b>				
Partially controlled	1.26 (0.76;2.11)	1.23 (0.69;2.19)	0.65 (0.39;1.08)	0.67 (0.37;1.20)
Uncontrolled	0.89 (0.39;2.03)	0.86 (0.32;2.32)	0.89 (0.39;2.03)	0.79 (0.29;2.19)
<b>Asthma symptom score (Pekkanen)</b>				
1 <i>versus</i> 0	1.30 (0.55;3.11)	1.14 (0.43;3.00)	1.70 (0.71;4.07)	1.94 (0.74;5.11)
≥ 2 <i>versus</i> 0	1.02 (0.48;2.19)	0.95 (0.41-2.21)	1.14 (0.53;2.44)	0.99 (0.43;2.30)
<b>Methacholine test,</b> <b>PD20≤4mg (N=189)</b>	1.13 (0.61;2.11)	0.90 (0.43;1.89)	<b>0.45 (0.24;0.84)</b>	<b>0.46 (0.23;0.91)</b>
<b>SPT+</b>	0.95 (0.51;1.79)	0.82 (0.38;1.78)	0.82 (0.42;1.60)	0.68 (0.30;1.58)
	<i>β (95% CI)</i>	<i>β (95% CI)</i>	<i>β (95% CI)</i>	<i>β (95% CI)</i>
<b>FEV<sub>1</sub> % predicted</b>	2.09 (-1.77;5.95)	1.75 (-2.27;5.77)	-1.34 (-5.20;2.53)	-0.41(-4.46;3.63)

\*OR /  $\beta$  (95% CI) were adjusted for age, sex, smoking status, BMI and center; GEE regression methods were performed, except for asthma symptom control and asthma symptom score where polytomous logistic regressions were applied. **Bolded results** are statistically significant at P-values < 0.05.

Abbreviations: FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV in 1 second from baseline; SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; PC, principal component; OR, odds ratio; CI, confidence interval.

Participants with higher PC1 score had significantly higher neutrophil counts, an association that remained significant after adjustment for respiratory infections in the past four weeks (P-value = 0.02). Participants with higher PC2 score had significantly lower total IgE levels, and lower neutrophil counts, an association of borderline significance. No associations were found between PC1 or PC2 and eosinophil counts (all P-values>0.2, Table 20).

**Table 20.** Cross-sectional associations between cytokines PC scores and neutrophil and eosinophil counts and IgE levels (at EGEA2)

	PC1 score		PC2 score	
	Unadjusted	Adjusted*	Unadjusted	Adjusted*
<b>Neutrophils, cells/mm<sup>3</sup></b>				
Below the median	3564 (3376-3764)	3590 (3357-3839)	3899 (3691-4120)	3906 (3637-4195)
Above the median	3937 (3728-4158)	3958 (3700-4234)	3602 (3410-3804)	3668 (3438-3912)
P-value	<b>0.01</b>	<b>0.02</b>	<b>0.04</b>	0.13
<b>Eosinophils, cells/mm<sup>3</sup></b>				
Below the median	209 (186-234)	207 (180-239)	226 (201-254)	227 (195-264)
Above the median	221 (197-248)	220 (190-254)	205 (183-230)	203 (178-233)
P-value	0.5	0.5	0.2	0.2
<b>IgE, IU/mL</b>				
Below the median	128 (101-163)	136 (102-181)	196 (154-250)	185 (137-251)
Above the median	185 (146-236)	166 (124-221)	122 (97-155)	128 (97-168)
P-value	<b>0.03</b>	0.26	<b>0.005</b>	<b>0.04</b>

All values are expressed as GM (min-max).

\*Results were adjusted for age, sex, smoking status, BMI and center; GEE regression methods were performed.

Abbreviations: GM, geometric mean; min, lower value; max, higher value; PC, principal component; IgE, immunoglobulin E.

### 3.7.2.4. Longitudinal associations between PC scores and asthma outcomes at EGEA3

Participants with higher PC1 score at baseline tended to have more asthma attacks at EGEA3, and higher subsequent risk of poor asthma symptom control, but the associations did not reach the level of significance (P-values < 0.10). Participants with higher PC2 score at baseline (EGEA2) reported significantly less asthma attacks 7 years later (at EGEA3), and were at lower subsequent risk of poor asthma symptom control (partly controlled and uncontrolled asthma) than those with lower PC2 score at baseline. Both PC1 and PC2 scores at EGEA2 were unrelated to asthma symptom score at EGEA3 and change in asthma symptom score between EGEA2 and EGEA3 (Table 21).

**Table 21.** Longitudinal associations between cytokines PC scores and asthma outcomes (EGEA3)

	PC1 score above the median		PC2 score above the median	
	Unadjusted <i>OR (95% CI)</i>	Adjusted* <i>OR (95% CI)</i>	Unadjusted <i>OR (95% CI)</i>	Adjusted* <i>OR (95% CI)</i>
<b>Asthma attacks</b> (past 12 months)	1.27 (0.78;2.08)	<i>1.71 (0.91;3.20)</i>	0.77 (0.47;1.27)	<b>0.56 (0.31;1.00)</b>
<b>Asthma symptom control, (controlled reference)</b>				
Partly controlled	0.81 (0.44;1.51)	0.80 (0.39;1.64)	0.64 (0.34;1.18)	<i>0.48 (0.23;1.02)</i>
Uncontrolled	2.24 (0.84;5.98)	<i>3.04 (0.92;9.98)</i>	<b>0.29 (0.10;0.81)</b>	<b>0.12 (0.03;0.45)</b>
<b>Asthma symptom score (Pekkanen)</b>				
1 versus 0	1.58 (0.74;3.37)	1.31 (0.57;3.03)	0.76 (0.36;1.61)	0.85 (0.37;1.95)
≥ 2 versus 0	1.01 (0.55;1.84)	1.02 (0.51;2.01)	0.74 (0.40;1.35)	0.59 (0.30;1.17)
<b>Change in the asthma symptom score (Pekkanen) between EGEA2 and EGEA3,</b>				
Better/stable versus worse	0.74 (0.42;1.30)	0.91 (0.49;1.69)	1.32 (0.74;2.36)	1.12 (0.56;2.24)

\*OR (95 % CI) were adjusted for age, sex, smoking, BMI and center. GEE regression methods were performed except for asthma symptom control and asthma symptom score evolution where polytomous logistic regressions were applied. **Bolded results** are statistically significant at P-values< 0.05, those in italic are at 0.05<P-values<0.10.

Abbreviations: PC, principal component; EGEA, epidemiological study on the genetics and environment of asthma; OR, odds ratio; CI, confidence interval.

### **3.7.2.5. *Associations between individual cytokines and asthma outcomes***

Associations between levels of individual cytokines and asthma control outcomes showed that participants with high levels of IL-13 reported significantly more asthma attacks at baseline (OR=2.41 [1.21; 4.79]), and that participants with high levels of IL-7 or IL-13 at baseline were at higher subsequent risk of uncontrolled asthma at EGEA3, those with high level of IL-13 at baseline reporting also significantly more asthma attacks at EGEA3. No other associations were found (Table 22).

**Table 22.** Longitudinal associations between individual cytokines and asthma outcomes (EGEA3)

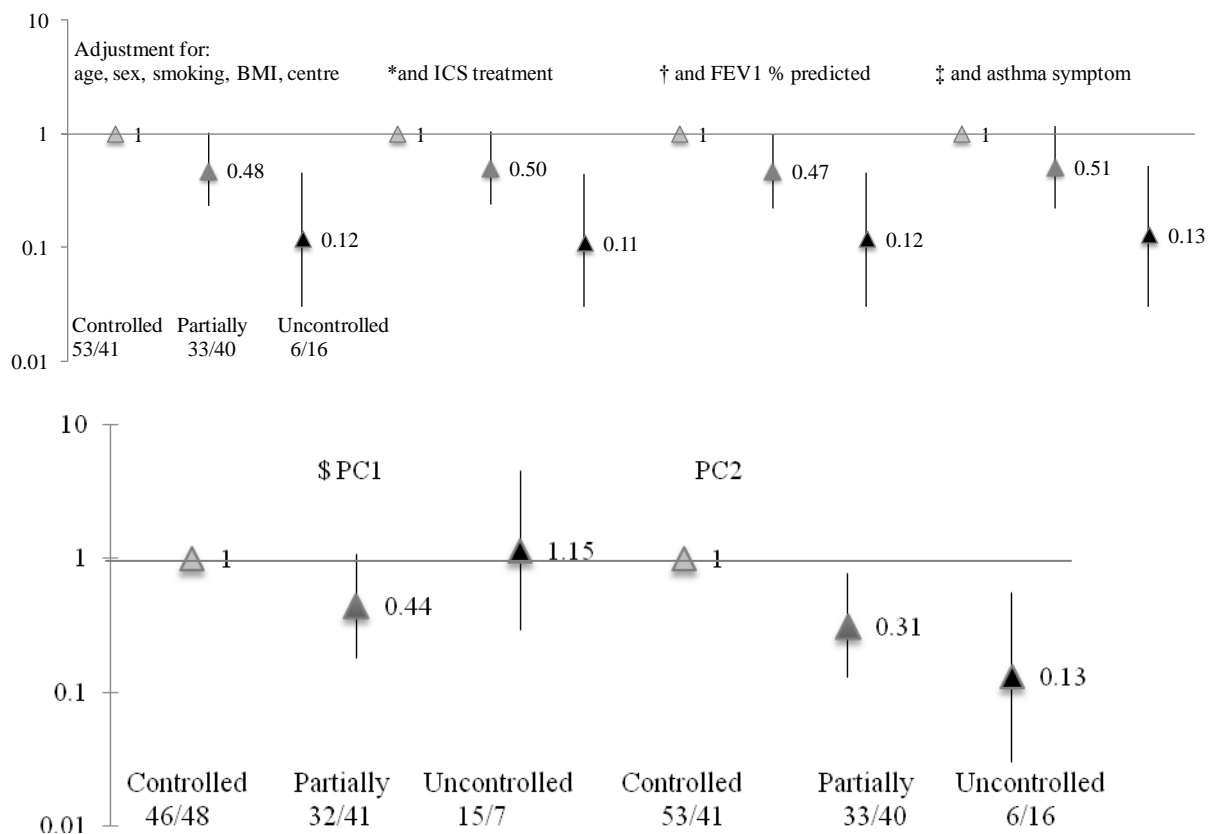
	<b>Unadjusted OR (95% CI)</b>	<b>Adjusted* OR (95% CI)</b>
<b>Ratio IL-1Ra/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	1.08 (0.66;1.75]	1.10 (0.62;1.98)
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	0.72 (0.39;1.33]	0.68 (0.33;1.37)
Uncontrolled vs. controlled	1.10 (0.43;2.80]	1.08 (0.35;3.32)
<b>Ratio IL-5/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	1.26 (0.77;2.05)	1.50 (0.80;2.82)
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	0.85 (0.46;1.57)	0.77 (0.38;1.59)
Uncontrolled vs. controlled	1.91 (0.73;4.97)	2.20 (0.70;6.93)
<b>Ratio IL-7/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	1.15 (0.71;1.87)	1.48 (0.82;2.69)
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	0.86 (0.47;1.59)	0.90 (0.44;1.85)
Uncontrolled vs. controlled	2.24 (0.84;5.98)	<b>3.42 (1.03;11.4)</b>
<b>Ratio IL-8/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	1.08 (0.66;1.75)	1.11 (0.62;1.97)
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	0.68 (0.37;1.26)	0.57 (0.27;1.21)
Uncontrolled vs. controlled	1.33 (0.52;3.40)	1.43 (0.42;4.85)
<b>Ratio IL-10/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	0.98 (0.61;1.60)	1.09 (0.62;1.92)
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	0.57 (0.31;1.06)	0.55 (0.27;1.10)
Uncontrolled vs. controlled	0.77 (0.31;1.96)	0.70 (0.23;2.11)
<b>Ratio IL-13/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	<b>1.67 (1.02;2.72)</b>	<b>2.51 (1.32;4.77)</b>
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	1.47 (0.79;2.71)	1.77 (0.83;3.77)
Uncontrolled vs. controlled	<b>3.60 (1.29;10.0)</b>	<b>7.53 (2.01;28.2)</b>
<b>Ratio TNF-<math>\alpha</math>/pool (above/below median)</b>		
<b>Asthma attacks</b> (past 12 months)	1.30 (0.80;2.11)	1.48 (0.81;2.70)
<b>Asthma symptom control</b>		
Partly controlled vs. controlled	0.74 (0.40;1.37)	0.68 (0.33;1.38)
Uncontrolled vs. controlled	1.75 (0.67;4.56)	2.06 (0.65;6.51)

\*OR (95 % CI) were adjusted for age, sex, smoking status, BMI and center. GEE regression methods were performed for asthma attacks and polytomous logistic regressions for asthma symptom control. **Bolded** results are statistically significant at P-values<0.05.

Abbreviations: IL, interleukin; TNF- $\alpha$ , tumor necrosis factor-alpha; OR, odds ratio; CI, confidence interval; vs, versus.

### 3.7.2.6. Sensitivity analyses

The association between higher PC2 score at baseline and lower subsequent risk of poor asthma symptom control remained significant after further adjustment for ICS use in the past 12 months, for lung function or asthma symptom control at EGEA2. Finally, this association was also consistently observed when analyses were performed after including PC1 in the model (Figure 13).



**Figure 13.** Association between PC2 score at EGEA2 and subsequent risk of asthma symptom control.

Odds Ratios (ORs) adjusted for age, sex, smoking, BMI and center. Further adjustment for \* ICS treatment, † FEV<sub>1</sub> % predicted at EGEA2, ‡ asthma symptom control at EGEA2. \$ both PC1 and PC2 scores were included in the same model.

Subjects were classified as controlled, partly controlled and uncontrolled according to the asthma symptom control as per GINA 2015.

Abbreviations: BMI, body mass index; ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in one second; PC, principal component.



### 3.7.3. Discussion

#### 3.7.3.1. *Summary of findings*

This study identified for the first time two serum cytokine profiles defined by PCA, the first profile including five cytokines involved either in eosinophilic inflammation (IL-5, IL-7, IL-13) or neutrophilic inflammation in asthma (IL-8 and TNF- $\alpha$ ) and the second one including two anti-inflammatory cytokines (IL-1Ra and IL-10). These profiles were differently associated with asthma control outcomes in adults with current asthma at baseline. The first cytokine profile was significantly associated with higher blood neutrophil counts at baseline, and positively associated at borderline significance with subsequent risk of asthma attacks and poor asthma control, but was not associated with any asthma outcomes cross sectionally. The second cytokine profile was significantly associated with low total IgE levels and lower BHR at baseline, and low subsequent risk of asthma attacks and poor asthma control in adults. Evidence for the main longitudinal associations is strengthened by consistency of the results obtained after further adjustments, showing that longitudinal effects on EGEA3 were not simply due to associations from EGEA2.

#### 3.7.3.2. *Comparisons with other studies and interpretation*

In the cross-sectional analyses, participants with high PC1 (above the median) scores had higher blood neutrophil counts than those with low PC1 scores. These results are consistent with the positive association between IL-8 levels and neutrophil counts found in sputum (115) and in blood (10). However, no associations between PC1 and blood eosinophil counts or total IgE levels were observed, in contrast with previous studies on IL-5 and IL-13 in sputum (153,224–226). PC1 profile was not correlated with asthma control outcomes, allergic sensitization, and lung function. In contrast, numerous reports found associations between sputum cytokines levels and clinical outcomes of asthma. Norzilla *et al.* reported an association between sputum IL-5 and IL-8 levels and exacerbations (227), and IL-8 levels in BAL were found to be inversely correlated with FEV<sub>1</sub> in a clinical study (155). It is to be noted that no association between PC1 and asthma symptom control were found, while participants with high levels of IL-13 reported significantly more asthma attacks at baseline, and were at higher subsequent risk of asthma attack and uncontrolled asthma at EGEA3, a result consistent with a previous study on 217 asthma patients that reported the threshold of

156 pg/mL of IL-13 in sputum to have good diagnostic accuracy for the detection of not-well-controlled asthma (157). Likewise, IL-8 levels in BAL were found to be higher in subjects with uncontrolled than in those with controlled asthma (155). To our knowledge, only one case-control study has studied the association between sputum levels of a panel of 12 cytokines including IL-5, IL-10 and TNF- $\alpha$  and asthma control in 106 asthma patients (156): uncontrolled asthma being more common among participants with the 'IL-5/IL-17A-high' profile than in those with the 'IL-5/IL-17A-low' profile. Although differences in study designs and protocols (profiles *versus* individual cytokines, biological compartments, asthma control assessment) prevent any direct between-study comparison, some consistencies in the result were found. In the present study, both T-helper type 1 lymphocytes (TH1) and TH2 pathways are covered by the panel of cytokines included in PC1. Interestingly, the recent review by Fatj and Wenzel (167) showed that therapies targeting the canonical type 2 cytokines IL-4, IL-5, and IL-13 have shown consistent efficacy, especially in asthma patients with evidence of TH2/type 2 inflammation. These results suggest that either the cytokine profile or individual cytokine approaches may be warranted and really complement each other, depending on the type of inflammation of the patients.

Interestingly, a high PC2 profile including IL-1Ra and IL-10 was significantly associated with low IgE levels. A negative association was also found with neutrophil counts but it was not significant. We also found that PC2 was significantly associated with lower BHR at baseline, and lower subsequent risk of asthma attacks and uncontrolled asthma seven years later. Hakonarson *et al.* were the first to show that the treatment of antigen-sensitized animals with IL-1Ra inhibited airway hyperresponsiveness and inflammation (228). Later, it was reported that sputum IL-1Ra levels were also significantly and positively correlated with lung function in patients with asthma (147,229). Recently (230), the IL-1Ra/IL-1 $\beta$  ratio was compared between four sputum inflammatory profiles (paucigranulocytic, eosinophilic, neutrophilic, mixed) in 80 adults with asthma, and was significantly reduced in neutrophilic asthma, suggesting an imbalance of available anti-inflammatory mediators. Moreover, in the large-scale consortium-based Genomewide Association Study of Asthma (GABRIEL), association of genomewide significance was found between asthma and a genetic variant implicating *IL1R1/IL18R1* (231). Previous studies have shown that IL-10 treatment reduced airway hyperresponsiveness (232,233) and that suppression of airway hyperresponsiveness by regulatory T cells is IL-10-dependent (234). Overall, these results highlight the importance of including IL-1Ra and IL-10, and possibly other anti-inflammatory cytokines, in future studies

of associations between cytokine profiles and asthma control outcomes with the possibility to monitor the evolution of the disease in uncontrolled patients.

### **3.7.3.3. *Strengths and limitations***

To our knowledge, our study is the first to investigate the association between serum cytokine profiles and asthma control outcomes in a prospective design. A careful procedure was set up to include true asthmatics in the study with a wide range of severity and response to methacholine. Asthma symptom control was defined according to GINA 2015. Cytokines were measured in serum, which represents a less invasive approach than sputum assays for further implementation in the context of epidemiological studies, or in clinical practice. Even though we have only explored a small part of the cytokines network, we have included key inflammatory, anti-inflammatory and TH2 cytokines as well as a receptor antagonist, cytokines often neglected in previous studies (97). Finally, since chronic inflammation in asthma is orchestrated by multiple cytokines through complex interactions (147), we performed a multiplex analysis, allowing the parallel analysis of multiple cytokines in one serum sample (146). It is interesting to note that IL-8 and TNF- $\alpha$  were clustered together with TH2 type cytokines, suggesting that systemic neutrophil activation and inflammation may result in asthma patients regardless of the type of local inflammatory profile.

### **3.7.3.4. *Conclusion***

In conclusion, the present study identified for the first time that serum cytokine profiles with high levels of IL1-Ra and IL-10 were associated with less asthma attacks and lower risk of poor asthma control outcomes in adults in a longitudinal design. More generally, this study indicates that assessing cytokine profiles when studying the associations with asthma control outcomes may be sometimes more efficient than assessing individual cytokine levels, and may help to target patients with uncontrolled asthma for therapy.

*As a result of the work performed in this part, an article entitled “Blood cytokine profiles and subsequent risk of asthma symptom control in adults from the EGEA study” was submitted to Allergy journal and is currently under review.*

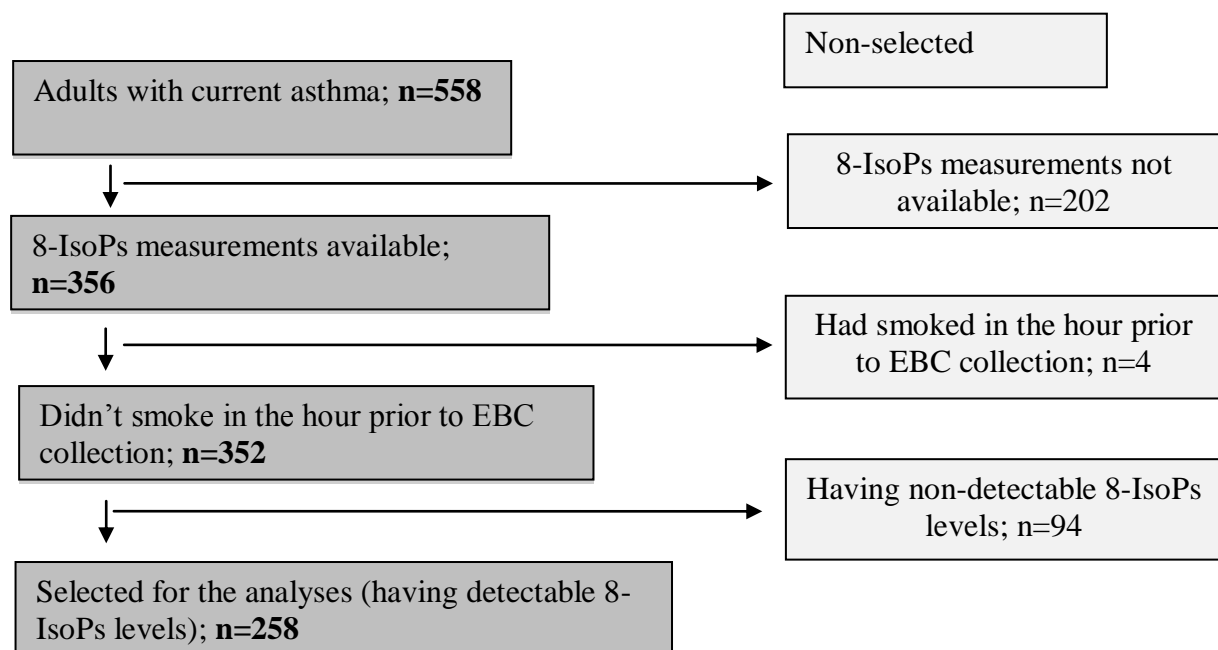
### 3.8. Third analysis: Preliminary findings for the association between 8-isoprostanes and current asthma

This analysis is devoted to find the associations between EBC 8-IsoPs levels and asthma outcomes using data from the EGEA study.

#### 3.8.1. Materials and methods

##### 3.8.1.1. Participants' selection

Among the 558 participants with current asthma who were adult at EGEA2 ( $\geq 16$  years old), the present analysis included 258 participants, excluding those who do not have available EBC 8-IsoPs measurements ( $n=202$ ), those who had smoked one hour prior to EBC collection ( $n=4$ ) or those who had non-detectable 8-IsoPs levels ( $n=94$ ) (Figure 14).



**Figure 14.** Flow chart of the participants – data on 8-IsoPs and current asthma

Abbreviations: EGEA, epidemiological study on the genetics and environment of asthma; 8-Isops, 8-isoprostanes.

### 3.8.1.2. *8-isoprostanes measurements*

8-IsoPs concentrations in EBC were measured with a specific enzyme immunoassay (EIA) kit (8-IsoPs EIA kit, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol between September 2012 and April 2013. Fifty microliters of EBC were assayed in duplicate and the 8-IsoPs concentrations were calculated from a calibration curve obtained from eight calibrator levels (0.8 – 500 pg/ mL). The LLD was 4.0 pg/ mL. The intra-assay coefficient of variation was less than 20%.

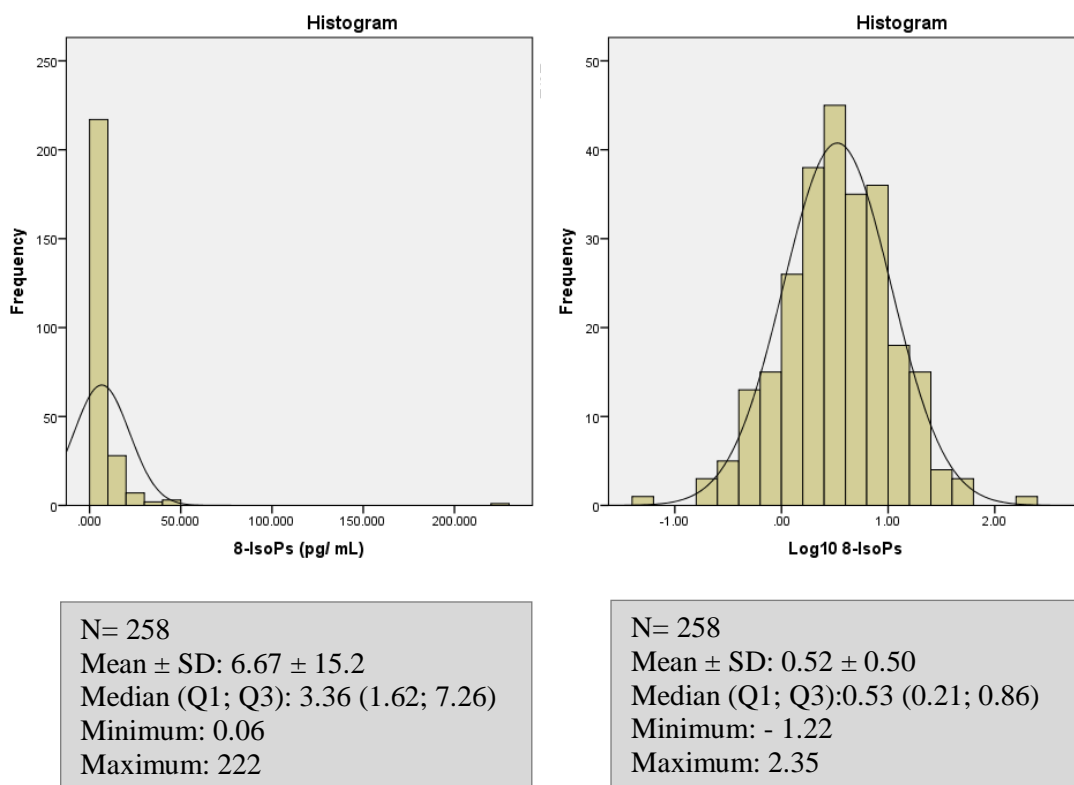
In order to "validate" the 8-IsoPs measurements, we first performed descriptive analyses for 8-IsoPs levels in non-asthmatics. Among them, 31% had non-detectable 8-IsoPs levels. No associations were found between 8-IsoPs levels and plate number, hour of EBC collection or storage time. 8-IsoPs levels were significantly and negatively associated with age ( $r = -0.17$ ,  $P$ -value = 0.001) and were higher in women ( $P$ -value = 0.04). 8-IsoPs levels were not associated with smoking ( $P$ -value = 0.5). No association was observed with BMI classes ( $P$ -value = 0.5), which is concordant with a previous study that reported no association with BMI in healthy non-asthmatics (235).

Comparison of 8-IsoPs levels between non-asthmatics and participants having current asthma was studied before selecting the participants with current asthma for the analyses. 8-IsoPs levels were significantly higher in participants having current asthma [ $n$ ; GM (Q1; Q3): 258; 3.34 (1.62; 7.26)] *versus* non-asthmatics [374; 2.46 (1.10; 5.69)],  $P=0.003$ . Moreover, 8-IsoPs levels were not significantly different between patients having current or ever asthma.

### 3.8.1.3. *Statistical analyses*

8-IsoPs values were log<sub>10</sub> transformed due to their skewed distribution and expressed as GMs (interquartile range) (Figure 15). The associations of 8-IsoPs levels with plate number, hour of EBC collection, storage time, and smoking within 24 hours prior to EBC collection and with age (continuous), sex, smoking (current, ex- and non-smokers), BMI (4 classes) and ICS use were assessed in patients with current asthma using standard statistical tests such as student T-test or ANOVA test and Pearson correlation coefficient or its non-parametric Spearman correlation coefficient. Multivariate analyses (except multinomial logistic regressions) were performed using GEE. 8-IsoPs levels were studied in association with asthma outcomes

(cross-sectional analyses). Age, sex, smoking status, BMI, and center were included as covariates. Multivariate analyses were as well conducted firstly further additional adjustment for ICS use in the past 12 months, secondly by adjusting for age, sex, smoking status, BMI, and center and stratifying by ICS use and thirdly by removing the outliers from the database. Results of the statistical tests were considered significant if P-values were < 0.05. All analyses were performed using SPSS software, version 20.0. Analyses are still in progress and will be finalized in the coming three months.



**Figure 15.** Histograms for 8-IsoPs levels before and after log10 transformation  
Abbreviations: 8-IsoPs, 8-isoprostanes.

## **3.8.2. Preliminary results**

### **3.8.2.1. Study participants**

#### *Participants selected for the analyses versus those non-selected*

Participants selected for the analyses (n=258) were similar to those non-selected (n=206) for age, sex, BMI status, smoking, exacerbations, asthma attacks, asthma symptom score, ICS use, FEV1, BHR, allergic sensitization and blood cell counts. Moreover, the 258 participants selected for the analyses were compared to those having non-detectable 8-IsoPs levels (n=94) as the levels of 8-IsoPs in this group were lower than the limit of detection, thus could be considered as an informative group. Participants having non-detectable 8-IsoPs levels had more asthma attacks and included more subjects with uncontrolled asthma. No other significant associations were observed (Table 23).

**Table 23.** Comparison of main characteristics between selected and non-selected participants and those having non-detectable 8-IsoPs levels

	<b>Selected; N=258</b>	<b>Non-selected; N=206</b>	<b>ND 8-IsoPs; n=94</b>	<b>P-value<sup>#</sup></b>	<b>P-value<sup>&amp;</sup></b>
<b>Age, years, Mean ± SD</b>	37.8 ± 16.6	39.8 ± 15.7	41.7 ± 18.2	0.1	0.06
<b>Women, n (%)</b>	127 (49.2)	99 (48.1)	44 (46.8)	0.8	0.7
<b>Body mass index (BMI), n (%)</b>				0.5	0.4
<20 kg/m <sup>2</sup>	37 (14.3)	16 (10.5)	10 (10.6)		
[20 – 25[ kg/m <sup>2</sup>	127 (49.2)	84 (54.9)	46 (48.9)		
[25 – 30[ kg/m <sup>2</sup>	61 (23.6)	37 (24.2)	29 (30.9)		
>=30 kg/m <sup>2</sup>	33 (12.8)	16 (10.5)	9 (9.60)		
<b>Smoking, n (%)</b>				0.3	0.1
Non-smokers	133 (51.6)	93 (45.4)	41 (43.6)		
Ex-smokers	55 (21.3)	54 (26.3)	30 (31.9)		
Smokers	70 (27.1)	58 (28.3)	23 (24.5)		
<b>Exacerbations, past 12 months, n (%)</b>	33 (14.3)	27 (18.9)	17 (19.8)	0.2	0.2
<b>Asthma attacks, past 12 months, n (%)</b>	106 (41.1)	88 (43.1)	57 (60.6)	0.7	<b>0.001</b>
<b>Asthma symptom control, n (%)</b>				0.06	<b>0.02</b>
Controlled	142 (58.0)	90 (50.6)	36 (40.9)		
Partly controlled	81 (33.1)	59 (33.1)	38 (43.2)		
Uncontrolled	22 (9.00)	29 (16.3)	14 (15.9)		
<b>Asthma symptom score (Pekkanen), n (%)</b>				0.7	0.08
0	28 (11.0)	21 (10.4)	6 (6.40)		
1	58 (22.7)	53 (26.4)	14 (14.9)		
≥2	169 (66.3)	127 (63.2)	74 (78.7)		
<b>ICS use, past 12 months, n (%)</b>	120 (46.9)	102 (50.7)	46 (50.0)	0.4	0.6
<b>FEV1% predicted, Mean ± SD</b>	96.5 ± 17.4	94.4 ± 20.8	93.7 ± 18.9	0.3	0.2
<b>Methacholine test*, PD20 ≤ 4 mg, n (%)</b>	103 (68.2)	62 (72.1)	41 (74.5)	0.5	0.4
<b>SPT+, n (%)</b>	197 (80.7)	115 (84.6)	70 (76.9)	0.4	0.4
<b>Eosinophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	213 (130 ; 300)	212 (138 ; 323)	216 (150 ; 360)	0.4	0.3
<b>Neutrophils, cells/mm<sup>3</sup>, GM (Q1;Q3)</b>	3754 (2910; 4700)	3951 (3068; 4860)	3980 (3138; 5058)	0.2	0.3

<sup>#</sup> For non-selected *versus* selected; <sup>&</sup> For having non-detectable (ND) 8-IsoPs *versus* selected.

Abbreviations: ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume in 1 second; PD20, dose of methacholine causing a 20% fall in FEV<sub>1</sub> in 1 second from baseline; \*Methacholine challenge test was performed if baseline FEV<sub>1</sub> ≥ 80% predicted (n=151 for selected participants); SPT+, a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; GM, geometric mean; (Q1; Q3), first and third quartiles; ND, non-detectable.



### Participants' characteristics

As shown in Table 23, the mean age of the selected 258 adults with current asthma was 38 years, half of them were women, 52% were non-smokers, and 41% reported asthma attacks in the past year. Moreover, 58% were classified as controlled according to GINA 2015 and 81% had an allergic sensitization.

#### 3.8.2.2. Association between EBC 8-IsoPs levels and potential confounding variables

No significant associations were observed between 8-IsoPs levels and plate number (P-value = 0.3), hour of EBC collection (P-value = 0.4), storage time (P-value = 0.1) or smoking in the 24 hours prior to EBC collection (P-value = 0.8). 8-IsoPs levels were negatively associated with age and were lower in participants who used ICS in the past 12 months *versus* not. No significant associations were observed with sex, BMI and smoking (Table 24).

**Table 24.** Associations between EBC 8-IsoPs levels and age, sex, BMI and smoking

	<b>8-Isoprostanes n; GM (Q1;Q3)*</b>	<b>P-value</b>
<b>Age, (years)</b>	258; - 0.19	<b>0.003</b>
<b>Age classes</b>		<b>0.01</b>
[16 – 25[	87; 4.20 (2.07;9.68)	
[25 – 35[	55; 3.87 (2.10;6.76)	
[35 – 45[	22; 2.35 (0.83;6.79)	
[45 – 55[	46; 3.38 (1.49;9.43)	
[55 – 80]	48; 2.15 (1.12;3.65)	
<b>Sex</b>		0.4
Women	127; 3.14 (1.62;6.88)	
Men	131; 3.53 (1.58;7.30)	
<b>Body mass index (BMI)</b>		0.3
<20 kg/m <sup>2</sup>	37; 3.30 (1.49;7.62)	
[20 – 25[kg/m <sup>2</sup>	127; 3.67 (1.90;7.83)	
[25 – 30[ kg/m <sup>2</sup>	61; 3.34 (1.59;8.96)	
>=30 kg/m <sup>2</sup>	33; 2.33 (1.35;5.16)	
<b>Smoking</b>		0.4
Non-smokers	133; 3.52 (1.74;7.62)	
Ex-smokers	55; 2.66 (0.99;6.88)	
Smokers	70; 3.61 (1.66;6.54)	
<b>ICS use, past 12 months</b>		<b>0.03</b>
No	136; 3.86 (2.07; 8.01)	
Yes	120; 2.82 (1.38; 6.58)	

\*All values are expressed as n; GM (Q1; Q3) except age that is expressed as n; correlation coefficient. Abbreviations: EBC, exhaled breath condensate; ICS, inhaled corticosteroids; GM, geometric mean; (Q1; Q3), first and third quartiles.

### 3.8.2.3. Cross-sectional associations between EBC 8-IsoPs and asthma outcomes at EGEA2

No associations were found between 8-IsoP levels and exacerbations or asthma attacks in the past 12 months, asthma symptom control, asthma symptom score (Pekkanen), BHR or FEV1. (Table 25).

**Table 25.** Cross-sectional associations between EBC 8-IsoPs levels and asthma outcomes

	<b>Unadjusted OR (95% CI)</b>	<b>Adjusted* OR (95% CI)</b>
<b>Exacerbations</b> , (past 12 months)	0.58 (0.28;1.19)	0.81 (0.34;1.95)
<b>Asthma attacks</b> , (past 12 months)	0.76 (0.46;1.25)	1.07 (0.55;2.09)
<b>Asthma symptom control</b> , (controlled reference)		
Partially controlled	0.80 (0.46;1.40)	1.05 (0.49;2.25)
Uncontrolled	0.64 (0.26;1.59)	0.96 (0.28;3.31)
<b>Asthma symptom score (Pekkanen)</b>		
1 versus 0	0.48 (0.19;1.19)	0.32 (0.09;1.18)
≥ 2 versus 0	0.80 (0.36;1.79)	0.74 (0.23;2.37)
<b>Methacholine test, PD20≤4mg</b> (N=103)	1.03 (0.52; 2.06)	0.97 (0.37; 2.59)
	<b>β (95% CI)</b>	<b>β (95% CI)</b>
<b>FEV<sub>1</sub> % predicted</b>	0.001 (-0.04; 0.04)	-0.004 (-0.06; 0.05)

\*OR/ β (95% CI) were adjusted for age, sex, smoking status, BMI, and center. GEE regression methods were performed, except for asthma symptom control and asthma symptom score where multinomial logistic regressions were applied.

Abbreviations: OR, odds ratio; CI, confidence interval; PD20, dose of methacholine causing a 20% fall in FEV in 1 second from baseline; FEV1, forced expiratory volume in 1 second.

Similar associations were observed after further adjustment for ICS use in the past 12 months, when adjusting for age, sex, BMI, smoking and center and stratifying by ICS use, or when removing the outliers from the database (n=2 for participants having current asthma).

### 3.8.3. Conclusion

No correlations were found between EBC 8-IsoPs levels measured at EGEA2 and variables like storage time and hour of blood test which is consistent with a good quality of the EGEA2 data collection.

The preliminary results showed no association with asthma outcomes. A previous study found no association between 8-IsoP levels and asthma control using ACT (170). Another study reported no significant differences in 8-IsoP levels between subjects exposed to oil spill *versus* those not and both groups included subjects with asthma or atopy (236). However, previous reports including children or adults having asthma, 8-IsoP levels were higher in severe patients and during exacerbations (237,238). Analyses will be pursued in order to confirm the initial findings. Moreover, additional analyses will be conducted by including participants having non-detectable 8-IsoPs levels as an informative group in the analyses and expressing the 8-IsoP levels by classes.

## 4. GENERAL DISCUSSION

This thesis has shown the usefulness of evaluating lung specific biological markers as clinical biomarkers for COPD as well as biological markers of systemic inflammation in assessing future risk of asthma outcomes.

### 4.1. Summary of findings and interpretation

In the first part of this thesis, SP-D was shown to be significantly associated with COPD but not with asthma. Moreover, a score for COPD diagnosis with excellent discriminant values (sensitivity, specificity, positive predictive value, and negative predictive value) was identified using serum SP-D levels.

Since COPD is still underdiagnosed and sometimes diagnosed too late, the early diagnosis of COPD is still quite a challenging task for the clinician, and efforts have been made to find specific markers that can identify disease phenotypes (239). In the study performed on the Lebanese population, COPD was defined as a post bronchodilator ratio of FEV<sub>1</sub>/FVC being < than 70%. In previous literature, diverse definitions were used to diagnose COPD. The most used definition relies on the GOLD guidelines, thus the presence of a post-bronchodilator FEV<sub>1</sub>/FVC ratio of < 70% to define irreversible airflow obstruction, regardless of the patients' age and sex. Since the FEV<sub>1</sub> value decreases more quickly with age than the FVC, the GOLD definition has the disadvantage of possible over-diagnosis in elderly patients. The proportion of false-positive diagnoses using the post-bronchodilator ratio was reported to be of 8.9% for 31–40 year-old group, 15.5% for those aged 41–50 years, 23.9% for those aged 51–60 years, 33.2% for those aged 61–70 years, 38.7% for those aged 71–80 years, and 42.7% for those aged over 81 years (240). Adjusting the FEV<sub>1</sub>/FVC ratio for normative ageing effects appears to reduce the false-positive rate reported in ageing patients (241). The ATS/ERS statement recommends using the lower limit of normal (LLN) procedure of the FEV<sub>1</sub>/FVC to diagnose COPD in place of using the post-bronchodilator ratio of FEV<sub>1</sub>/FVC. The LLN is based on age-stratified pre-bronchodilator cut-off values of the FEV<sub>1</sub>/FVC ratio. Patients having a value below the lower 5<sup>th</sup> percentile of an aged-matched healthy reference group are diagnosed as having COPD (242,243). This will result in lower prevalence estimates of COPD compared to the GOLD definition in the elderly (242). However, Güder *et al.* have reported that the LLN led to more false negative diagnoses and that adding FEV<sub>1</sub> and the

residual volume/total lung capacity ratio (RV/TLC) to GOLD or LLN improved the diagnostic accuracy, with a reduction of up to 50% of the number of misdiagnoses (244). Simple and robust tools are needed to identify subjects with an obstructive ventilator condition even if they have few symptoms. Using the correct diagnosis is very important in order to avoid misclassification biases and especially for the differentiation of COPD from asthma patients. Lung specific biological markers, such as SP-D could be used as clinical biomarkers for COPD. Moreover, these biological markers or scores generated from them could provide a surrogate for spirometry by using equipment that is cost-effective and readily available in the clinical settings. Moreover, this could make an early detection possible in order to prevent the decline in respiratory function, for which current pharmacological treatment is ineffective.

Similarly, there is a need to find asthma biological markers able to identify clinically relevant asthma phenotypes, optimize diagnosis, and guide treatment. In the second part of this thesis, we found that the cytokine profiles were associated differently with subsequent asthma outcomes.

Different asthma biological markers are already available for clinical practice such as airway eosinophilia and airway remodeling measured from BAL or bronchial biopsy. Even though biopsies and BAL are considered as the GOLD standard methods to assess airway inflammation, they are invasive methods, thus their use in the clinical routine is limited (97,245,246). Inflammatory phenotypes, namely eosinophilic, neutrophilic, mixed and paucigranulocytic types, have been identified in sputum (247). In addition to being invasive, the reproducibility of sputum induction measurements over time has been debated (248,249). A less invasive procedure can be used such as peripheral blood collection, which is practical among participants of all ages for large epidemiological studies and for further routine clinical practice (151,158). Single serum or plasma biological markers have been previously measured and associated with asthma outcomes but integrating a multidimensional approach could strengthen the diagnostic ability of a clinically applicable algorithm to phenotype asthma (97). Moreover, approaching phenotypes by using more statistically based approaches such as cluster analysis or PCA could capture key patterns of marker profiles from a panel of multiple analytes (10,250), thus offering new possibilities for the disease management, such as the development of targeted asthma treatment. Among key serum/plasma biological markers, we will cite the cytokines since they play important roles in both eosinophilic and non-eosinophilic asthma, and anti-interleukin therapy with anti-IL-4, IL-5 and IL-13 have shown

success among specific populations of asthma patients (251). Hence, more research in the field may allow a personalized approach to asthma management. Moreover, since asthma is associated with oxidative stress, measuring biological markers of damage due to oxidative stress from EBC could provide a non-invasive alternative for BAL, sputum, biopsy, and even blood. In the last two decades, there has been increasing interest in measuring EBC compounds in subjects with pulmonary diseases (252,253) since this approach is feasible in children and adults, could provide access to volatile and nonvolatile respiratory compounds without the need of performing bronchoscopy with BAL, and offers a 100% successful collection (254). However, even though research has focused on the measurement of non-invasive biological markers such as those found in EBC or in saliva and their association with asthma outcomes (159,250), they are still in their research phase. Once the clinical value of these biological markers (especially the ones from EBC since closer to the lung) is established, they could provide a practical and non-invasive method to use in care centers. Lastly, whether from blood or EBC, the current need is for profiles of markers that best predict treatment response in asthma patients.

#### **4.2. Common strengths and limitations**

The strengths of this work lie firstly in the well-defined classification of the patients included in both parts of the thesis, which is spirometry for COPD patients and recruiting asthma patients in chest clinics. Secondly, they lie in the statistical methods used and /or the study designs. This includes the ROC curve analysis, the longitudinal design of the EGEA study, which can provide information about continuity, prediction, and within-individual change (255), and the choice of mediators studied in the multiplex analyses that could determine the methods' success (97). Finally, all reported outcomes in this thesis are patient-centered. This reflects the pragmatic nature of the research, for which the hypothesis and study design are formulated, based on information needed to make a clinical and/or public health decision (256).

An important component not approached in this thesis is the effect of the environment on the occurrence and severity of COPD and asthma. However, assessing individual exposure with pollutants from the environment remains an important challenge in epidemiology studies (257). Considering the occurrence of COPD or asthma as the clinical consequence of an interaction between environmental factors on the one hand and the existence of a not yet

properly understood genetic predisposition on the other is needed. This will enable us to intervene before the disease is developed.

### **4.3. Implications for public health**

Hundreds of millions of people are burdened with chronic respiratory conditions. The disease burden from COPD is in the number of deaths that it causes while the greatest burden of asthma is the morbidity and disruption of lives that it causes. The ability to control and eliminate respiratory diseases worldwide relies on public health measures, which include increasing awareness, education and capacity, thus a better management of the disease (2,3). This could be accomplished through research. Research helps to improve the understanding of disease processes, which then allows for better diagnoses, treatment, and prevention. In order to promote the respiratory health of the population, we need to identify new initiatives. In this thesis, integrating the study of biological markers in respiratory epidemiology has been explored. Some of the results might have public health implications that are discussed below, firstly regarding the global burden of COPD, and secondly regarding asthma management.

#### **Global burden of COPD**

Given the emergence of COPD in the last 20 years, especially in developing countries, the global burden of COPD is projected to increase substantially in Asia and Africa in the coming decades, mostly as a result of increased tobacco use. Tobacco is the single most preventable cause of death in the world (2); however, it may not be possible to reduce its use in some regions due to the lack of government oversight and regulations regarding the sale of tobacco products or the use of tobacco in public places. Moreover, the tobacco industry has insistently fought the efforts done to reduce the use of its products. In this context, trying to identify COPD by using biological markers, especially in developing countries or in the regions where spirometry is not always available has an important public health implication. It may allow the fast implementation of treatment, thus the improvement of the lung health of COPD patients. Additionally, scores generated from biological markers could be used in the primary care settings in order to ensure a smoother transition from research to practice.

## **Asthma management**

Despite advances in asthma therapies, a proportion of asthma patients are still having difficulty in gaining adequate asthma control (258). Moreover, asthma prevalence is increasing with urbanization which is due to changes in the lifestyles and in the socioeconomic factors (60). Thus, the number of patients having uncontrolled asthma is as well increasing. Therefore, achieving asthma control remains a challenge. Assessing biological markers profiles that are related to subsequent asthma outcomes could help to target patients for therapy and might reduce future risks of uncontrolled asthma. Additionally, this will have an impact on the national economic implications and will lead to a reduction in the cost of future interventions.

Overall, our findings are useful for health care professionals and public health decision makers since they can assist them in making decisions to allocate resources toward research in the field of respiratory epidemiology. They can help in ensuring an early diagnosis for COPD patients, especially in developing countries and in achieving and maintaining an adequate control for asthma patients. This will lead to an improvement in the quality of life of these patients, especially at a time where active ageing is central to political agendas in the industrialized world (259).

### **4.4. Recommendations and perspectives**

Several recommendations and perspectives might be raised from this thesis. Some of them are presented below.

- Since SP-D is a lung specific biological marker, its predictive value should be evaluated within a large scale study with prospective follow-up in Lebanon. In fact, this part was planned to be pursued after the case-control study but was interrupted due to financial issues. However, we hope we will be able to finalize this work in the future.
- The association between serum cytokines profiles, by including a large number of inflammatory and anti-inflammatory cytokines, and asthma outcomes using a



prospective design needs to be further studied. This could improve our understanding of the pathophysiology of asthma by opening new possibilities for disease management, especially since cytokines are currently being implemented as targeted therapies for asthma patients (167,168). However, these therapies showed encouraging results in some patients while they remained ineffective in others, thus need to be further investigated in future research.

- Studying the associations of biological markers from different compartments and asthma outcomes is of interest. In this thesis, we studied the associations between biological markers from blood and from EBC and asthma outcomes. However, the analyses on EBC 8-IsoPs and asthma outcomes as well as the article are still in progress and will be finalized in the coming three months.

Finally, with the enormous global morbidity and mortality rates that chronic respiratory diseases are causing, further research is needed in the field of respiratory epidemiology. The transition from research to public health practices has the best chance of reducing the burden of COPD and asthma around the world in the coming years.

## 5. REFERENCES

1. 001-156\_ARP.indd - GARD Book 2007.pdf [Internet]. [cited 2015 Jul 14]. Available from: <http://www.who.int/gard/publications/GARD%20Book%202007.pdf>
2. Schluger NW, Koppaka R. Lung disease in a global context. A call for public health action. *Ann Am Thorac Soc*. 2014 Mar;11(3):407–16.
3. Ferkol T, Schraufnagel D. The global burden of respiratory disease. *Ann Am Thorac Soc*. 2014 Mar;11(3):404–6.
4. Varkey B, Maier LA. Chronic respiratory diseases: challenges in diagnosis and prevention. *Curr Opin Pulm Med*. 2015 Mar;21(2):111–3.
5. GBD 2013 DALYs and HALE Collaborators, Murray CJL, Barber RM, Foreman KJ, Ozgoren AA, Abd-Allah F, et al. Global, regional, and national disability-adjusted life years (DALYs) for 306 diseases and injuries and healthy life expectancy (HALE) for 188 countries, 1990-2013: quantifying the epidemiological transition. *Lancet Lond Engl*. 2015 Aug 27;
6. Layout 1 - GARD\_actionplan\_FINAL.pdf [Internet]. [cited 2015 Nov 24]. Available from: [http://www.who.int/gard/publications/GARD\\_actionplan\\_FINAL.pdf?ua=1](http://www.who.int/gard/publications/GARD_actionplan_FINAL.pdf?ua=1)
7. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet Lond Engl*. 2012 Dec 15;380(9859):2095–128.
8. GOLD\_Report\_2015\_Apr2.pdf [Internet]. [cited 2015 Jul 15]. Available from: [http://www.goldcopd.org/uploads/users/files/GOLD\\_Report\\_2015\\_Apr2.pdf](http://www.goldcopd.org/uploads/users/files/GOLD_Report_2015_Apr2.pdf)
9. Bousquet J, Mantzouranis E, Cruz AA, Ait-Khaled N, Baena-Cagnani CE, Bleecker ER, et al. Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma. *J Allergy Clin Immunol*. 2010 Nov;126(5):926–38.
10. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med*. 2012 May;18(5):716–25.
11. Antó JM. Recent advances in the epidemiologic investigation of risk factors for asthma: a review of the 2011 literature. *Curr Allergy Asthma Rep*. 2012 Jun;12(3):192–200.
12. Postma DS, Rabe KF. The Asthma-COPD Overlap Syndrome. *N Engl J Med*. 2015 Sep 24;373(13):1241–9.
13. Carraro S, Scheltema N, Bont L, Baraldi E. Early-life origins of chronic respiratory diseases: understanding and promoting healthy ageing. *Eur Respir J*. 2014 Dec;44(6):1682–96.
14. Stockley RA. Biomarkers in chronic obstructive pulmonary disease: confusing or useful? *Int J Chron Obstruct Pulmon Dis*. 2014;9:163–77.
15. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol*. 2015 Jan;16(1):45–56.
16. Girdhar A, Kumar V, Singh A, Menon B, Vijayan VK. Systemic inflammation and its response to treatment in patients with asthma. *Respir Care*. 2011 Jun;56(6):800–5.

17. Jaw JE, Sin DD. Unifying thoracic biomarkers: surfactant protein-D and beyond. *Expert Rev Respir Med.* 2012 Apr;6(2):147–54.
18. Janicka M, Kot-Wasik A, Kot J, Namieśnik J. Isoprostanes-biomarkers of lipid peroxidation: their utility in evaluating oxidative stress and analysis. *Int J Mol Sci.* 2010;11(11):4631–59.
19. Kessler R, Partridge MR, Miravittles M, Cazzola M, Vogelmeier C, Leynaud D, et al. Symptom variability in patients with severe COPD: a pan-European cross-sectional study. *Eur Respir J.* 2011 Feb;37(2):264–72.
20. Zwar NA, Marks GB, Hermiz O, Middleton S, Comino EJ, Hasan I, et al. Predictors of accuracy of diagnosis of chronic obstructive pulmonary disease in general practice. *Med J Aust.* 2011 Aug 15;195(4):168–71.
21. Faner R, Tal-Singer R, Riley JH, Celli B, Vestbo J, MacNee W, et al. Lessons from ECLIPSE: a review of COPD biomarkers. *Thorax.* 2014 Jul;69(7):666–72.
22. WHO | COPD management [Internet]. WHO. [cited 2015 Dec 1]. Available from: <http://www.who.int/respiratory/copd/management/en/>
23. Athanazio R. Airway disease: similarities and differences between asthma, COPD and bronchiectasis. *Clin São Paulo Braz.* 2012 Nov;67(11):1335–43.
24. Prevalence in EU | COPD [Internet]. [cited 2015 Dec 29]. Available from: <http://www.copdcoalition.eu/about-copd/prevalence>
25. Waked M, Khayat G, Salameh P. Chronic obstructive pulmonary disease prevalence in Lebanon: a cross-sectional descriptive study. *Clin Epidemiol.* 2011;3:315–23.
26. Løkke A, Lange P, Scharling H, Fabricius P, Vestbo J. Developing COPD: a 25 year follow up study of the general population. *Thorax.* 2006 Nov;61(11):935–9.
27. Kim WJ, Oh Y-M, Lee JH, Park C-S, Park SW, Park JS, et al. Genetic variants in HHIP are associated with FEV1 in subjects with chronic obstructive pulmonary disease. *Respirol Carlton Vic.* 2013 Nov;18(8):1202–9.
28. Pabst S, Bradler O, Gillissen A, Nickenig G, Skowasch D, Grohe C. Toll-like receptor-9 polymorphisms in sarcoidosis and chronic obstructive pulmonary disease. *Adv Exp Med Biol.* 2013;756:239–45.
29. Stoller JK, Brantly M. The challenge of detecting alpha-1 antitrypsin deficiency. *COPD.* 2013 Mar;10 Suppl 1:26–34.
30. Hunninghake GM, Cho MH, Tesfaigzi Y, Soto-Quiros ME, Avila L, Lasky-Su J, et al. MMP12, lung function, and COPD in high-risk populations. *N Engl J Med.* 2009 Dec 31;361(27):2599–608.
31. Raad D, Gaddam S, Schunemann HJ, Irani J, Abou Jaoude P, Honeine R, et al. Effects of water-pipe smoking on lung function: a systematic review and meta-analysis. *Chest.* 2011 Apr;139(4):764–74.
32. Kohansal R, Martinez-Camblor P, Agustí A, Buist AS, Mannino DM, Soriano JB. The natural history of chronic airflow obstruction revisited: an analysis of the Framingham offspring cohort. *Am J Respir Crit Care Med.* 2009 Jul 1;180(1):3–10.

33. Naidoo RN. Occupational exposures and chronic obstructive pulmonary disease: incontrovertible evidence for causality? *Am J Respir Crit Care Med*. 2012 Jun 15;185(12):1252–4.
34. Ko FWS, Hui DSC. Air pollution and chronic obstructive pulmonary disease. *Respirol Carlton Vic*. 2012 Apr;17(3):395–401.
35. Atkinson RW, Carey IM, Kent AJ, van Staa TP, Anderson HR, Cook DG. Long-term exposure to outdoor air pollution and the incidence of chronic obstructive pulmonary disease in a national English cohort. *Occup Environ Med*. 2015 Jan;72(1):42–8.
36. WHO | Burden of COPD [Internet]. WHO. [cited 2015 Nov 29]. Available from: <http://www.who.int/respiratory/copd/burden/en/>
37. de Marco R, Accordini S, Marcon A, Cerveri I, Antó JM, Gislason T, et al. Risk factors for chronic obstructive pulmonary disease in a European cohort of young adults. *Am J Respir Crit Care Med*. 2011 Apr 1;183(7):891–7.
38. Svanes C, Sunyer J, Plana E, Dharmage S, Heinrich J, Jarvis D, et al. Early life origins of chronic obstructive pulmonary disease. *Thorax*. 2010 Jan;65(1):14–20.
39. Voraphani N, Stern DA, Wright AL, Guerra S, Morgan WJ, Martinez FD. Risk of current asthma among adult smokers with respiratory syncytial virus illnesses in early life. *Am J Respir Crit Care Med*. 2014 Aug 15;190(4):392–8.
40. Postma DS, Bush A, van den Berge M. Risk factors and early origins of chronic obstructive pulmonary disease. *Lancet Lond Engl*. 2015 Mar 7;385(9971):899–909.
41. Silva GE, Sherrill DL, Guerra S, Barbee RA. Asthma as a risk factor for COPD in a longitudinal study. *Chest*. 2004 Jul;126(1):59–65.
42. Guerra S, Sherrill DL, Venker C, Ceccato CM, Halonen M, Martinez FD. Chronic bronchitis before age 50 years predicts incident airflow limitation and mortality risk. *Thorax*. 2009 Oct;64(10):894–900.
43. GINA\_Report\_2015\_May19.pdf [Internet]. [cited 2015 Jul 14]. Available from: [http://www.ginasthma.org/local/uploads/files/GINA\\_Report\\_2015\\_May19.pdf](http://www.ginasthma.org/local/uploads/files/GINA_Report_2015_May19.pdf)
44. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal*. 2014 Mar 1;20(7):1126–67.
45. Chung KF, Marwick JA. Molecular mechanisms of oxidative stress in airways and lungs with reference to asthma and chronic obstructive pulmonary disease. *Ann N Y Acad Sci*. 2010 Aug;1203:85–91.
46. Taylor DR, Cowan JO, Greene JM, Willan AR, Sears MR. Asthma in remission: can relapse in early adulthood be predicted at 18 years of age? *Chest*. 2005 Mar;127(3):845–50.
47. Melgert BN, Ray A, Hylkema MN, Timens W, Postma DS. Are there reasons why adult asthma is more common in females? *Curr Allergy Asthma Rep*. 2007 May;7(2):143–50.
48. Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? *Thorax*. 1999 Mar;54(3):268–72.
49. de Nijs SB, Venekamp LN, Bel EH. Adult-onset asthma: is it really different? *Eur Respir Rev Off J Eur Respir Soc*. 2013 Mar 1;22(127):44–52.

50. Xie M, Wenzel SE. A global perspective in asthma: from phenotype to endotype. *Chin Med J (Engl)*. 2013 Jan;126(1):166–74.
51. Siroux V, Garcia-Aymerich J. The investigation of asthma phenotypes. *Curr Opin Allergy Clin Immunol*. 2011 Oct;11(5):393–9.
52. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med*. 2010 Feb 15;181(4):315–23.
53. Siroux V, Basagaña X, Boudier A, Pin I, Garcia-Aymerich J, Vesin A, et al. Identifying adult asthma phenotypes using a clustering approach. *Eur Respir J*. 2011 Aug;38(2):310–7.
54. Schatz M, Hsu J-WY, Zeiger RS, Chen W, Dorenbaum A, Chipps BE, et al. Phenotypes determined by cluster analysis in severe or difficult-to-treat asthma. *J Allergy Clin Immunol*. 2014 Jun;133(6):1549–56.
55. Prospero MCF, Sahiner UM, Belgrave D, Sackesen C, Buchan IE, Simpson A, et al. Challenges in identifying asthma subgroups using unsupervised statistical learning techniques. *Am J Respir Crit Care Med*. 2013 Dec 1;188(11):1303–12.
56. Siroux V, Boudier A, Bousquet J, Bresson J-L, Cracowski J-L, Ferran J, et al. Phenotypic determinants of uncontrolled asthma. *J Allergy Clin Immunol*. 2009 Oct;124(4):681–687.e3.
57. Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention possible? *Lancet Lond Engl*. 2015 Sep 12;386(9998):1075–85.
58. Dixon AE. Long-acting  $\beta$ -agonists and asthma: the saga continues. *Am J Respir Crit Care Med*. 2011 Dec 1;184(11):1220–1.
59. Masoli M, Fabian D, Holt S, Beasley R, Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy*. 2004 May;59(5):469–78.
60. Robinson CL, Baumann LM, Romero K, Combe JM, Gomez A, Gilman RH, et al. Effect of urbanisation on asthma, allergy and airways inflammation in a developing country setting. *Thorax*. 2011 Dec;66(12):1051–7.
61. Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med*. 2006 Nov 23;355(21):2226–35.
62. To T, Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA, et al. Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC Public Health*. 2012;12:204.
63. Bousquet J, Bousquet PJ, Godard P, Daures J-P. The public health implications of asthma. *Bull World Health Organ*. 2005 Jul;83(7):548–54.
64. L'asthme en France en 2006 : prévalence et contrôle des symptômes - Qes138.pdf [Internet]. [cited 2015 Aug 28]. Available from: <http://www.irdes.fr/Publications/Qes/Qes138.pdf>
65. Musharrafieh U, Al-Sahab B, Zaitoun F, El-Hajj MA, Ramadan F, Tamim H. Prevalence of asthma, allergic rhinitis and eczema among Lebanese adolescents. *J Asthma Off J Assoc Care Asthma*. 2009 May;46(4):382–7.

66. Waked M, Salameh P. Asthma, allergic rhinitis and eczema in 5-12-year-old school children across Lebanon. *Public Health*. 2008 Sep;122(9):965–73.
67. Murray CJL, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet Lond Engl*. 2012 Dec 15;380(9859):2197–223.
68. Pawankar R, Canonica GW, Holgate ST, Lockey RF. Allergic diseases and asthma: a major global health concern. *Curr Opin Allergy Clin Immunol*. 2012 Feb;12(1):39–41.
69. Anandan C, Nurmatov U, van Schayck OCP, Sheikh A. Is the prevalence of asthma declining? Systematic review of epidemiological studies. *Allergy*. 2010 Feb;65(2):152–67.
70. Cazzoletti L, Marcon A, Janson C, Corsico A, Jarvis D, Pin I, et al. Asthma control in Europe: a real-world evaluation based on an international population-based study. *J Allergy Clin Immunol*. 2007 Dec;120(6):1360–7.
71. Braman SS. The global burden of asthma. *Chest*. 2006 Jul;130(1 Suppl):4S–12S.
72. Programme d'action, de prévention et de prise en charge de l'asthme (2002-2005) - Ministère des Affaires sociales, de la Santé et des Droits des femmes - [www.sante.gouv.fr](http://www.sante.gouv.fr) [Internet]. [cited 2015 Aug 28]. Available from: <http://www.sante.gouv.fr/programme-d-action-de-prevention-et-de-prise-en-charge-de-l-asthme-2002-2005.html>
73. Bahadori K, Doyle-Waters MM, Marra C, Lynd L, Alasaly K, Swiston J, et al. Economic burden of asthma: a systematic review. *BMC Pulm Med*. 2009;9:24.
74. Accordini S, Corsico AG, Braggion M, Gerbase MW, Gislason D, Gulsvik A, et al. The cost of persistent asthma in Europe: an international population-based study in adults. *Int Arch Allergy Immunol*. 2013;160(1):93–101.
75. Bousquet J, Gern JE, Martinez FD, Anto JM, Johnson CC, Holt PG, et al. Birth cohorts in asthma and allergic diseases: report of a NIAID/NHLBI/MeDALL joint workshop. *J Allergy Clin Immunol*. 2014 Jun;133(6):1535–46.
76. Laprise C, Bouzigon E. To define the biological nature of asthma. *Curr Opin Allergy Clin Immunol*. 2011 Oct;11(5):391–2.
77. Kauffmann F, Demenais F. Gene-environment interactions in asthma and allergic diseases: challenges and perspectives. *J Allergy Clin Immunol*. 2012 Dec;130(6):1229-1240-1242.
78. Rava M, Smit LAM, Nadif R. Gene-environment interactions in the study of asthma in the postgenomewide association studies era. *Curr Opin Allergy Clin Immunol*. 2015 Feb;15(1):70–8.
79. Le Moual N, Jacquemin B, Varraso R, Dumas O, Kauffmann F, Nadif R. Environment and asthma in adults. *Presse Médicale Paris Fr* 1983. 2013 Sep;42(9 Pt 2):e317-333.
80. Sozańska B, Pearce N, Dudek K, Cullinan P. Consumption of unpasteurized milk and its effects on atopy and asthma in children and adult inhabitants in rural Poland. *Allergy*. 2013;68(5):644–50.
81. Varraso R, Oryszczyn MP, Mathieu N, Le Moual N, Boutron-Ruault MC, Clavel-Chapelon F, et al. Farming in childhood, diet in adulthood and asthma history. *Eur Respir J*. 2012 Jan;39(1):67–75.

82. Lemiere C, Ameille J, Boschetto P, Labrecque M, Pralong J-A. Occupational asthma: new deleterious agents at the workplace. *Clin Chest Med.* 2012 Sep;33(3):519–30.
83. Zock J-P, Vizcaya D, Le Moual N. Update on asthma and cleaners. *Curr Opin Allergy Clin Immunol.* 2010 Apr;10(2):114–20.
84. Pietinalho A, Pelkonen A, Ryttilä P. Linkage between smoking and asthma. *Allergy.* 2009 Dec;64(12):1722–7.
85. Varraso R. Nutrition and asthma. *Curr Allergy Asthma Rep.* 2012 Jun;12(3):201–10.
86. Eijkemans M, Mommers M, Draaisma JMT, Thijs C, Prins MH. Physical activity and asthma: a systematic review and meta-analysis. *PloS One.* 2012;7(12):e50775.
87. Sonnenschein-van der Voort AMM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, et al. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. *J Allergy Clin Immunol.* 2014 May;133(5):1317–29.
88. Rantala A, Jaakkola JJK, Jaakkola MS. Respiratory infections precede adult-onset asthma. *PloS One.* 2011;6(12):e27912.
89. AsthmaCOPDOverlap.pdf [Internet]. [cited 2015 Dec 17]. Available from: <http://www.goldcopd.org/uploads/users/files/AsthmaCOPDOverlap.pdf>
90. Gibson PG, McDonald VM. Asthma-COPD overlap 2015: now we are six. *Thorax.* 2015 Jul;70(7):683–91.
91. Vineis P, Chadeau-Hyam M. Integrating biomarkers into molecular epidemiological studies. *Curr Opin Oncol.* 2011 Jan;23(1):100–5.
92. Council TC on BM of the NR. Biological Markers in Environmental Health Research. *Environ Health Perspect.* 1987;74:3–9.
93. Perera FP, Weinstein IB. Molecular epidemiology and carcinogen-DNA adduct detection: new approaches to studies of human cancer causation. *J Chronic Dis.* 1982;35(7):581–600.
94. Schulte PA. A conceptual framework for the validation and use of biologic markers. *Environ Res.* 1989 Apr;48(2):129–44.
95. Pritzker KP, Pritzker LB. Bioinformatics advances for clinical biomarker development. *Expert Opin Med Diagn.* 2012 Jan;6(1):39–48.
96. Schulte PA. Biomarkers in epidemiology: scientific issues and ethical implications. *Environ Health Perspect.* 1992 Nov;98:143–7.
97. Vijverberg SJ, Hilvering B, Raaijmakers JA, Lammers J-WJ, Maitland-van der Zee A-H, Koenderman L. Clinical utility of asthma biomarkers: from bench to bedside. *Biol Targets Ther.* 2013;7:199–210.
98. Voynow JA, Kummarapurugu A. Isoprostanes and asthma. *Biochim Biophys Acta.* 2011 Nov;1810(11):1091–5.
99. Allam MH, Said AF, El Samie Omran AA, Abd El-Reheim DM, Kasem AH. High sensitivity C-reactive protein: its correlation with sputum cell counts in bronchial asthma. *Respir Med.* 2009 Dec;103(12):1878–84.

100. Kony S, Zureik M, Driss F, Neukirch C, Leynaert B, Neukirch F. Association of bronchial hyperresponsiveness and lung function with C-reactive protein (CRP): a population based study. *Thorax*. 2004 Oct;59(10):892–6.
101. Olafsdottir IS, Gislason T, Thjodleifsson B, Olafsson I, Gislason D, Jögi R, et al. C reactive protein levels are increased in non-allergic but not allergic asthma: a multicentre epidemiological study. *Thorax*. 2005 Jun;60(6):451–4.
102. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest*. 2003 Jun;111(12):1805–12.
103. Man SFP, Connett JE, Anthonisen NR, Wise RA, Tashkin DP, Sin DD. C-reactive protein and mortality in mild to moderate chronic obstructive pulmonary disease. *Thorax*. 2006 Oct;61(10):849–53.
104. Dahl M, Vestbo J, Zacho J, Lange P, Tybjaerg-Hansen A, Nordestgaard BG. C reactive protein and chronic obstructive pulmonary disease: a Mendelian randomisation approach. *Thorax*. 2011 Mar;66(3):197–204.
105. Stockley RA, O'Brien C, Pye A, Hill SL. Relationship of sputum color to nature and outpatient management of acute exacerbations of COPD. *Chest*. 2000 Jun;117(6):1638–45.
106. Peng C, Tian C, Zhang Y, Yang X, Feng Y, Fan H. C-reactive protein levels predict bacterial exacerbation in patients with chronic obstructive pulmonary disease. *Am J Med Sci*. 2013 Mar;345(3):190–4.
107. de Torres JP, Pinto-Plata V, Casanova C, Mullerova H, Córdoba-Lanús E, Muros de Fuentes M, et al. C-reactive protein levels and survival in patients with moderate to very severe COPD. *Chest*. 2008 Jun;133(6):1336–43.
108. Thorleifsson SJ, Margretardottir OB, Gudmundsson G, Olafsson I, Benediktsdottir B, Janson C, et al. Chronic airflow obstruction and markers of systemic inflammation: results from the BOLD study in Iceland. *Respir Med*. 2009 Oct;103(10):1548–53.
109. Garcia-Rio F, Miravittles M, Soriano JB, Muñoz L, Duran-Tauleria E, Sánchez G, et al. Systemic inflammation in chronic obstructive pulmonary disease: a population-based study. *Respir Res*. 2010;11:63.
110. Dickens JA, Miller BE, Edwards LD, Silverman EK, Lomas DA, Tal-Singer R, et al. COPD association and repeatability of blood biomarkers in the ECLIPSE cohort. *Respir Res*. 2011;12:146.
111. Lock-Johansson S, Vestbo J, Sorensen GL. Surfactant protein D, Club cell protein 16, Pulmonary and activation-regulated chemokine, C-reactive protein, and Fibrinogen biomarker variation in chronic obstructive lung disease. *Respir Res*. 2014;15:147.
112. Büyüköztürk S, Gelincik AA, Genç S, Koçak H, Oneriyidogan Y, Erden S, et al. Acute phase reactants in allergic airway disease. *Tohoku J Exp Med*. 2004 Nov;204(3):209–13.
113. Bafadhel M, Clark TW, Reid C, Medina M, Batham S, Barer MR, et al. Procalcitonin and C-reactive protein in hospitalized adult patients with community-acquired pneumonia or exacerbation of asthma or COPD. *Chest*. 2011 Jun;139(6):1410–8.
114. Zietkowski Z, Tomasiak-Lozowska MM, Skiepkó R, Mroczo B, Szmitkowski M, Bodzenta-Lukaszyk A. High-sensitivity C-reactive protein in the exhaled breath condensate and serum in stable and unstable asthma. *Respir Med*. 2009 Mar;103(3):379–85.



115. Wood LG, Baines KJ, Fu J, Scott HA, Gibson PG. The neutrophilic inflammatory phenotype is associated with systemic inflammation in asthma. *Chest*. 2012 Jul;142(1):86–93.
116. Qian F-H, Zhang Q, Zhou L-F, Liu H, Huang M, Zhang X-L, et al. High-sensitivity C-reactive protein: a predicative marker in severe asthma. *Respirol Carlton Vic*. 2008 Sep;13(5):664–9.
117. Takemura M, Matsumoto H, Niimi A, Ueda T, Matsuoka H, Yamaguchi M, et al. High sensitivity C-reactive protein in asthma. *Eur Respir J*. 2006 May;27(5):908–12.
118. Ramirez D, Patel P, Casillas A, Cotelingam J, Boggs P, Bahna SL. Assessment of high-sensitivity C-reactive protein as a marker of airway inflammation in asthma. *Ann Allergy Asthma Immunol Off Publ Am Coll Allergy Asthma Immunol*. 2010 Jun;104(6):485–9.
119. Panaszek B, Liebhart E, Liebhart J, Pawłowicz R, Fal AM. Serum concentration of C-reactive protein is not a good marker of bronchial hyperresponsiveness. *Arch Immunol Ther Exp (Warsz)*. 2007 Oct;55(5):341–5.
120. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999 Feb 11;340(6):448–54.
121. Takeda Y. Studies of the metabolism and distribution of fibrinogen in healthy men with autologous 125-I-labeled fibrinogen. *J Clin Invest*. 1966 Jan;45(1):103–11.
122. Fibrinogen Studies Collaboration, Kaptoge S, White IR, Thompson SG, Wood AM, Lewington S, et al. Associations of plasma fibrinogen levels with established cardiovascular disease risk factors, inflammatory markers, and other characteristics: individual participant meta-analysis of 154,211 adults in 31 prospective studies: the fibrinogen studies collaboration. *Am J Epidemiol*. 2007 Oct 15;166(8):867–79.
123. Eickhoff P, Valipour A, Kiss D, Schreder M, Cekici L, Geyer K, et al. Determinants of systemic vascular function in patients with stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2008 Dec 15;178(12):1211–8.
124. Engström G, Segelstorm N, Ekberg-Aronsson M, Nilsson PM, Lindgärde F, Löfdahl C-G. Plasma markers of inflammation and incidence of hospitalisations for COPD: results from a population-based cohort study. *Thorax*. 2009 Mar;64(3):211–5.
125. Duvoix A, Dickens J, Haq I, Mannino D, Miller B, Tal-Singer R, et al. Blood fibrinogen as a biomarker of chronic obstructive pulmonary disease. *Thorax*. 2013 Jul;68(7):670–6.
126. Jiang R, Burke GL, Enright PL, Newman AB, Margolis HG, Cushman M, et al. Inflammatory markers and longitudinal lung function decline in the elderly. *Am J Epidemiol*. 2008 Sep 15;168(6):602–10.
127. Cockayne DA, Cheng DT, Waschki B, Sridhar S, Ravindran P, Hilton H, et al. Systemic biomarkers of neutrophilic inflammation, tissue injury and repair in COPD patients with differing levels of disease severity. *PloS One*. 2012;7(6):e38629.
128. Sin DD, Leung R, Gan WQ, Man SP. Circulating surfactant protein D as a potential lung-specific biomarker of health outcomes in COPD: a pilot study. *BMC Pulm Med*. 2007;7:13.
129. Liu W, Ju C-R, Chen R-C, Liu Z-G. Role of serum and induced sputum surfactant protein D in predicting the response to treatment in chronic obstructive pulmonary disease. *Exp Ther Med*. 2014 Oct;8(4):1313–7.

130. Winkler C, Atochina-Vasserman EN, Holz O, Beers MF, Erpenbeck VJ, Krug N, et al. Comprehensive characterisation of pulmonary and serum surfactant protein D in COPD. *Respir Res.* 2011;12:29.
131. Lomas DA, Silverman EK, Edwards LD, Locantore NW, Miller BE, Horstman DH, et al. Serum surfactant protein D is steroid sensitive and associated with exacerbations of COPD. *Eur Respir J.* 2009 Jul;34(1):95–102.
132. Sorensen GL, Husby S, Holmskov U. Surfactant protein A and surfactant protein D variation in pulmonary disease. *Immunobiology.* 2007;212(4–5):381–416.
133. Hartl D, Griese M. Surfactant protein D in human lung diseases. *Eur J Clin Invest.* 2006 Jun;36(6):423–35.
134. Hoegh SV, Lindegaard HM, Sorensen GL, Høj A, Bendixen C, Junker P, et al. Circulating surfactant protein D is decreased in early rheumatoid arthritis: a 1-year prospective study. *Scand J Immunol.* 2008 Jan;67(1):71–6.
135. Hoegh SV, Sorensen GL, Tornøe I, Lottenburger T, Ytting H, Nielsen HJ, et al. Long-term stability and circadian variation in circulating levels of surfactant protein D. *Immunobiology.* 2010 Apr;215(4):314–20.
136. Sørensen GL, Hjelmberg J v B, Kyvik KO, Fenger M, Høj A, Bendixen C, et al. Genetic and environmental influences of surfactant protein D serum levels. *Am J Physiol Lung Cell Mol Physiol.* 2006 May;290(5):L1010-1017.
137. Zhao XM, Wu YP, Wei R, Cai HX, Tornøe I, Han JJ, et al. Plasma surfactant protein D levels and the relation to body mass index in a chinese population. *Scand J Immunol.* 2007 Jul;66(1):71–6.
138. Sorensen GL, Hjelmberg JVB, Leth-Larsen R, Schmidt V, Fenger M, Poulain F, et al. Surfactant protein D of the innate immune defence is inversely associated with human obesity and SP-D deficiency infers increased body weight in mice. *Scand J Immunol.* 2006 Dec;64(6):633–8.
139. Ju C-R, Liu W, Chen R-C. Serum surfactant protein D: biomarker of chronic obstructive pulmonary disease. *Dis Markers.* 2012;32(5):281–7.
140. Shakoori TA, Sin DD, Ghafoor F, Bashir S, Bokhari SNH. Serum surfactant protein D during acute exacerbations of chronic obstructive pulmonary disease. *Dis Markers.* 2009;27(6):287–94.
141. Moré JM, Voelker DR, Silveira LJ, Edwards MG, Chan ED, Bowler RP. Smoking reduces surfactant protein D and phospholipids in patients with and without chronic obstructive pulmonary disease. *BMC Pulm Med.* 2010;10:53.
142. Mutti A, Corradi M, Goldoni M, Vettori MV, Bernard A, Apostoli P. Exhaled metallic elements and serum pneumoproteins in asymptomatic smokers and patients with COPD or asthma. *Chest.* 2006 May;129(5):1288–97.
143. Engström G, Lindberg C, Gerhardsson de Verdier M, Nihlén U, Anderson M, Svartengren M, et al. Blood biomarkers and measures of pulmonary function--a study from the Swedish twin registry. *Respir Med.* 2012 Sep;106(9):1250–7.
144. Vestbo J, Edwards LD, Scanlon PD, Yates JC, Agustí A, Bakke P, et al. Changes in forced expiratory volume in 1 second over time in COPD. *N Engl J Med.* 2011 Sep 29;365(13):1184–92.

145. Koopmans JG, van der Zee JS, Krop EJM, Lopuhaä CE, Jansen HM, Batenburg JJ. Serum surfactant protein D is elevated in allergic patients. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2004 Dec;34(12):1827–33.
146. Burska A, Boissinot M, Ponchel F. Cytokines as biomarkers in rheumatoid arthritis. *Mediators Inflamm*. 2014;2014:545493.
147. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest*. 2008 Nov;118(11):3546–56.
148. Eberl G, Colonna M, Di Santo JP, McKenzie ANJ. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science*. 2015 May 22;348(6237):aaa6566.
149. Kelly EAB, Koziol-White CJ, Clay KJ, Liu LY, Bates ME, Bertics PJ, et al. Potential contribution of IL-7 to allergen-induced eosinophilic airway inflammation in asthma. *J Immunol Baltim Md 1950*. 2009 Feb 1;182(3):1404–10.
150. Walsh GM. Targeting eosinophils in asthma: current and future state of cytokine- and chemokine-directed monoclonal therapy. *Expert Rev Clin Immunol*. 2010 Sep;6(5):701–4.
151. Kattan M, Kumar R, Bloomberg GR, Mitchell HE, Calatroni A, Gergen PJ, et al. Asthma control, adiposity, and adipokines among inner-city adolescents. *J Allergy Clin Immunol*. 2010 Mar;125(3):584–92.
152. Arend WP, Guthridge CJ. Biological role of interleukin 1 receptor antagonist isoforms. *Ann Rheum Dis*. 2000 Nov;59 Suppl 1:i60-64.
153. Trifunović J, Miller L, Debeljak Ž, Horvat V. Pathologic patterns of interleukin 10 expression--a review. *Biochem Medica*. 2015;25(1):36–48.
154. Andreev K, Graser A, Maier A, Mousset S, Finotto S. Therapeutical measures to control airway tolerance in asthma and lung cancer. *Front Immunol*. 2012;3:216.
155. Hosoki K, Ying S, Corrigan C, Qi H, Kurosky A, Jennings K, et al. Analysis of a Panel of 48 Cytokines in BAL Fluids Specifically Identifies IL-8 Levels as the Only Cytokine that Distinguishes Controlled Asthma from Uncontrolled Asthma, and Correlates Inversely with FEV1. *PloS One*. 2015;10(5):e0126035.
156. Seys SF, Grabowski M, Adriaensen W, Decraene A, Dilissen E, Vanoirbeek JA, et al. Sputum cytokine mapping reveals an “IL-5, IL-17A, IL-25-high” pattern associated with poorly controlled asthma. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2013 Sep;43(9):1009–17.
157. Tsiligianni Z, Bakakos P, Papaioannou AI, Papaporfyriou A, Hillas G, Papiris S, et al. Sputum interleukin (IL)-13 as a biomarker for the evaluation of asthma control. *Clin Transl Allergy*. 2015 Mar 23;5(Suppl 2):O9.
158. Kato M, Yamada Y, Maruyama K, Hayashi Y. Serum eosinophil cationic protein and 27 cytokines/chemokines in acute exacerbation of childhood asthma. *Int Arch Allergy Immunol*. 2010;152 Suppl 1:62–6.
159. Sood A, Qualls C, Seagrave J, McDonald J, Shohreh R, Chiavaroli A, et al. Effect of allergen inhalation on airway oxidant stress, using exhaled breath condensate 8-isoprostane, in mild asthma. *J Asthma Off J Assoc Care Asthma*. 2013 Jun;50(5):449–56.

160. Zanconato S, Carraro S, Corradi M, Alinovi R, Pasquale MF, Piacentini G, et al. Leukotrienes and 8-isoprostane in exhaled breath condensate of children with stable and unstable asthma. *J Allergy Clin Immunol.* 2004 Feb;113(2):257–63.
161. Vestbo J, Anderson W, Coxson HO, Crim C, Dawber F, Edwards L, et al. Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE). *Eur Respir J.* 2008 Apr;31(4):869–73.
162. Celli BR, MacNee W, ATS/ERS Task Force. Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *Eur Respir J.* 2004 Jun;23(6):932–46.
163. Thomsen M, Ingebrigtsen TS, Marott JL, Dahl M, Lange P, Vestbo J, et al. Inflammatory biomarkers and exacerbations in chronic obstructive pulmonary disease. *JAMA.* 2013 Jun 12;309(22):2353–61.
164. Man SFP, Connett JE, Anthonisen NR, Wise RA, Tashkin DP, Sin DD. C-reactive protein and mortality in mild to moderate chronic obstructive pulmonary disease. *Thorax.* 2006 Oct;61(10):849–53.
165. Sigari N, Ghasri H. Correlation between hs-CRP and Asthma Control Indices. *Tanaffos.* 2013;12(3):44–8.
166. Kilic H, Karalezli A, Hasanoglu HC, Erel O, Ates C. The relationship between hs-CRP and asthma control test in asthmatic patients. *Allergol Immunopathol (Madr).* 2012 Dec;40(6):362–7.
167. Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. *J Allergy Clin Immunol.* 2015 Feb;135(2):299–310; quiz 311.
168. Hilvering B, Pavord ID. What goes up must come down: biomarkers and novel biologicals in severe asthma. *Clin Exp Allergy J Br Soc Allergy Clin Immunol.* 2015 Jul;45(7):1162–9.
169. Galera R, Casitas R, Martínez-Cerón E, Romero D, García-Río F. Does airway hyperresponsiveness monitoring lead to improved asthma control? *Clin Exp Allergy J Br Soc Allergy Clin Immunol.* 2015 Mar 26;
170. Piotrowski WJ, Majewski S, Marczak J, Kurmanowska Z, Górski P, Antczak A. Exhaled breath 8-isoprostane as a marker of asthma severity. *Arch Med Sci AMS.* 2012 Jul 4;8(3):515–20.
171. GOLD\_Report\_2013.pdf [Internet]. [cited 2015 Aug 18]. Available from: [http://www.goldcopd.org/uploads/users/files/GOLD\\_Report\\_2013.pdf](http://www.goldcopd.org/uploads/users/files/GOLD_Report_2013.pdf)
172. Antoniu SA. Effects of inhaled therapy on biomarkers of systemic inflammation in stable chronic obstructive pulmonary disease. *Biomark Biochem Indic Expo Response Susceptibility Chem.* 2010 Mar;15(2):97–103.
173. GINA\_Pocket2013\_May15\_1.pdf [Internet]. [cited 2015 Sep 17]. Available from: [http://www.ginasthma.org/local/uploads/files/GINA\\_Pocket2013\\_May15\\_1.pdf](http://www.ginasthma.org/local/uploads/files/GINA_Pocket2013_May15_1.pdf)
174. Husby S, Herskind AM, Jensenius JC, Holmskov U. Heritability estimates for the constitutional levels of the collectins mannan-binding lectin and lung surfactant protein D. A study of unselected like-sexed mono- and dizygotic twins at the age of 6-9 years. *Immunology.* 2002 Jul;106(3):389–94.

175. Ferris BG. Epidemiology Standardization Project (American Thoracic Society). *Am Rev Respir Dis.* 1978 Dec;118(6 Pt 2):1–120.
176. Stenton C. The MRC breathlessness scale. *Occup Med Oxf Engl.* 2008 May;58(3):226–7.
177. Leth-Larsen R, Nordenbaek C, Tornoe I, Moeller V, Schlosser A, Koch C, et al. Surfactant protein D (SP-D) serum levels in patients with community-acquired pneumonia. *Clin Immunol Orlando Fla.* 2003 Jul;108(1):29–37.
178. Akobeng AK. Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta Paediatr Oslo Nor* 1992. 2007 May;96(5):644–7.
179. The Area Under an ROC Curve [Internet]. [cited 2016 Feb 8]. Available from: <http://gim.unmc.edu/dxtests/roc3.htm>
180. Ilumets H, Mazur W, Toljamo T, Louhelainen N, Nieminen P, Kobayashi H, et al. Ageing and smoking contribute to plasma surfactant proteins and protease imbalance with correlations to airway obstruction. *BMC Pulm Med.* 2011;11:19.
181. Motulsky H. *Intuitive Biostatistics.* 1 edition. New York: Oxford University Press; 1995. 408 p.
182. Madsen J, Kliem A, Tornoe I, Skjodt K, Koch C, Holmskov U. Localization of lung surfactant protein D on mucosal surfaces in human tissues. *J Immunol Baltim Md* 1950. 2000 Jun 1;164(11):5866–70.
183. Ou C-Y, Chen C-Z, Hsiue T-R, Lin S-H, Wang J-Y. Genetic variants of pulmonary SP-D predict disease outcome of COPD in a Chinese population. *Respirol Carlton Vic.* 2015 Feb;20(2):296–303.
184. de Torres JP, Cordoba-Lanus E, López-Aguilar C, Muros de Fuentes M, Montejo de Garcini A, Aguirre-Jaime A, et al. C-reactive protein levels and clinically important predictive outcomes in stable COPD patients. *Eur Respir J.* 2006 May;27(5):902–7.
185. Kushner I, Rzewnicki D, Samols D. What does minor elevation of C-reactive protein signify? *Am J Med.* 2006 Feb;119(2):166.e17-28.
186. Inase N, Ohtani Y, Sumi Y, Umino T, Usui Y, Miyake S, et al. A clinical study of hypersensitivity pneumonitis presumably caused by feather duvets. *Ann Allergy Asthma Immunol Off Publ Am Coll Allergy Asthma Immunol.* 2006 Jan;96(1):98–104.
187. Tsushima K, Fujimoto K, Yoshikawa S, Kawakami S, Koizumi T, Kubo K. Hypersensitivity pneumonitis due to Bunashimeji mushrooms in the mushroom industry. *Int Arch Allergy Immunol.* 2005 Jul;137(3):241–8.
188. Um SJ, Lam S, Coxson H, Man SFP, Sin DD. Budesonide/formoterol enhances the expression of pro Surfactant Protein-B in lungs of COPD patients. *PloS One.* 2013;8(12):e83881.
189. Ozyurek BA, Ulasli SS, Bozbas SS, Bayraktar N, Akcay S. Value of serum and induced sputum surfactant protein-D in chronic obstructive pulmonary disease. *Multidiscip Respir Med.* 2013;8(1):36.
190. Johansson SL, Tan Q, Holst R, Christiansen L, Hansen NCG, Hojland AT, et al. Surfactant protein D is a candidate biomarker for subclinical tobacco smoke-induced lung damage. *Am J Physiol Lung Cell Mol Physiol.* 2014 May 1;306(9):L887-895.

191. Dahl M, Tybjaerg-Hansen A, Vestbo J, Lange P, Nordestgaard BG. Elevated plasma fibrinogen associated with reduced pulmonary function and increased risk of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2001 Sep 15;164(6):1008–11.
192. Salameh P, Khayat G, Waked M. Could symptoms and risk factors diagnose COPD? Development of a Diagnosis Score for COPD. *Clin Epidemiol*. 2012;4:247–55.
193. Foreman MG, Kong X, DeMeo DL, Pillai SG, Hersh CP, Bakke P, et al. Polymorphisms in surfactant protein-D are associated with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol*. 2011 Mar;44(3):316–22.
194. Sin DD, Man SFP, Marciniuk DD, Ford G, FitzGerald M, Wong E, et al. The effects of fluticasone with or without salmeterol on systemic biomarkers of inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2008 Jun 1;177(11):1207–14.
195. Kauffmann F, Dizier MH, Pin I, Paty E, Gormand F, Vervloet D, et al. Epidemiological study of the genetics and environment of asthma, bronchial hyperresponsiveness, and atopy: phenotype issues. *Am J Respir Crit Care Med*. 1997 Oct;156(4 Pt 2):S123-129.
196. Kauffmann F, Dizier MH, Annesi-Maesano I, Bousquet J, Charpin D, Demenais F, et al. EGEA (Epidemiological study on the Genetics and Environment of Asthma, bronchial hyperresponsiveness and atopy)-- descriptive characteristics. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 1999 Dec;29 Suppl 4:17–21.
197. Bouzigon E, Nadif R, Le Moual N, Dizier M-H, Aschard H, Boudier A, et al. [Genetic and environmental factors of asthma and allergy: Results of the EGEA study]. *Rev Mal Respir*. 2015 Mar 17;
198. ECRHS II Questionnaires and Protocols [Internet]. [cited 2015 Dec 21]. Available from: <http://www.ecrhs.org/Quests.htm>
199. Français [Internet]. The Union. [cited 2015 Dec 21]. Available from: <http://www.theunion.org/francais>
200. Institute NH Lung, and Blood. Considerations for diagnosing and managing asthma in the elderly: NAEPP working group report. National Institutes of Health, National Heart, Lung, and Blood Institute; 1995. 68 p.
201. chronique CEDCEDL [Luxembourg ] G de travail ad hoc É de la bronchite. Commentaires relatifs au “Questionnaire pour l’étude de la bronchite chronique et de l’emphysème pulmonaire (1967)”: établi, sous l’égide de la Commission de recherches Affections respiratoires chroniques, par un Groupe de travail ad hoc Épidémiologie de la bronchite chronique. Brille, D., Casula, D., Lende, R. Van Der, Smidt, U. Rapporteur: Minette, A. Office des publications officielles des Communautés européennes; 1971. 106 p.
202. [Air pollution and chronic respiratory disease. I. Methods and material (author’s transl)]. *Bull Eur Physiopathol Respir*. 1982 Feb;18(1):87–99.
203. Pekkanen J, Sunyer J, Anto JM, Burney P, European Community Respiratory Health Study. Operational definitions of asthma in studies on its aetiology. *Eur Respir J*. 2005 Jul;26(1):28–35.
204. Sunyer J, Pekkanen J, Garcia-Esteban R, Svanes C, Künzli N, Janson C, et al. Asthma score: predictive ability and risk factors. *Allergy*. 2007 Feb;62(2):142–8.
205. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. *Eur Respir J*. 2005 Aug;26(2):319–38.

206. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur Respir J*. 2012 Dec 1;40(6):1324–43.
207. Hermans C, Aly O, Nyberg BI, Peterson C, Bernard A. Determinants of Clara cell protein (CC16) concentration in serum: a reassessment with two different immunoassays. *Clin Chim Acta Int J Clin Chem*. 1998 Apr 27;272(2):101–10.
208. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009 May 5;150(9):604–12.
209. Common Statistical Methods for Clinical Research with SAS Examples, Third Edition - common statistical methods for clinical research with sas examples third edition 9781607642282 c20100518 [37].pdf [Internet]. [cited 2015 Aug 15]. Available from: <http://electronicsandbooks.com/eab1/manual/Publisher/S/SAS%20Institute/common%20statistical%20methods%20for%20clinical%20research%20with%20sas%20examples%20third%20edition%209781607642282%20c20100518%20%5B37%5D.pdf>
210. Woloshin S, Schwartz LM. Distribution of C-reactive protein values in the United States. *N Engl J Med*. 2005 Apr 14;352(15):1611–3.
211. Ishii S, Karlamangla AS, Bote M, Irwin MR, Jacobs DR, Cho HJ, et al. Gender, obesity and repeated elevation of C-reactive protein: data from the CARDIA cohort. *PLoS One*. 2012;7(4):e36062.
212. Butland BK, Strachan DP, Rudnicka AR. C-reactive protein, obesity, atopy and asthma symptoms in middle-aged adults. *Eur Respir J*. 2008 Jul;32(1):77–84.
213. Luna RCP, do Nascimento CCC, Ascitti LSR, Franceschini S do CC, Filizola RG, Diniz A da S, et al. Relation between glucose levels, high-sensitivity C-reactive protein (hs-CRP), body mass index (BMI) and serum and dietary retinol in elderly in population-based study. *Arch Gerontol Geriatr*. 2012 Jun;54(3):462–8.
214. Choi J, Joseph L, Pilote L. Obesity and C-reactive protein in various populations: a systematic review and meta-analysis. *Obes Rev Off J Int Assoc Study Obes*. 2013 Mar;14(3):232–44.
215. Khan UI, Rastogi D, Isasi CR, Coupey SM. Independent and synergistic associations of asthma and obesity with systemic inflammation in adolescents. *J Asthma Off J Assoc Care Asthma*. 2012 Dec;49(10):1044–50.
216. DeBoer MD. Obesity, systemic inflammation, and increased risk for cardiovascular disease and diabetes among adolescents: a need for screening tools to target interventions. *Nutr Burbank Los Angel Cty Calif*. 2013 Feb;29(2):379–86.
217. Hoshino M, Ohtawa J, Akitsu K. Increased C-reactive protein is associated with airway wall thickness in steroid-naïve asthma. *Ann Allergy Asthma Immunol Off Publ Am Coll Allergy Asthma Immunol*. 2014 Jul;113(1):37–41.
218. Wang T-N, Lin M-C, Wu C-C, Huang M-S, Leung SY, Huang C-C, et al. Role of gender disparity of circulating high-sensitivity C-reactive protein concentrations and obesity on asthma in Taiwan. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2011 Jan;41(1):72–7.
219. Asthma Control Test (ACT) - Adult - 337 Asthma Control Test Adult pdf.pdf [Internet]. [cited 2015 Jul 18]. Available from: <http://www.allergyassoc.net/337%20Asthma%20Control%20Test%20Adult%20pdf.pdf>

220. Razi E, Ehteram H, Akbari H, Chavoshi V, Razi A. Evaluation of high-sensitivity C-reactive protein in acute asthma. *Tanaffos*. 2012;11(1):32–7.
221. Shimoda T, Obase Y, Kishikawa R, Iwanaga T. Serum high-sensitivity C-reactive protein can be an airway inflammation predictor in bronchial asthma. *Allergy Asthma Proc Off J Reg State Allergy Soc*. 2015 Apr;36(2):e23-28.
222. 203-30: Principal Component Analysis versus Exploratory Factor Analysis - 203-30.pdf [Internet]. [cited 2015 Dec 30]. Available from: <http://www2.sas.com/proceedings/sugi30/203-30.pdf>
223. Kaiser HF. The application of electronic computers to factor analysis. *Educ Psychol Meas*. 1960;20:141–51.
224. Finkelman FD, Hogan SP, Hershey GKK, Rothenberg ME, Wills-Karp M. Importance of cytokines in murine allergic airway disease and human asthma. *J Immunol Baltim Md* 1950. 2010 Feb 15;184(4):1663–74.
225. Manise M, Holtappels G, Van Crombruggen K, Schleich F, Bachert C, Louis R. Sputum IgE and cytokines in asthma: relationship with sputum cellular profile. *PloS One*. 2013;8(3):e58388.
226. Ghebre MA, Bafadhel M, Desai D, Cohen SE, Newbold P, Rapley L, et al. Biological clustering supports both “Dutch” and “British” hypotheses of asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 2015 Jan;135(1):63–72.
227. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med*. 2000 Mar;161(3 Pt 1):769–74.
228. Hakonarson H, Herrick DJ, Serrano PG, Grunstein MM. Autocrine role of interleukin 1beta in altered responsiveness of atopic asthmatic sensitized airway smooth muscle. *J Clin Invest*. 1997 Jan 1;99(1):117–24.
229. Chung KF, Barnes PJ. Cytokines in asthma. *Thorax*. 1999 Sep;54(9):825–57.
230. Gao P, Gibson PG, Baines KJ, Yang IA, Upham JW, Reynolds PN, et al. Anti-inflammatory deficiencies in neutrophilic asthma: reduced galectin-3 and IL-1RA/IL-1 $\beta$ . *Respir Res*. 2015;16:5.
231. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. 2010 Sep 23;363(13):1211–21.
232. Oh J-W, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol*. 2002 Sep;110(3):460–8.
233. Tournoy KG, Kips JC, Pauwels RA. Endogenous interleukin-10 suppresses allergen-induced airway inflammation and nonspecific airway responsiveness. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2000 Jun;30(6):775–83.
234. Presser K, Schwinge D, Wegmann M, Huber S, Schmitt S, Quaas A, et al. Coexpression of TGF-beta1 and IL-10 enables regulatory T cells to completely suppress airway hyperreactivity. *J Immunol Baltim Md* 1950. 2008 Dec 1;181(11):7751–8.



235. Komakula S, Khatri S, Mermis J, Savill S, Haque S, Rojas M, et al. Body mass index is associated with reduced exhaled nitric oxide and higher exhaled 8-isoprostanes in asthmatics. *Respir Res.* 2007;8:32.
236. Zock J-P, Rodríguez-Trigo G, Rodríguez-Rodríguez E, Souto-Alonso A, Espinosa A, Pozo-Rodríguez F, et al. Evaluation of the persistence of functional and biological respiratory health effects in clean-up workers 6 years after the prestige oil spill. *Environ Int.* 2014 Jan;62:72–7.
237. Montuschi P, Corradi M, Ciabattini G, Nightingale J, Kharitonov SA, Barnes PJ. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med.* 1999 Jul;160(1):216–20.
238. Baraldi E, Carraro S, Alinovi R, Pesci A, Ghiro L, Bodini A, et al. Cysteinyl leukotrienes and 8-isoprostane in exhaled breath condensate of children with asthma exacerbations. *Thorax.* 2003 Jun;58(6):505–9.
239. Nobakht M Gh BF, Aliannejad R, Rezaei-Tavirani M, Taheri S, Oskouie AA. The metabolomics of airway diseases, including COPD, asthma and cystic fibrosis. *Biomark Biochem Indic Expo Response Susceptibility Chem.* 2015 Feb;20(1):5–16.
240. Schermer TRJ, Smeele IJM, Thoonen BPA, Lucas AEM, Grootens JG, van Boxem TJ, et al. Current clinical guideline definitions of airflow obstruction and COPD overdiagnosis in primary care. *Eur Respir J.* 2008 Oct;32(4):945–52.
241. Vollmer WM, Gíslason T, Burney P, Enright PL, Gulsvik A, Kocabas A, et al. Comparison of spirometry criteria for the diagnosis of COPD: results from the BOLD study. *Eur Respir J.* 2009 Sep;34(3):588–97.
242. Hansen JE, Sun X-G, Wasserman K. Spirometric criteria for airway obstruction: Use percentage of FEV1/FVC ratio below the fifth percentile, not < 70%. *Chest.* 2007 Feb;131(2):349–55.
243. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. *Eur Respir J.* 2005 Nov;26(5):948–68.
244. Güder G, Brenner S, Angermann CE, Ertl G, Held M, Sachs AP, et al. GOLD or lower limit of normal definition? A comparison with expert-based diagnosis of chronic obstructive pulmonary disease in a prospective cohort-study. *Respir Res.* 2012;13(1):13.
245. Sofia M, Maniscalco M, de Laurentiis G, Paris D, Melck D, Motta A. Exploring airway diseases by NMR-based metabolomics: a review of application to exhaled breath condensate. *J Biomed Biotechnol.* 2011;2011:403260.
246. Bossley CJ, Fleming L, Gupta A, Regamey N, Frith J, Oates T, et al. Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines. *J Allergy Clin Immunol.* 2012 Apr;129(4):974–982.e13.
247. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirol Carlton Vic.* 2006 Jan;11(1):54–61.
248. Fleming L, Tsartsali L, Wilson N, Regamey N, Bush A. Sputum inflammatory phenotypes are not stable in children with asthma. *Thorax.* 2012 Aug;67(8):675–81.
249. Wang F, He XY, Baines KJ, Gunawardhana LP, Simpson JL, Li F, et al. Different inflammatory phenotypes in adults and children with acute asthma. *Eur Respir J.* 2011 Sep;38(3):567–74.

250. Little FF, Delgado DM, Wexler PJ, Oppenheim FG, Mitchell P, Feldman JA, et al. Salivary inflammatory mediator profiling and correlation to clinical disease markers in asthma. *PloS One*. 2014;9(1):e84449.
251. Dunn RM, Wechsler ME. Anti-interleukin therapy in asthma. *Clin Pharmacol Ther*. 2015 Jan;97(1):55–65.
252. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. *J Allergy Clin Immunol*. 2002 Jul;110(1):28–34.
253. Kharitonov SA, Barnes PJ. Exhaled markers of inflammation. *Curr Opin Allergy Clin Immunol*. 2001 Jun;1(3):217–24.
254. Baraldi E, Ghiro L, Piovan V, Carraro S, Zacchello F, Zanconato S. Safety and success of exhaled breath condensate collection in asthma. *Arch Dis Child*. 2003 Apr;88(4):358–60.
255. Farrington DP. Longitudinal research strategies: advantages, problems, and prospects. *J Am Acad Child Adolesc Psychiatry*. 1991 May;30(3):369–74.
256. Schwartz D, Lellouch J. Explanatory and pragmatic attitudes in therapeutical trials. *J Chronic Dis*. 1967 Aug;20(8):637–48.
257. Kostrzewa A, Reungoat P, Raheison C. Validity of a traffic air pollutant dispersion model to assess exposure to fine particles. *Environ Res*. 2009 Aug;109(6):651–6.
258. Sheehan WJ, Phipatanakul W. Difficult-to-control asthma: epidemiology and its link with environmental factors. *Curr Opin Allergy Clin Immunol*. 2015 Oct;15(5):397–401.
259. Social Care Online | Active ageing: a strategic policy solution to demographic ageing in the European Union [Internet]. [cited 2015 Dec 14]. Available from: <http://www.scie-socialcareonline.org.uk/active-ageing-a-strategic-policy-solution-to-demographic-ageing-in-the-european-union/r/a1CG0000000GR0BMAW>