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Diagnosis and clinical interest of asthma inflammatory phenotypes

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“*Coming together is a beginning;*

keeping together is progress; working

together is success».

Henry Ford

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List of Abbreviations

ACQ: Asthma Control Questionnaire.

AQLQ: Asthma Quality of Life Questionnaire.

AUC: Area Under the Curve.

CRP: C-Reactive Protein.

eNose: Electronic nose.

FENO: Exhaled nitric oxide.

FEV_1: Forced expiratory volume in 1 second.

FRC: Functional residual capacity

ICS: Inhaled Corticosteroids. Low dose: ≤500µg/day beclomethasone; Moderate dose: 500-1000µg/day beclomethasone; High dose: >1000µg/day beclomethasone.

LABA: Long Acting β-2 Agonist.

LTRA: Leukotriene Receptor Antagonist.

NPV: Negative Predictive Value

PC20M: Concentration of methacholine required to provoke a fall in FEV_1 of 20% or more

PEF: Peak expiratory flow

PPV: Positive Predictive Value.

SA: Severe asthma.

SRA: Severe refractory asthma.

TLC: Total lung capacity.

VC: Vital Capacity

VOCs: Volatile organic compounds

Summary

Asthma is a complex airway disease with many different underlying mechanisms. Therefore it tends to be considered as a syndrome containing several subtypes sharing important similarities but also displaying differences caused by variable aetiologies. We propose that a phenotype should be comprised of those clinical characteristics of a disease that have prognostic or therapeutic implications that are disease-specific. The classification of asthmatics in different inflammatory phenotypes according to induced sputum cell count has already been shown to predict the response to targeted therapy.

In the first part of this work, we have confirmed on a large unselected asthmatic cohort the heterogeneity of airway inflammation and provided the distribution of each inflammatory phenotype as encountered in patients seen in a university hospital. The most frequently observed inflammatory subtypes were eosinophilic and paucigranulocytic phenotypes. The same approach for severe asthmatics showed that the proportion of paucigranulocytic asthma was very low in this subgroup, supporting the role of uncontrolled airway inflammation as a key factor in determining asthma severity.

In the second part of this thesis, we described the demographic, functional and inflammatory characteristics of the different inflammatory phenotypes. We showed that eosinophilic asthma was more prone to exacerbations.

In the third part, we have shown that factors associated with airway eosinophilic differ from those associated with airway neutrophilic phenotype. Independent predictors of

eosinophilic asthma were blood eosinophils, elevated FENO levels, high serum IgE levels and low FEV_1/FVC ratio while for neutrophilic asthma we found higher FRC and age. We have been able to show that FENO and blood eosinophil thresholds that predict airway eosinophilic inflammation may be influenced by the dose of inhaled corticosteroids and smoking history for the former. We have also found that Volatile Organic Compounds are able to discriminate between airway eosinophilic and neutrophilic inflammatory cell types.

In the fourth part of this work, we have evaluated the value of exhaled nitric oxide as a marker of airway hyperresponsiveness. We have shown that FENO measurement may be useful in diagnosing asthma in patients with chronic respiratory symptoms in whom bronchodilation test failed or was not appropriate to demonstrate asthma. In this pre-specified asthma population, the value of FENO essentially lies in its high specificity, and a FENO level lower than the best threshold (34ppb) should prompt the clinician to ask for a methacholine challenge to confirm asthma diagnosis.

In the fifth part of this thesis we have proposed a new way to classify asthma based on compartmental eosinophilic inflammation. By looking simultaneously at blood and sputum eosinophil count we have identified a subgroup of asthmatics exhibiting both blood and airway eosinophilic inflammation. This subgroup included a majority of males, had lower asthma control, poorer lung function and was more prone to exacerbation. With respect to co-morbidities, this group also had nasal polyposis more often. We believe our new classification may be useful as we now have available treatments targeting either airway (ICS) or systemic (OCS, anti-IL5) eosinophilic inflammation.

Conclusion

It is becoming increasingly clear that classification of asthmatics according to various inflammatory phenotypes will guide the choice of treatment. Induced sputum is the gold standard non-invasive tool for inflammatory phenotyping. We have provided evidence that this technique may be routinely applied in a university centre. We have also proposed a new classification of inflammatory phenotypes based on blood and airway eosinophilia, a classification which identified a subgroup of patients with diffuse eosinophilic inflammation with poor asthma control and higher risk of exacerbation. As we understand induced sputum may never be applied in primary or some secondary care settings we have also identified surrogate markers for inflammatory phenotypes.

Résumé

L'asthme est une maladie complexe des voies respiratoires avec de nombreux mécanismes sous-jacents. Pour cette raison, l'asthme est souvent considéré comme un syndrome avec plusieurs sous-types partageant des similitudes importantes mais également des différences liées à des étiologies variables. Nous proposons que chaque phénotype corresponde à un ensemble de caractéristiques cliniques d'une maladie qui a des implications pronostiques ou thérapeutiques et qui est spécifique de cette pathologie. La classification des asthmatiques en différents phénotypes inflammatoires en fonction du nombre de cellules présentes dans l'expectoration induite a prouvé son efficacité dans la prédiction de la réponse à une thérapie ciblée.

Dans la première partie de ce travail, nous avons confirmé l'hétérogénéité de l'inflammation des voies aériennes sur une large cohorte d'asthmatiques non sélectionnés. Nous avons également présenté la distribution de chaque phénotype inflammatoire sachant que les patients étudiés ont été recrutés dans un hôpital universitaire. Les sous-types inflammatoires les plus fréquemment observés sont le phénotype éosinophilique et paucigranulocytique. Nous avons utilisé la même approche pour les asthmatiques sévères et avons observé que la proportion d'asthmatiques paucigranulocytiques était très faible dans ce sous-groupe. Cette observation renforce le rôle de l'inflammation non contrôlée des voies aériennes comme facteur clé dans la détermination de la sévérité de l'asthme.

Dans la deuxième partie de cette thèse, nous avons décrit les caractéristiques démographiques, fonctionnelles et inflammatoires des différents phénotypes inflammatoires. Nous avons montré que l'asthme éosinophilique était plus enclin aux

exacerbations.

Dans la troisième partie, nous avons montré que les facteurs associés à l'inflammation éosinophilique des voies respiratoires diffèrent de ceux associés au phénotype neutrophilique. Les facteurs prédictifs indépendants de l'asthme éosinophilique étaient les éosinophiles sanguins, un taux élevé de FENO, des taux d'IgE sériques élevés et un faible rapport VEMS/CVF tandis que pour l'asthme neutrophilique les facteurs de prédiction étaient une capacité résiduelle fonctionnelle plus élevée et un âge plus avancé. Nous avons pu montrer que les seuils de FENO et d'éosinophiles sanguins qui permettent de prédire l'inflammation éosinophilique peuvent être influencés par la dose de corticoïdes inhalés et les antécédents de tabagisme pour le FENO. Nous avons également trouvé que les composés organiques volatiles sont capables de discriminer le phénotype éosinophilique du neutrophilique.

Dans la quatrième partie de ce travail, nous avons évalué la valeur du monoxyde d'azote exhalé comme marqueur de l'hyperréactivité bronchique. Nous avons montré que la mesure du FENO peut être utile dans le diagnostic de l'asthme chez les patients présentant des symptômes respiratoires chroniques pour lesquels un test de bronchodilatation a échoué à démontrer l'asthme ou n'était pas approprié vu des valeurs spirométriques de base supranormales. Dans cette population spécifique d'asthmatique, la valeur du FENO tient surtout à sa spécificité élevée mais une valeur de FENO inférieure à 34ppb devrait convaincre le clinicien de demander un test de provocation à la méthacholine pour confirmer la pathologie asthmatique.

Dans la cinquième partie de cette thèse, nous avons proposé une nouvelle façon de classer l'asthme, basée sur la compartimentalisation de l'inflammation éosinophilique. En regardant simultanément le nombre d'éosinophiles du sang et des expectorations, nous avons identifié un sous-groupe d'asthmatiques présentant à la fois une inflammation éosinophilique systémique et bronchique. Ce sous-groupe incluait une majorité d'hommes, avait le moins bon contrôle de l'asthme, la fonction pulmonaire la plus mauvaise et était plus enclin à présenter des exacerbations. En ce qui concerne les comorbidités, ce groupe présentait aussi plus souvent de la polypose nasale. Nous pensons que notre nouvelle classification peut être utile vu la disponibilité de traitements ciblant l'inflammation éosinophilique des voies aériennes (CSI) ou systémique (CSO, anti-IL5).

Conclusion

Il est de plus en plus évident que la classification des asthmatiques selon divers phénotypes inflammatoires guidera le choix du traitement. L'analyse de l'expectoration induite est l'outil non invasif de choix pour le phénotypage inflammatoire. Nous avons apporté la preuve que cette technique peut être appliquée en routine dans un centre universitaire. Nous avons également proposé une nouvelle classification des phénotypes inflammatoires sur base de l'éosinophilie des voies aérienne et systémique, une classification qui a permis d'identifier un sous-groupe de patients présentant une inflammation éosinophilique diffuse avec un mauvais contrôle de l'asthme et un risque plus élevé d'exacerbation. Comme nous sommes conscients du fait que la technique de l'expectoration induite ne pourra jamais être appliquée en pratique clinique dans les centres non universitaires, nous avons

également identifié des marqueurs de substitution pour l'identification des phénotypes inflammatoires.

Samenvatting

Astma is een complexe luchtwegaandoening met veel verschillende onderliggende mechanismen. Daarom wordt astma vaak beschouwd als een syndroom bestaande uit meerdere subtypes die enerzijds belangrijke overeenkomsten vertonen maar anderzijds ook belangrijke verschillen, die geassocieerd zijn met de verscheidenheid in de ontstaansmechanismen. Wij stellen voorop dat elk astma fenotype zou moeten bestaan uit een reeks klinische kenmerken van de ziekte die een prognostische of therapeutische waarde hebben, en die ziektespecifiek zijn.

Er werd reeds aangetoond dat de classificatie van astma patiënten in verschillende inflammatoire fenotypes op basis van de cellulaire samenstelling van het geïnduceerd sputum de reactie op een gerichte therapie kan voorspellen.

In het eerste deel van dit proefschrift, hebben we de heterogeniteit van de luchtweginflammatie bevestigd op een grote cohorte van ongeselecteerde astmapatiënten en hebben we de distributie van elk inflammatoire fenotype beschreven, zoals deze werd waargenomen bij de patiënten die werden gerekruteerd in een universitair ziekenhuis. De meest frequent geobserveerde inflammatoire subtypes waren het eosinofiele en het paucigranulocitaire fenotype.

Wanneer dezelfde onderzoeksbenadering werd gebruikt bij ernstig astmapatiënten bleek dat het aandeel van paucigranulocytic astma heel laag was in deze subgroep. Deze observatie versterkt de rol van ongecontroleerde luchtweginflammatie als bepalende factor in de ernst van astma.

In een tweede deel van dit proefschrift beschreven we de demografische, functionele en inflammatoire kenmerken van de verschillende inflammatoire fenotypes. We toonden aan dat eosinofiel astma meer gevoelig is voor exacerbaties.

In een derde deel, hebben we aangetoond dat de factoren die geassocieerd zijn met eosinofiele luchtweginflammatie verschillend zijn van deze die geassocieerd zijn met het neutrofiele fenotype. Onafhankelijke predictors van eosinofiel astma waren bloedeosinofielen, verhoogde FENO waarden, hoge niveaus van serum IgE en een lage FEV_1/FVC verhouding. Voor neutrofiel astma was dit hogere FRC en hogere leeftijd.

We konden aantonen dat de drempelwaarden voor bloedeosinofielen en FENO, die een eosinofilie luchtweginflammatie voorspellen, beïnvloed kunnen worden door de dosis inhalatiecorticosteroïden. De FENO waarden worden ook beïnvloed door de rookgeschiedenis. We konden eveneens aantonen dat er een onderscheid kan gemaakt worden tussen eosinofiele en neutrofiele fenotype op basis van vluchtige organische componenten in de uitgeademde lucht.

In een vierde deel van dit proefschrift, hebben we de waarde van uitgeademd stikstofoxide als merker van luchtweghyperresponsiviteit geëvalueerd.

We toonden aan dat FENO bepalingen nuttig kunnen zijn in de diagnose van astma bij patiënten met chronische respiratoire symptomen waarbij de bronchodilatortest mislukte of niet van toepassing was gezien de supra normale longfunctiewaarden. In deze specifieke astmapopulatie ligt het belang van FENO voornamelijk in zijn hoge specificiteit. Toch een FENO waarde onder 34ppb zou de clinicus moeten overtuigen

een methacholine provocatietest aan te vragen om de diagnose alsnog te bevestigen.

In het vijfde deel van deze thesis hebben we een nieuwe methode voorgesteld om astma te classificeren, gebaseerd op de compartimentalisatie van de eosinofiele inflammatie. Door gelijktijdig eosinofiel aantallen in bloed en sputum te bestuderen hebben we een subgroep van astmatici geïdentificeerd, die gekarakteriseerd wordt door een eosinofiele inflammatie, zowel systemisch als in de luchtwegen.

Deze subgroep bestond voornamelijk uit mannen, had een slechtere astmacontrole, een slechtere longfunctie en was gevoeliger voor exacerbaties. Wat betreft de comorbiditeiten, had deze groep ook vaak neuspoliepen.

We denken dat deze nieuwe classificatie nuttig kan zijn, daar er nu behandelingen bestaan die enerzijds gericht zijn op de eosinofiele luchtweginflammatie (inhalatiecorticosteroïden) of anderzijds op systemische eosinofiele inflammatie (orale corticosteroïden, anti-IL-5).

Conclusie

Het wordt steeds duidelijker dat de classificatie van astmapatiënten op basis van inflammatoire fenotypes zal bijdragen tot de keuze van behandeling.

Geïnduceerd sputum is de gouden standaard als niet-invasieve methode voor inflammatoire fenotypering. We hebben aangetoond dat deze techniek als routineonderzoek kan gebruikt worden in een universitair centrum.

We hebben ook een nieuwe classificatie van inflammatoire fenotypes voorgesteld, gebaseerd op bloed- en luchtwegeosinofilie, een classificatie die een subgroep van patiënten identificeerde met een diffuse eosinofiele inflammatie, slechte astmacontrole en hoger risico op exacerbaties.

Vermits we begrijpen dat de techniek van geïnduceerd sputum mogelijks nooit in de klinische praktijk zal worden toegepast in niet-universitaire centra, hebben we eveneens surrogaat merkers geïdentificeerd voor de identificatie van inflammatoire fenotypes.

Chapter 1. Introduction

1.1 Definition of asthma

Asthma is a heterogeneous disease of the airways resulting from a complex interaction between genetic and environmental factors. It is a chronic inflammatory disease of the airways in which various cells and mediators play a role (1). Type 2 high asthma is characterised by increased cytokines following a TH2 pattern. The traditional guidelines for asthma diagnosis include suggestive clinical symptoms and the demonstration of airflow variability. However, symptoms and lung function are relatively insensitive in reflecting the underlying airway inflammation (2;3).

1.2 Asthma management

The aim of asthma management is to obtain asthma control and to reduce the risks of exacerbations (4). Treatment schemes follow a step-wise approach based on the level of severity and on the level of symptoms reported by asthmatics (5). All these parameters are subjective and involve patients' perception. Monitoring of airway inflammation provides objective markers of assessment but requires time, as well as technical and financial resources. The importance of managing asthma on the basis of inflammatory biomarkers is highlighted in patients with discordance between symptoms and airway inflammation (6). Patients with discordance between symptoms and eosinophilic inflammation were the ones who benefited from inflammation-guided management (Fig 1).

1.3 Asthma inflammatory phenotypes

As knowledge of airways disease has grown it has become apparent that asthma is not a simple, easily defined disease. Bronchial asthma is a complex disease of the airways with many different underlying mechanisms. It is now considered as a syndrome containing several subtypes with similarities and differences caused by variable underlying aetiologies (7). In the past, treatment options for asthma were limited; hence the need to define subtypes was not present. According to GINA guidelines, asthma treatment must be adjusted according to asthma control, rescue medication consumption, lung function and the risk of exacerbations. However, the relationship between airway inflammation and asthma symptoms or lung function is uncertain. As treatment options have grown so has our need to predict who will best respond to therapies, and optimise quality of life by reducing the risk of future events (such as exacerbations) that impair it. There is a need to focus on asthma subtypes by detailed clinical characterisation or differences in pathobiology. These subtypes are commonly called ‘phenotypes’. Progression in asthma is known to be much more complex than deterioration in lung function alone and harder to compare effectively between subtypes. The inherent variability of asthma also complicates the assessment of overall prognosis. Several phenotypes described in the literature are based on clinical features found in asthmatics databases and cluster analyses. Molecular and genetic approaches have also been widely used to categorise patients (8). This merging of clinical and pathophysiological features has been reviewed extensively elsewhere (9), often using the word endotype to delineate this way of defining disease.

1.3.1 Clinical interest of phenotyping

Studies based on factor analysis tell us that airway inflammation is an independent domain of the asthma syndrome, distinct from airways hyperresponsiveness, abnormal lung function and respiratory symptoms (2;6). Common maintenance treatments of asthma such as anti-leukotrienes or inhaled corticosteroids and new biotherapies are directed towards modifying the underlying inflammatory process rather than improving symptoms. An example was given by the studies on treatment targeting IL-5, a very specific pathway in asthma. This treatment did not significantly improve an unselected population of severe asthmatics while it improved asthma control and reduced exacerbation in selected patients exhibiting eosinophilic phenotype despite a treatment with high doses of inhaled corticosteroids, in which IL-5 pathway is important (10;11).

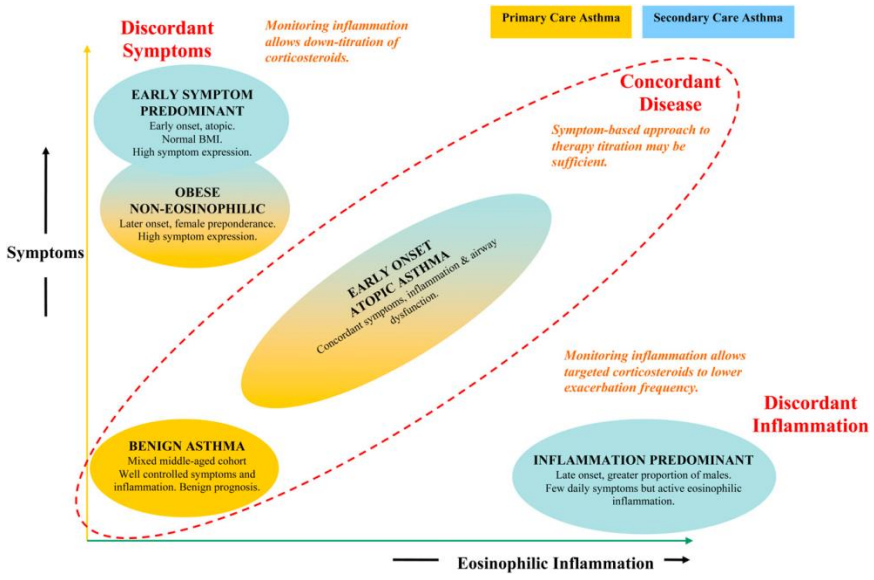

Fig 1. Cluster analysis to identify clinical phenotypes of asthma in primary- and secondary-care asthma populations. Although reasons for this dissociation between symptoms and eosinophilic inflammation are unclear, the use of measures of airway inflammation in these subgroups is clinically informative (Reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society. Haldar *et al.*, 2008, Am J Respir Crit Care Med Vol 178. pp 218–224. Official Journal of the American Thoracic Society (6)).

1.3.2 Inflammatory pathways

Based on sputum cell analysis, four inflammatory phenotypes have been described in the literature: eosinophilic, neutrophilic, paucigranulocytic and mixed granulocytic phenotype. The importance of these inflammatory phenotypes is that the underlying molecular mechanisms are different. While the eosinophilic phenotype is likely to reflect ongoing adaptive immunity in response to allergen with Th2 cytokine IL-4, IL-5 and IL-13 playing a key role, the neutrophilic is thought to reflect innate immune system activation in response to pollutants or infectious agents (9;12) (Fig 2). Therefore it is conceivable that the two phenotypes actually require different therapeutic molecular approaches.

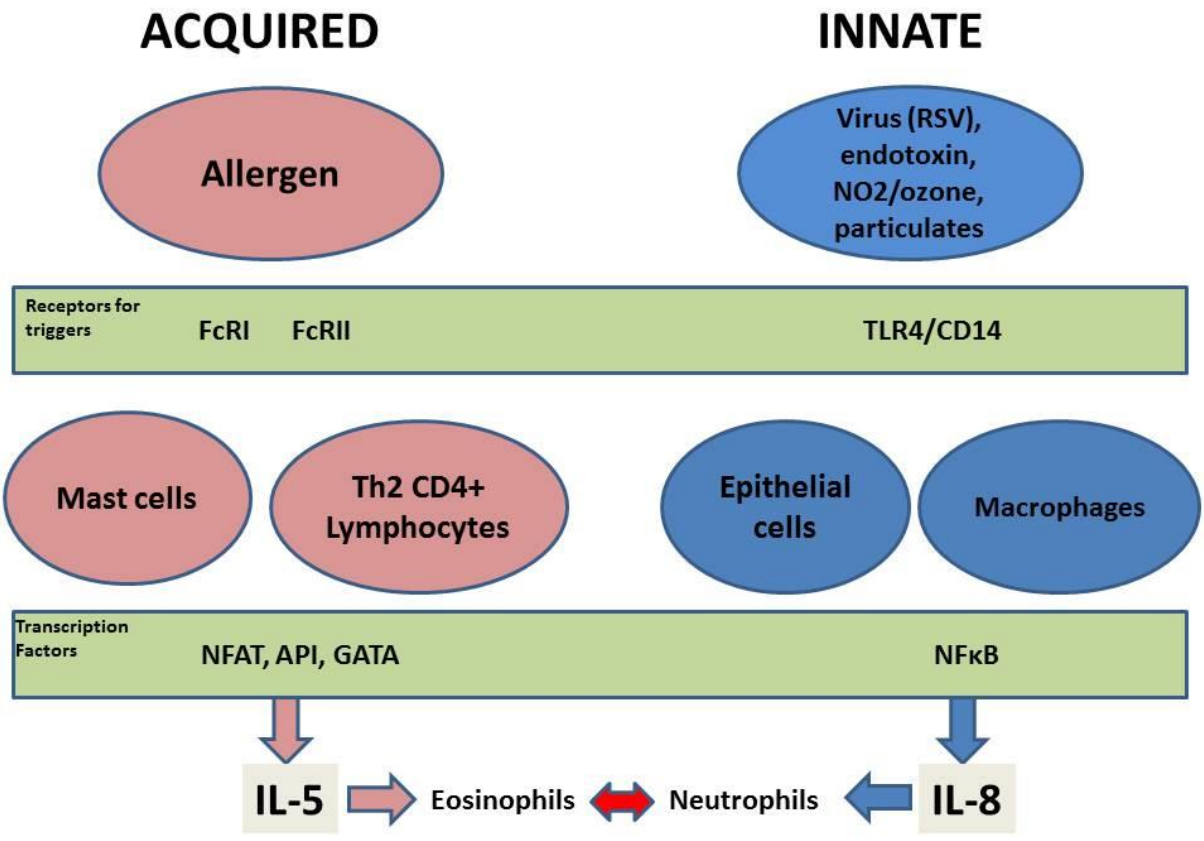

Fig 2. Acquired and innate immune pathways leading to IL-5 mediated eosinophil inflammation (acquired pathway) or IL-8 mediated neutrophil inflammation (innate pathway). Adapted from Douwes *et al.*Thorax 2002.

1.3.3 Targeted therapy

Phenotyping asthma according to airway inflammation can thus allow the identification of subgroups of patients who are more likely to respond to targeted therapy.

1.3.3.1 Eosinophilic phenotype

In particular, important studies have confirmed that eosinophilic airway inflammation most reliably predicts the response to anti-inflammatory treatment such as inhaled corticosteroids (13;14) and anti-IL5 (15;16). Numerous studies showed that regular treatments with ICS sharply and quickly reduce the percentage of eosinophils contained in the sputum from asthmatics (17;18), repress the release of Th2 cytokines from lymphocytes (19), and eotaxin from epithelial cells (20). ICS are particularly effective in combating Th2-driven inflammation featuring mast cell and eosinophilic airway infiltration. Their effect on innate immunity-driven neutrophilic inflammation is rather poor.

Several studies have demonstrated the usefulness of induced sputum to guide asthma treatment (21;22). These studies showed that targeting the sputum eosinophil count <2-3% for adjustment in the dose of ICS in moderate to severe asthma results in a marked reduction in asthma exacerbations and hospitalisations. This reduction of exacerbations was obtained without an increase in total dose of inhaled corticosteroids. Sputum eosinophil count follow-up has allowed selecting patients requiring high doses of inhaled corticosteroids and those with non-eosinophilic asthma in whom corticosteroid doses have been decreased without worsening of asthma control (21). Another study including patients with refractory

eosinophilic asthma (sputum eosinophil count >2%) has shown that triamcinolone intramuscular administration was able to abolish sputum eosinophilic inflammation. This suggests that patients in whom asthma remains uncontrolled despite high-dose inhaled corticosteroids may be improved by systemic corticosteroid administration (23). This important study points to a reduced sensitivity rather than to a real resistance of eosinophilic inflammation to corticosteroids.

1.3.3.2 Non-eosinophilic phenotypes

There is no evidence that inhaled corticosteroids may improve short-term asthma control in the absence of uncontrolled eosinophilic inflammation as encountered in pauci-granulocytic asthma (14;24). On the other hand data suggest that severe neutrophilic asthma could be best targeted by using clarithromycin (25;26). In neutrophilic asthma, absolute value and percentages of sputum neutrophils have been associated with altered FEV_1 suggesting that neutrophilic inflammation could play a role in lung function decline in asthmatics (27;28). Anti-inflammatory properties of macrolides include a decrease in IL-8 and a reduction in neutrophils recruitment and activation.

Characterising the inflammatory phenotype in patients with chronic respiratory symptoms can thus be more important than giving an “asthma” label to predict response to anti-inflammatory treatment.

1.3.4 Prediction of the risk of exacerbations and lung function decline

Induced sputum analysis can allow the clinician to use inflammatory cell characteristics to interpret the aetiology of a patient’s symptoms and to predict the risk of exacerbations and prognosis. It has been shown that induced sputum was

able to detect an increase in eosinophilic inflammation after allergen challenge in sensitised asthmatics (29;30). Exacerbations are indeed associated with increased eosinophilic inflammation in case of intense allergen exposure (31) or with increased neutrophilic inflammation in case of infectious aetiology (32). A prospective study has found a correlation between sputum eosinophils percentage and FEV_1 decline after 5 years (33) suggesting a prognostic value of sputum eosinophil count in asthma follow-up. Jatakanon has shown that the percentage of sputum eosinophils was a predicting factor of loss of asthma control (34) when stepping down ICS. In another study, Leuppi reported that sputum eosinophil percentage >6.3% was a predictor of loss of asthma control after inhaled corticosteroid cessation with 90% sensitivity and 63% specificity (35). Persistent airway eosinophilic inflammation despite intensive anti-inflammatory treatment has been associated with more severe asthma (36;37), more frequent exacerbations (21) and accelerated lung function decline (38). The association of increased eosinophilic and neutrophilic inflammation (mixed granulocytic asthma) is often associated with more severe disease (39;40). Neutrophils seem to play a role in airway calibre while eosinophils are associated with bronchial hyperresponsiveness (27;41). It has been shown that sputum eosinophil count increases several weeks before exacerbation (34;42) so a treatment strategy based on airway inflammation seems to be appropriate to prevent exacerbations (21;34).

1.3.5 Distribution of inflammatory phenotypes

Gibson *et al.* found that 41% of non-smoking asthmatics had a sputum eosinophil count >2.5% (43). Simpson *et al.* showed in a population of 93 asthmatics that 41% had eosinophilic asthma (>1%), 20% neutrophilic asthma (>61%), 31%

paucigranulocytic asthma and 8% mixed granulocytic asthma. The proportion of eosinophilic asthma reported by Louis (44) and Green (13) was higher (69% and 52% respectively) but the thresholds used in those studies were 2 and 1.9% respectively and patients recruited in the former study were essentially mild corticosteroid-naïve atopic asthmatics. A recent American multicentre study found that paucigranulocytic asthma accounted for more than 50% of patients while eosinophilic asthma (≥2%) represented roughly 25%. In this study, neutrophilic phenotype and mixed granulocytic asthma represented less than 15% and 5% respectively.

1.3.6 Biomarkers available to classify asthmatics in inflammatory phenotypes

1.3.6.1 Induced sputum

Currently available data seem to underline the robustness of induced sputum as a non-invasive method of collection of airway cells and lining fluid for assessing airway inflammation in asthma to identify inflammatory phenotype. Since its first description as a diagnostic tool for Pneumocystis carinii in AIDS patients (45), the technique has been used in chronic respiratory disease research and to monitor airway inflammation in clinical practice (46). It is however technically demanding and time consuming. There is a need for surrogate markers to identify neutrophilic and eosinophilic phenotypes to allow for personalised medicine. A biomarker is a surrogate measurement designed to characterise and quantify an underlying disease process (47). The use of biomarkers in exhaled air has the potential to unravel disease heterogeneity (48).

1.3.6.2 Surrogate markers for eosinophilic phenotype

1.3.6.2.1 Exhaled nitric oxide

The role of exhaled nitric oxide (FENO) as a valid marker of airway eosinophilic inflammation has been debated. Online measurement of FENO has the advantage of being simple and providing immediate results. Some studies (49-53), have shown a correlation between FENO and eosinophil in both mucosal biopsies and induced sputum. Berry *et al.* found that a FENO value ≥8.3 ppb in non-smokers predicted a sputum eosinophil count >3% when measured at a flow rate of 250 ml/s(49). The importance of correlations between FENO and induced sputum eosinophil counts varies widely in the literature and the clinical utility of FENO remains doubtful (54;55) as FENO was shown to be less successful than induced sputum (21;22) in guiding therapy in asthma management.

1.3.6.2.2 Systemic biomarkers

It is unclear whether systemic inflammation is able to predict airway inflammatory phenotypes. This alternative test is biologically plausible since the infiltrating granulocytes in the airway are bone marrow-derived cells, which access the airway through diapedesis from the circulation. The appeal of the approach comes from the ease of sample collection of peripheral blood from subjects of all ages and clinical characteristics.

1.3.6.2.3 Other exhaled biomarkers

Nitric oxide measure in exhaled air may not be the only diagnostically relevant molecule in exhaled breath. Breath VOCs might originate from either inside or outside the body, as endogenous products of metabolism or from exogenous sources

such as air, food and water. Volatile organic compounds (VOCs) present in the exhaled air were shown to be able to discriminate between various lung pathologies (56;57). Several studies have already suggested the usefulness of VOCs detection in exhaled air as a diagnostic tool in brain, prostate and lung cancer as well as in tuberculosis, asthma and COPD. Electronic nose (eNose) has been shown to be able to discriminate between asthma and controls with similar accuracy to FENO and sputum eosinophils (58). However, eNose identifies a breathprint pattern associated with a disease without precise identification of the compounds present in the exhaled air.

We sought to determine the ability of VOCs to discriminate between paucigranulocytic asthma and eosinophilic asthma. Moreover, non-invasive assessment of breath metabolites offers the potential for providing insight into inflammatory pathways. Inflammation-associated oxidative stress leads to peroxidation of polyunsaturated fatty acids thereby generating volatile organic compounds (VOCs) excreted in exhaled air. We hypothesised that inflamed lungs secrete different patterns of VOCs than normal healthy lungs. Asthma is characterised by specific biomarkers that reflect an altered airway redox chemistry, including lower levels of pH, and increased reactive oxygen species (ROS) (59) and reactive nitrogen species (RNS) (60). A potential source for the generation of microbicidal and pathological levels of superoxide anion ($O2^{\bullet-}$) is the NADPH oxidase, which is found in neutrophils, eosinophils, monocytes, and macrophages. Most of the $O2^{\bullet-}$ generated in vivo undergoes a nonenzymatic or superoxide dismutase (SOD-) catalysed reaction resulting in its dismutation into hydrogen peroxide (H2O2). Once formed, the oxidising potential of H2O2 may be amplified by eosinophil- and neutrophil-derived peroxidases, EPO and MPO, respectively. Recent

evidence supports the key roles of endogenous NO and NO-derived reactive nitrogen species (RNS) in modulating airway function and inducing asthma (60). Previous studies have shown that some NO metabolic by-products exhibit cytotoxic effects in the central and peripheral bronchioles and alveoli. For instance, the rapid reaction between superoxide and NO produces a highly unstable RNS, peroxynitrite ($ONOO^-$), which has been involved in cellular damage and airway hyper-responsiveness (61). Highly bioactive NO and NO-derived RNS can inflame the airways and could be measured in exhaled air. The oxidative and nitrosative stresses can be elicited by allergens and irritants (62).

Because the field of breath analysis is relatively new and the advances in analytic technology occur so fast, many compounds in exhaled breath have been detected by gas chromatography and mass spectometry. Analysis of exhaled breath for recognition of asthma phenotype using endogenous volatile organic compounds offers the possibility of noninvasive diagnosis and therapeutic monitoring. Several studies have shown that eNose was able to discriminate between various diseases (63-68). However eNose cannot identify individual VOCs. To unravel which VOCs drive the distinctive patterns between different asthma phenotype, specific analysis of individual compounds with GC-MS is necessary.

1.3.6.3 Surrogate markers for neutrophilic phenotype

We currently lack of marker predicting airway neutrophilic inflammation. There is evidence that non-eosinophilic asthma is not improved by treatment with inhaled corticosteroids (14;24) while macrolides were shown to decrease the number of exacerbations in severe non-eosinophilic asthma (25;26). Analysis of exhaled air

using chromatography and mass spectrometry could allow identification of breath prints specific to neutrophilic phenotype.

1.4 Exhaled nitric oxide and airway hyperresponsiveness

Some early studies suggested that high FENO might reflect the presence of bronchial hyperresponsiveness (69). As FENO was identified as a surrogate marker for eosinophilic phenotype, we wondered if FENO was also a marker of airway hyperresponsiveness. Some studies have suggested FENO may be helpful in diagnosing asthma but it remains debated (69;70). Both sputum eosinophil count and FENO have been proposed as a useful diagnostic tool (71) in mild to moderate asthma. In this group of patients airway inflammatory markers proved to be superior to classic FEV_1 reversibility to β2-agonist or to peak expiratory flow (PEF) variability criteria but slightly less efficient than methacholine challenge (70;71). Asthma diagnosis remains a challenge in clinical practice (72) and either reversibility test or bronchial provocation challenge is required to confirm the diagnosis according to GINA guidelines (5). There is a need for a simple, quick and reliable test in those patients with symptoms suggestive of asthma. Early studies have suggested that fractional exhaled nitric oxide, measured with a cut-off of 16ppb at a flow rate of 200ml/s may help to identify patients with bronchial hyperresponsiveness to histamine or reversibility to β2-agonist among those presenting with chronic respiratory symptoms and normal baseline lung function (specificity of 90% and PPV >90%) (73). FENO alone is not a diagnostic test for asthma as elevated levels of FENO have been found in other pathologies such as eosinophilic bronchitis (74), allergic rhinitis and COPD (75). Moreover, factor analysis has revealed that airway inflammation and bronchial hyperresponsiveness towards methacholine load in different clusters in patients with long disease duration (2;76). On the other hand,

FENO has been shown to correlate with new-onset wheeze in a longitudinal population study (77). Airway hyperresponsiveness assessed by methacholine challenge is time consuming and unpleasant to the patient whereas FENO measurement is easy to perform and provides immediate results. In patients exhibiting unspecific respiratory symptoms, a low level of FENO could suggest alternative diagnosis and the absence of response to anti-inflammatory treatment.

1.5 Clinical implications of the site of eosinophilic inflammation in asthma

The eosinophilic feature is recognised as a pivotal trait of bronchial asthma (36). However, there are patients who show discordance between local and systemic inflammation. There is a huge controversy about the role of eosinophils as a key player in asthma severity (34;40;50;78-86). Some studies have looked at airway eosinophils in bronchoalveolar lavage (BAL) (86), induced sputum (34;79;80;82;83) and bronchial biopsies (84;85), while others focused on systemic inflammation through blood eosinophil count measurement (85). Discrepancies between studies may be linked to the different compartments sampled and, for some of the studies, to the limited number of subjects investigated. While inhaled corticoids, the recommended mainstay treatment of asthma, have been consistently shown to reduce airway eosinophilic inflammation and improve asthma control (87), their effect on systemic eosinophilia was shown to be rather weak at usual doses (88). By contrast, the new biologicals directed towards interleukin-5 were shown to dramatically decrease circulating blood eosinophils, an effect that was associated with the reduction of asthma exacerbation (11) and improvement in asthma control (10). To the best of our knowledge, there has never been detailed investigation of the

relationship between blood and sputum eosinophil in a large population of asthmatics.

1.6 Severe asthma phenotype

1.6.1 Severe asthma definition

It is recognised that the majority of asthmatics may be controlled by regular treatment with ICS/LABA. Yet a small fraction of asthmatics called refractory or severe asthmatics escape this treatment (89;90). Severe asthma accounts for a major part of the financial burden asthma poses to the health care system (91). This phenotype is defined by inadequate asthma control or frequent severe exacerbations despite high-dose inhaled corticosteroids or the need for oral corticosteroids often associated with other controller medication such as long-acting β2 agonists, leukotriene receptor antagonists or theophylline (92;93). Refractory asthmatics are patients in whom alternative diagnoses have been excluded, co-morbidities have been treated, trigger factors have been removed and adherence with treatment has been checked. By itself this phenotype clearly points out the inability of corticosteroids to control disease expression in some asthmatics. A functional variant of glucocorticosteroid transcript 1 gene (GLCCI1) was found to be associated with decreased response to ICS in several randomised clinical trials (94). Reduced eosinophil apoptosis in induced sputum despite a high dose of ICS was shown to be related to disease severity (95).

1.6.2 Severe asthma management

While evaluation and monitoring of airway inflammation does not seem to be warranted in everyday clinical management of controlled mild-to-moderate asthma,

the assessment of the inflammatory phenotype is required in severe asthma that is resistant to conventional treatment and may help to reduce exacerbation rates. The recent ERS/ATS guidelines suggest that induced sputum can be used in the management of severe asthma in specialised centres with a dedicated laboratory (93).

1.6.3 Severe asthma inflammatory subphenotypes

Severe asthma is not a single disease itself but can be divided into several subphenotypes according to inflammatory, clinical and functional characteristics (96). Those phenotypes may have prognostic value and therapeutic implications. However, severe asthma subphenotypes have not yet been fully characterised. Early reports suggested that patients with more severe asthma had increased eosinophil (40) or neutrophil (39) counts in induced sputum and in bronchial biopsies (97).

1.6.4 Targeted therapies available for severe asthma

Most of the new biological treatments available for severe asthma are monoclonal antibodies directed against specific immunologic processes and in particular against cytokines. Several cytokines are involved in the pathophysiology of asthma including Th2 cytokines. It is critical to assess inflammation in severe asthma to predict the response to targeted therapies.

In those refractory asthmatics with moderately elevated total serum IgE and sensitisation to a perennial allergen, omalizumab, a humanised monoclonal antibody against IgE has proved to be effective in reducing exacerbation rate and improving quality of life (98;99) although part of the effect in quality of life improvement seen in clinical practice is likely to be due to a placebo effect and a careful follow-up of the

patient inherent in the mode of drug administration (100). The importance of IL-5 in driving the persistent systemic and airway eosinophilic inflammation has been demonstrated by the efficacy of mepolizumab, an anti-IL-5 monoclonal antibody, to further decrease eosinophilic inflammation in those patients with refractory asthma despite high dose of corticosteroids (15;16). The clinical relevance of the persistent eosinophilic inflammation is demonstrated by the reduction in exacerbation rate and the improvement in quality of life observed in those patients receiving mepolizumab, even if no effect is observed on airway calibre and bronchial hyperresponsiveness (15). IL-13 is increased in severe asthma and causes corticosteroid resistance so is a logical target. Anti-IL-13 such as lebrikizumab has been disappointing with little physiological effect and no effect on symptoms or exacerbations (101). A recent study with anti-IL-17 has shown no effect on ACQ score, FEV_1 and symptom-free days (102).

Anti-TSLP, which was found effective in the model of acute bronchial allergen challenge, still has to prove its validity in improving asthma control and reducing asthma exacerbation. It is indeed worth noting that anti-IL5 whose effect on asthma control and exacerbation in severe eosinophilic asthma has been recently established, was shown to fail in protecting against acute and late bronchospasm after allergenic challenge. Blocking antibodies to other cytokines including IL-9, IL-25 or IL-33 are also in development for asthma. The major drawback of such treatments is the high cost which weakens the cost-effectiveness relationship.

The studies focusing on neutrophilic inflammation in refractory asthma have been limited so far. Studies using clarithromycin or azithromycin have shown a significant reduction of sputum neutrophil count together with improved quality of life and decreased severe exacerbation rate in non-eosinophilic severe asthmatics (25;26).

Long-lasting studies on larger asthma cohorts are warranted before claiming macrolides may be used as maintenance treatment in severe neutrophilic or at least non-eosinophilic asthma.

Bronchial thermoplasty is an innovative non-pharmacological treatment approach to reduce the bronchoconstrictor response in asthma (103). During this bronchoscopic procedure, all visible and reachable airways are treated with radiofrequency energy provided by the Alair catheter (Asthmatx, Inc., Mountain View, CA, USA) introduced via a flexible bronchoscope in order to reduce airway smooth muscle mass (104) and decrease bronchial reactivity. It has been shown to improve asthma control and quality of life and to be safe in moderate to severe asthmatics (105;106).

1.6.5 Severe asthma registries

Studying large numbers of severe asthmatics recruited in daily practice is critical to better understand and treat severe asthma. Several severe asthma cohorts and registries aimed to collect data and information on severe asthma in Europe and USA. The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) (107), the BIOAIR European study (108), the TENOR (109) and the SARP study (110) and the UK multicentre registry on refractory asthma (111) have yielded recent insights into the demographics, and functional and inflammatory features of asthma. This information helps guide effective management of patients with severe asthma by improved understanding of factors associated with poor asthma control and high levels of healthcare resource use.

Chapter 2. Aim of the thesis

2.1 Description of inflammatory phenotypes

The aim of the present thesis was first to look at the distribution of inflammatory profiles based on induced sputum. We wanted to describe the distribution of inflammatory phenotypes in a large asthmatic population encompassing the whole disease severity spectrum and in a population of severe asthmatics recruited from 9 Belgian academic centres in the Belgian Severe Asthma Registry. We also looked at the demographic, functional and inflammatory characteristics of the different inflammatory phenotypes.

2.2 Value of FENO measurement in clinical practice

Exhaled nitric oxide is an easily applicable and widely available measurement. We wanted to highlight the link between this biomarker and eosinophilic inflammation, bronchial hyperresponsiveness and respiratory symptoms. As we thought that the doubt on the utility of FENO to identify eosinophilic phenotype was due to the use of inappropriate cut-off points, we further searched for series of predictive cut-off points taking into account factors that were found to influence FENO levels.

2.3 Factors associated with eosinophilic and neutrophilic inflammation

We wondered whether patients with eosinophilic versus neutrophilic inflammation may be distinguished by demographic, functional and immunologic features. As induced sputum is a complex and expensive technique, we wanted to identify surrogate markers for airway inflammation. We wanted to test the ability of volatile organic compounds in differentiating between asthma inflammatory phenotypes.

2.4 Clinical interest of a new asthma classification based on compartmental eosinophilic inflammation

As there are patients exhibiting discordance between local and systemic inflammation, we wanted to study the added value of measuring blood and sputum eosinophil cell counts. We wondered whether patients' characteristics varied according to the amount and site of eosinophilic inflammation.

2.5 Setting up the severe asthma registry in Belgium

The Belgian Severe Asthma Registry (SAR) is a national Belgian secured web database for severe asthma initiated by the Asthma Working Group of the Belgian Thoracic Society. We wanted to collect epidemiological and clinical data from the Belgian Severe Asthma population in order to raise awareness of severe asthma, to identify several phenotypes, to promote optimal care for these patients and to be a valuable platform of patients for new severe asthma drug testing. We also sought to compare the findings to that of ENFUMOSA(107) and BIOAIR European study(108), the TENOR study (109), the SARP study (110) and the UK multicentre registry on refractory asthma (111).

Chapter 3. Methods

3.1 Subject characteristics and study design

Patients with asthma were recruited from the University Asthma Clinic of CHU Liege between October 2005 and January 2014 while severe refractory asthmatics were recruited from 9 Belgian centres (CHU Liege, Ghent University Hospital, Mont-Godinne, Erasme, Cliniques universitaires de Saint-Luc, CHU Vesale, CHU St Pierre, Brugman and UZ Leuven) between March 2009 and January 2014.

3.2 Visit to the asthma clinic of CHU Liege

Patients attended the clinic on two days at an interval of one week. On day 1, each patient underwent FENO measurement at a flow rate of 50 ml/s followed by spirometry with bronchodilation, sputum induction, blood sampling and validated asthma control and quality of life questionnaires. All tests were performed on the same day. On day 2 the subjects underwent methacholine challenge after refraining from using bronchodilators for the appropriate time (8h for short-acting bronchodilators and 24h for long-acting bronchodilators) as long as the baseline forced expiratory volume in 1s (FEV_1) value was not less than 70% predicted. Asthma was diagnosed based on the presence of chronic respiratory symptoms such as cough, breathlessness or dyspnea together with the demonstration of airflow variability. The latter was defined by airway hyper-responsiveness shown by one or more of the following: increase in Forced expiratory volume in 1 s (FEV_1) of >12% and 200 ml following inhalation of 400 µg salbutamol or inhaled concentration of methacholine provoking a 20% fall in FEV_1 of <16mg/ml. Methacholine challenge was performed according to a standardised methodology as previously described (44). Subjects were characterised as atopic if they had at least one positive specific IgE

(>0.35 kU/l; Phadia) for at least one common aeroallergen (cat, dog, house dust mites, grass pollen, tree pollen and a mixture of moulds). Quality of life was assessed using the self-administered Asthma Quality of Life Questionnaire (AQLQ) (112) and asthma control by the Juniper Asthma Control Questionnaire (ACQ) (113) and Asthma control test (ACT) (114).

Our studies were conducted with the approval of the ethics committee of CHU Liege.

3.3 Investigation of bronchial inflammation

As bronchial endoscopy is an invasive technique, inflammatory cell count on bronchial biopsies and bronchoalveolar lavage are not widely used to assess inflammation in asthmatics. Several non-invasive techniques have been developed to evaluate airway inflammation. Induced sputum has been shown to be able to reflect airway inflammation and its evolution.

3.3.1 Sputum induction

Sputum was induced and processed as previously reported (115). The patients received inhaled salbutamol 400µg administered through pMDI and Volumatic (Glaxo Smith Kline, UK) 20 minutes before sputum induction. Saline was inhaled through an ultrasonic nebuliser (Devilbiss 2000), the mean output of which was calculated to be 0.93 ml/min. The cup of the nebuliser was filled with 50 ml hypertonic or isotonic (if FEV_1 value was less than 65% predicted (116)) saline to which was added 1.75 ml salbutamol solution at 5 mg/ml. FEV_1 was measured at 5, 10, 15 and 20minutes after starting inhalation or earlier in case of worsening respiratory symptoms. Inhalation of saline was stopped after 20 minutes or when a fall in FEV_1 of 20% from baseline had occurred. After performing spirometric measurements at 5, 10, 15 and 20 minutes the

subjects were asked to rinse their mouth with tap water and to cough up sputum into a plastic container. For safety reasons, FEV_1 was measured 10 minutes after the end of the induction in every patient. Sputum should be processed within two hours in order to ensure optimum cell counting and staining. For sputum processing, samples were poured into a 50ml polypropylene tube, weighed, and diluted with a threefold weight of a phosphate buffered saline (PBS) solution for homogenisation. The samples were then rocked at room temperature for 20 minutes and centrifuged at 400g for 10 minutes at 4°C. The supernatant was stored at –80°C until biochemical analyses for albumin and histamine. The cellular phase was dispersed in 1 ml PBS without Ca2+ and Mg2+ solution for total cell counts using a manual haemocytometer. The differential cell count was performed on cytospins stained with Diff-Quick by counting 500 cells under a light microscope (Dade, Brussels, Belgium). Cellular viability was evaluated using trypan blue.

Sputum induction was successful in 78% of the patients encountered in our asthma clinic which is similar to previous reports (95;117). As the technique is non-invasive, it has been applied to a large series of healthy subjects in order to derive reference values. A series of 113 healthy subjects including 24 current smokers was sampled in University of Liege hospital (118). There are three other detailed published studies that investigated the sputum cell counts in large numbers of non-smoking healthy subjects (119-121). When deriving the upper limit of the 90% reference interval (mean +1.7SD), abnormally high neutrophil count ranges from 49% according to Spanavello (120) to 93% according to Thomas (121), most of the authors setting the threshold between 61% and 76%. It is recognised that the number of neutrophils is influenced by age and may rise rapidly in the sputum as a consequence of airways irritation (122) or exposure to pollutants (123). This could explain the variability of

normal range according to the studies conducted in different countries. In our experience, an abnormally high sputum neutrophil count is defined as a percentage ≥76% calculated as mean + 1.7 SD (mean±SD: 34.9+/-24.3%; n=113) (118).

As opposed to what has been found for neutrophils, there is much less variation according to the centres with respect to the eosinophil percentage. The abnormal threshold ranges from 1% according to Simpson (124) to 2.6% according to our reference values (118). To give a safe margin in the cell counting, it has been accepted that an abnormal sputum eosinophil count is ≥3%.

In our studies, eosinophilic phenotype was defined as ≥3% sputum eosinophil count while neutrophilic phenotype consisted of ≥76% sputum neutrophil counts (118).

It has been shown that early sputum (after 5 minutes) contains more granulocytes but less mononuclear cells than late sputum (after 20 minutes).

As compared to bronchoalveolar lavage, induced sputum in healthy subjects displays more neutrophils and less lymphocytes and macrophages (125-127). Induced sputum is more indicative of proximal inflammation but sputum eosinophil count has been shown to be well correlated to distal eosinophil count in bronchoalveolar lavage. Comparison of bronchial biopsies and sputum in the same subject showed that sputum eosinophils reflect airway wall eosinophil content while this did not hold true for the neutrophils (125;127). The latter are present in greater number in airway lumen, and therefore in sputum, than in airway walls.

3.3.2 Exhaled nitric oxide

The technique of induced sputum is complex and time consuming. Moreover, in our experience, only 80% of asthmatics are able to cough up sputum (128). FENO has been considered as a good surrogate marker for sputum eosinophil count (49).

NO is mainly produced by epithelial cells in asthma but can also be released by mast cells, macrophages, endothelial cells and vascular smooth muscle cells. The synthesis of NO is mediated by constitutive (eNOS or nNOS) and inducible NO synthase. The production is due to oxidation of L-arginin to L-citrullin. iNOS is the only isoform correlated with exhaled nitric oxide (129).

FENO is measured by chemoluminescence using a nitric oxide monitor set at an exhalation flow rate of 50ml/s according to the ERS/ATS recommendations (NIOX, Aerocrine, Sweden). Nitric oxide reacts with ozone to form hydrogen dioxide with photon emission that can be detected. The signal is proportional to the concentration of nitric oxide. NO concentration is inversely related to expiration flow. Nitric oxide measurement is precise, reproducible and gives immediate results. Nitric oxide is measured in part per billion (ppb).

FENO has been shown to be correlated with blood eosinophils (130), sputum eosinophils (49), eosinophil counts in bronchial mucosa (131) and bronchoalveolar lavage (73). Adult asthmatics exhibiting FENO levels lower than 25ppb have a high probability of non-eosinophilic asthma (51;132) while patients with FENO levels >47ppb show a good response to inhaled corticosteroids.

It is however highly likely that FENO and induced sputum are different facets of bronchial inflammation. Jatakanon has indeed shown that the change in sputum eosinophil count was better than the change in FENO in predicting loss of asthma control (34). Michils *et al*. have also shown that as opposed to induced sputum, it is more the individual change in FENO value than the absolute value of FENO that is predictive of asthma control. In this study, a reduction of FENO of 40% predicted improvement of asthma control with a PPV of 83% and a NPV of 79%. As opposed to

what is seen in studies evaluating sputum eosinophil count, FENO levels did not differ between mild to moderate asthma and severe refractory asthma (110).

Indeed it has been shown that in the absence of elevated eosinophil count, FENO was highly predictive of a response to inhaled corticosteroids with a cut-off value of 33 ppb (133). This could be due to eosinophils phagocytosis by macrophages whose presence could become undetectable in induced sputum but still reflected by FENO measurement (134).

3.3.3 VOCs sample collection and analysis

Exhaled air was sampled from patients in whom asthma had been diagnosed according to GINA guidelines and who were classified in asthma inflammatory phenotypes according to the results of sputum inflammatory cell count. Induced sputum was sampled on the same day as VOCs measurement. The recruitment of asthmatics stopped when eosinophilic, paucigranulocytic and neutrophilic phenotypes reached at least 50 asthmatics in each group. The mixed granulocytic asthmatics were quite rare so we decided not to include them in the statistical analysis.

All breath samples were donated between 9 and 11a.m. in the same room, to minimise the effect of variation in background air. Exhaled air was collected by exhaling into inert Tedlar bags (5L). Subjects were asked to inhale, hold their breath for 5 seconds and subsequently fully exhale into the Tedlar bag. All Tedlar bags were washed twice with high-grade nitrogen as described by the manufacturer before usage to make sure all contaminants were eliminated. The content of the Tedlar bag was transported under standardised conditions onto desorption tubes; stainless steel two-bed sorption tubes, filled with carbograph 1TD/Carbopack X. These desorption

tubes were placed inside the thermal desorption unit and quickly heated to 270°C in order to release all VOCs and transport the released VOCs onto the GC-capillary. The used desorption unit was highly suitable for repeated, quantitative and reproducible measurements. Ten percent of the sample was injected into the GC, the remaining 90% transported to another adsorption tube for storage and may be used for later reanalysis. Just before the sample enters the GC, it is trapped by a cold trap at 5 degrees Celsius in order to concentrate the sample. Next, VOCs were separated by capillary gas chromatography (column: RTX-5ms, 30m x 0.25mm 5% diphenyl, 95% dimethylsiloxane, film thickness 1.0µm, Thermo Electron Trace GC Ultra, Thermo Electron Corporation, Waltham, USA). The temperature of the chromatograph was programmed as follows: 40°C during 5 minutes, then raised with 10°C/min until a final maximum temperature of 270°C in the final step, this temperature was maintained for 5 min. Time-of-flight mass spectrometry was used to detect and identify components available in the samples. Electron ionisation mode was set at 70eV and the mass range m/z 35-350 was measured. Sample frequency of the mass spectrometer was set to 5Hz and analysis run time to 33 minutes.

3.4 Statistical analysis

The results were expressed as mean ± SD for continuous variables; median and interquartile ranges (IQR) were preferred for skewed distributions. For categorical variables, the number of observations and percentages were given in each category. Comparisons between different subgroups were performed with a Kruskal-Wallis test. The Spearman correlation coefficient was used to measure the association between clinical parameters. The receiver-operating characteristic (ROC) curve was constructed to determine cut-offs for variables in order to distinguish between various subgroups. Logistic regression analysis was used to assess the relationship between

binary outcomes and sets of covariates, individually or in combination. We established formula taking into account independent predictors to predict the probability of inflammatory subphenotypes. The validity of the equations was tested in independent populations. The agreement between predicted and observed value was tested by Cohen Kappa's coefficient. Calculations were done using SAS version 9.1 (SAS Institute, Cary, North Carolina, USA). The results were considered to be significant at the 5% critical level ($p < 0.05$).

To identify volatile organic compounds from the exhaled air able to discriminate between three asthma inflammatory subtypes (paucigranulocytic, eosinophilic and neutrophilic asthma), we used conditional Inference Forests (CIFs) (135) to build an ensemble of conditional inference trees and to rank features based on the ability of components to predict asthma inflammatory subtype. The advantage of CIF framework is that the node variable selection and its posterior splitting are two separate steps. The CIFs do not show bias towards variables with many possible splits and are scale-independent due to association measure based on Strasser and Weber's work (136). Thus CIFs implemented in party and party-kit packages (137;138) had been shown to provide a superior performance compared to traditional classification and regression trees (CART) including the widely-used Random Forests by Breiman (139). Briefly, the aim of the CIFs in this study was to find the components with the strongest association to the inflammatory phenotype in each of the three tested scenarios including eosinophilic/neutrophilic, eosinophilic/paucigranulocytic and neutrophilic/paucigranulocytic. The association of a particular component to asthma inflammatory subtype via CIFs allowed to identify only components that are deemed to be associated to specific phenotype, but did not

provide enough information to form a robust classifier. In order to extract further information and to see the direction of each component impact on the asthma sub-type, we had conducted Student's t-test on the top ranked components from CIFs analysis. The original data consisted of 276 asthmatics and 3328 components with 122 patients exhibiting eosinophilic asthma, 50 with neutrophilic asthma, 14 with mixed granulocytic asthma and 90 with paucigranulocytic asthma. In order to improve power and reduce dimensionality of the dataset, we had filtered out the components that had < 30 samples (i.e. the mixed granulocytic group was not analysed). After filtering the eosinophilic/neutrophilic subset contained 172 samples and 561 components, the neutrophilic/paucigranulocytic subset contained 140 samples and 429 components, and the eosinophilic/paucigranulocytic subset contained 212 samples and 714 components. The parameters to build Conditional Inference Forests included c_{quad} test statistic (teststat="quad"), multiple-testing correction via Monte Carlo resampling (testtype="MonteCarlo", nresample=9999), 65% of the dataset was used to build trees and the remaining one to calculate variable importance (fraction=0.65), the minimum criteria to continue splitting the tree node was set at p-value ≤ 0.01 (mincriterion=0.99), a minimum of 30 samples in a node were required to execute split (minsplit=30), a total of 999 trees were built (ntree=999), all predictor variables/components had a chance to be assigned to a tree node (mtry=0), the CI trees could have unlimited number of levels (maxdepth=0). The variable importance was calculated using the default settings of the *varimp* function. The importance of the variable/component was measured via the standard decrease in MSE ("%IncMSE") measure. The statistical significance of the components identified across two asthma subgroups was calculated with the Student's t-test assuming different variances in two groups.

All the studies were conducted with the approval of the ethics committee of CHU Liege B70720096732, reference Liege 2009/161.

Chapter 4. Results

4.1 Distribution of inflammatory phenotypes in asthma

4.1.1 Distribution in a large asthmatic population encompassing the entire disease severity spectrum

We sought to assess the proportion of asthmatic patients displaying eosinophilic vs neutrophilic vs paucigranulocytic phenotypes based on sputum cell analysis.

508 subjects were recruited from the University Asthma Clinic of Liege between 1st October 2005 and 27th June 2011. Their demographic and functional characteristics are summarised in Table 1. All the asthmatics recruited had given a successful sputum induction. From those patients, 211 (41%) had eosinophilic inflammation (≥3% eosinophils), 80 (16%) neutrophilic inflammation (≥76% neutrophils), 14 (3%) mixed granulocytic and 203 (40%) paucigranulocytic (sputum eosinophil count <3% and sputum neutrophil count <76%) inflammation (Fig 3). The proportions of inflammatory subtypes were rather similar after exclusion of steroid-treated patients (Table 2).

The proportion of asthmatics with raised sputum eosinophil counts was 44% in our series, similar to Gibson *et al.* (41%) (43). The proportion of eosinophilic asthma reported by Louis (44) and Green (13) was higher but with thresholds of 2% and 1.9% respectively. Our results are similar to Simpson's study conducted on 93 subjects using thresholds of 1 and 61% for eosinophilic and neutrophilic inflammation respectively. This group found 41% eosinophilic asthma, 20% neutrophilic asthma, 31% paucigranulocytic asthma and 8% mixed granulocytic asthma (124). The higher thresholds used in our study certainly explain on the one hand the lower proportion of mixed granulocytic and neutrophilic asthma and on the other hand, the higher proportion of paucigranulocytic asthma in our patients. The American multicentre

study from McGrath *et al.* showed that the dominant phenotype was paucigranulocytic asthma (>50% of patients) while eosinophilic asthma (sputum eosinophil count ≥2%) represented roughly 25%. As in our study, the neutrophilic phenotype (sputum neutrophil count >61%) was quite rare representing less than 15% of the patients while the mixed granulocytic phenotype was less than 5% (140).

Table 1. Demographic, functional and inflammatory characteristics for the whole population.

Characteristics	
N.	508
Gender (M/F)	201/307
Age, yrs	52 (19-88)
Height, cm	167 ± 9
Weight, kg	74 ± 16
Atopy (Y/N)	296/212 (58%)
Current Smoker (n) (pack-yr)	101 (22 (0.5-60))
Ex-smokers (n) (pack-yr)	99 (15 (0.5-90))
FEV_1, % predicted	84 ± 19
Sputum eosinophils, %	2 (0-94)
Sputum neutrophils, %	45 (0-100)
ICS therapy	
Steroid-naïve	153 (30%)
Low-dose ICS	73 (15%)
Moderate-dose ICS	138 (27%)
High-dose ICS	144 (28%)

Data are presented as mean ± SD or median (range). FEV_1: forced expiratory volume in 1s. ICS: inhaled corticosteroids. Low dose: ≤500µg/day beclomethasone; Moderate dose: 500-1000µg/day beclomethasone; High dose: >1000µg/day beclomethasone.

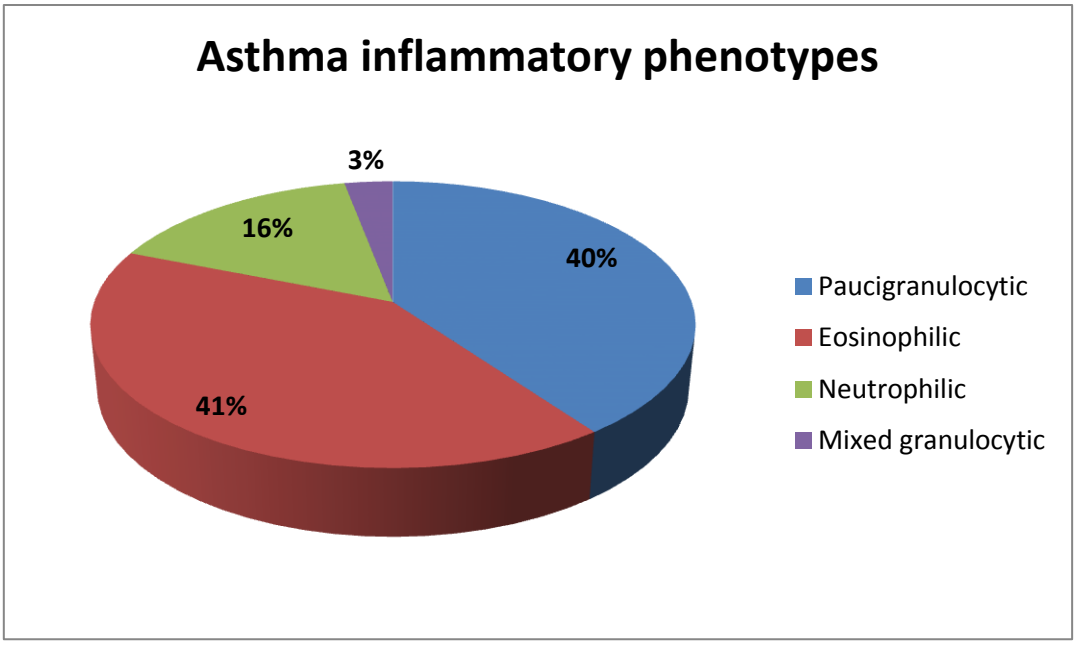

Fig 3. Proportion of various inflammatory phenotypes according to cellularity of induced sputum in a large cohort of asthmatics.

Table 2. Proportion of asthma inflammatory phenotypes in steroid-naïve and steroid-treated patients

	Paucigranulocytic phenotype	**Eosinophilic phenotype**	**Neutrophilic phenotype**	**Mixed granulocytic phenotype**
Steroid-naïve (n=153)	65 (42.5%)	60 (39.2%)	25 (16.3%)	3 (2%)
Steroid-treated (n=355)	138 (38%)	151 (43%)	55 (15%)	11 (3%)*

*p<0.05.

4.1.2 Distribution of phenotypes in a population of severe refractory asthma

Sputum was induced in 86 out of 111 severe asthmatics (SA) in CHU Liege with a success rate of 77%. By comparison, Ten Brincke *et al.* found a success rate in severe asthma of 74% (116). In severe asthma, eosinophilic phenotype (sputum Eos≥3%) was the predominant phenotype (55%) while neutrophilic (sputum Neu≥76%) and paucigranulocytic asthma were 22% and 17% respectively (Fig 4).

The raised airway granulocytic inflammation is a common finding in severe asthma (40). The median sputum eosinophil count was higher than that observed in the UK SA cohort (111). We found a lower proportion of paucigranulocytic asthma in the severe asthma population than the 40% observed in the general population of asthmatics (128). Eosinophilic asthma was more frequent in severe asthma (55% vs 41%) and neutrophilic asthma followed the same trend (21% vs 16% in the general population of asthmatics). Duncan (95) has demonstrated that both reduced eosinophil apoptosis and increased sputum eosinophilia significantly correlate with asthma severity. Neutrophilic inflammation was found to be increased in severe asthma (39). It has been suggested that this could be due to a protection of neutrophils from apoptosis by corticosteroids. The mixed granulocytic asthma was two times more frequently encountered in severe asthmatics than in the general population of asthmatics. Eosinophilic asthma was more frequently encountered in late onset asthma in our registry data. This is in line with previous reports (141).

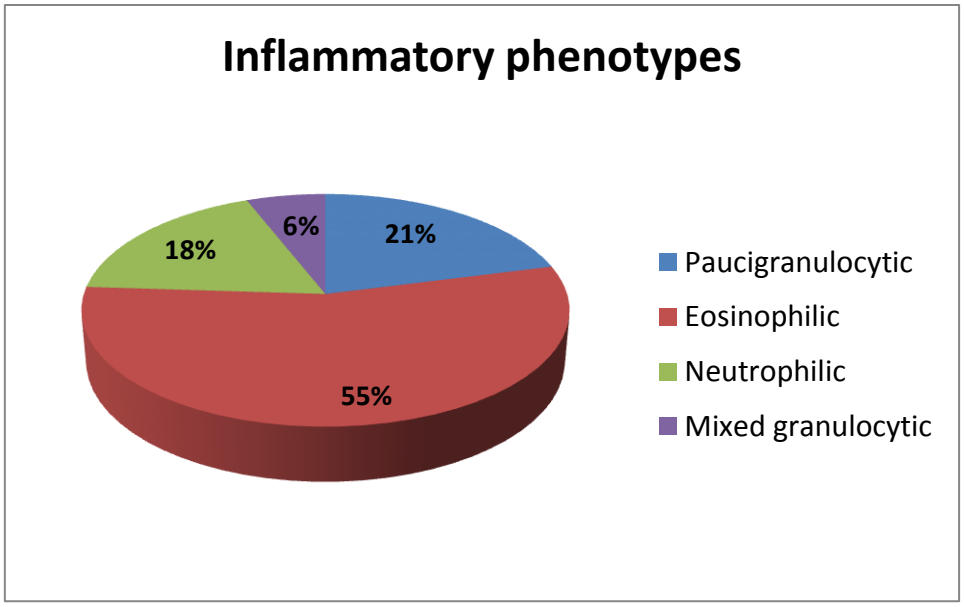

Fig 4. Distribution of sputum cellular phenotypes in severe asthma (n=88). Eosinophilic asthma (≥3% sputum eosinophils, <76% sputum neutrophils); Neutrophilic asthma (<3% sputum eosinophils, ≥76% sputum neutrophils); Paucigranulocytic asthma (<3% eosinophils and <76% neutrophils in induced sputum); Mixed granulocytic asthma (≥3% eosinophils and ≥76% neutrophils in induced sputum).

4.2 Demographic, functional and inflammatory characteristics of asthma phenotypes based on sputum cell analysis

The demographic, functional and inflammatory characteristics are summarised in Tables 3 and 4. Compared to the paucigranulocytic phenotype, eosinophilic, neutrophilic and mixed granulocytic phenotypes were characterised by a poorer lung function.

The eosinophilic phenotype exhibited higher frequency of atopy, higher levels of IgE, higher bronchial hyperresponsiveness to methacholine, higher FENO levels and lower asthma control compared to paucigranulocytic. Those results are in accordance with previous studies (28;41;44;50;142).

The mixed granulocytic phenotype had higher levels of fibrinogen, the lowest lung function and the highest degree of bronchial hyperresponsiveness to methacholine (Table 3 and 4). Like Hastie *et al.* (143), we identified patients with mixed granulocytic sputum inflammation exhibiting the lowest lung function. Moreover, the mixed granulocytic phenotype had higher serum fibrinogen values pointing to systemic inflammation in this subgroup. This interesting finding has not been reported so far in asthma but raised fibrinogen levels have been demonstrated to be associated with reduced FEV_1 and increased risk of Chronic Obstructive Pulmonary Disease (COPD) in a population study (144). As for neutrophilic asthma there was no special characteristic that distinguishes this group from the other inflammatory patterns. In particular neutrophilic asthmatics did not display higher serum C-Reactive Protein and fibrinogen levels.

We looked at the exacerbation rate in the previous year according to asthma inflammatory phenotype. As compared to paucigranulocytic asthma (0.41 (0.29-0.43), n=203) the only inflammatory phenotype associated with increased exacerbation rate in the previous year was the eosinophilic phenotype (1.07 (0.76-1.37), n=211,

p<0.0001) while there was a trend for mixed granulocytic asthma (0.83 (0.24 – 1.43), n=14, p=0.08). Neutrophilic asthma did not differ significantly from paucigranulocytic and mixed granulocytic asthma (0.54 (0.24 – 0.83), n=80) but exhibited significantly lower exacerbation rate as compared to eosinophilic asthma (p=0.04).

Table 3. Demographic characteristics according to inflammatory phenotypes.

	Paucigranulocytic phenotype	Eosinophilic phenotype	Neutrophilic phenotype	Mixed granulocytic phenotype
N.	203 (40%)	211 (41.5%)	80 (15.7%)	14 (2.8%)
Gender (M/F)	72/131	101/110	21/59	7/7
Age, yrs	51 (21-86)	51 (19-87)	57 (21-84)	68 (31-88)
Atopy (Y/N)	100/103 (49%)	140/71 (66%)*	48/32 (60%)	8/6 (57%)
Smoking (Y/N)	48/155 (24%)	38/173 (18%)	12/68 (15%)	3/11 (21%)
ICS therapy				
Steroid naïve	66 (32.5%)	59 (28%)	25 (31.3%)	3 (21.4%)
Low dose	31 (15.3%)	37 (17.5%)	4 (5%)*	1 (7.2%)
Moderate dose	58 (28.6%)	54 (25.6%)	23 (28.7%)	3 (21.4%)
High dose	48 (23.6%)	61 (28.9%)	28 (35%)	7 (50%)

*p<0.05, **p<0.001, ***p<0.0001 *Paucigranulocytic asthma is used as the comparator. ICS: inhaled corticosteroids. Low dose: ≤500µg/day beclomethasone; Moderate dose: 500-1000µg/day beclomethasone; High dose: >1000µg/day beclomethasone.

Table 4. Functional and inflammatory characteristics according to inflammatory phenotypes.

	Paucigranulocytic phenotype	Eosinophilic phenotype	Neutrophilic phenotype	Mixed granulocytic phenotype
N.	203 (40%)	211 (41.5%)	80 (15.7%)	14 (2.8%)
IgE, kU/l	84 (1-7338)	211 (3-17183)***	107 (2-7338)	346 (1-2063)
Blood eosinophils, /mm³	160 (0-1220)	360 (0-3220)***	170 (20-1020)	420 (190-3040)***
Blood eosinophils, %	2 (0-13)	4.5 (0-26)***	1.9 (0.2-15)	5 (1.3-30)***
Blood neutrophils, /mm³	4030 (76-11080)	4220 (1820-15410)	5000 (2070-10440)	4245 (3520-6170)
Blood neutrophils, %	59 (27-82)	55 (32-91)*	62 (42-80)	59 (43-68)
FEV_1, % predicted	90 ± 17	80 ± 20***	79 ± 20***	72 ± 14***
FEV_1/FVC, %	77 ± 9	71 ± 10***	72 ± 11***	69 ± 9***
TLC, % predicted	99 ± 16	102 ± 18	102 ± 18	101 ± 14
FRC, % predicted	103 ± 27	104 ± 19	119 ± 32	111 ± 22
KCO, % predicted	90 ± 19	92 ± 21	91 ± 19	100 ± 10
PC_{20}, mg/ml	4.42 (0.13-16)	2.02 (0.025-16)**	3.22 (0.05-16)	1.08 (0.53-2.2)**
Reversibility, %	8 ± 9	15 ± 17**	8 ± 10	12 ± 10
Sputum eosinophils, %	0.4 (0-2.9)	18 (3-94)***	0.2 (0-2.8)	4.3 (3-8)***
Sputum neutrophils, %	41 (0-76)	33 (0-76)	87 (77 – 100)	82 (76-92)
Fibrinogen, g/l	3.1 (2-6.3)	3.1 (2-7.2)	3.3 (1.9-10)	4.1 (2.7-6.3)*
CRP, mg/l	1.6 (0.2-14)	1.8 (0.2-14)	2.3 (0.2-10)	1.9 (1.1-6)
FENO, ppb	16 (1-128)	53 (2-247)***	22 (0-192)	41 (12-161)*
ACQ	1.82 ± 1.15	2.16 ± 1.36*	2.09 ± 1.88	2.09 ± 1.16
Global AQLQ	4.6 ± 1.3	4.58 ± 1.34	4.76 ± 1.46	4.45 ± 1.74
- Emotion	4.92 ± 1.35	4.57 ± 1.63	4.9 ± 1.76	4.64 ± 1.83
- Symptoms	4.46 ± 1.46	4.42 ± 1.43	4.65 ± 1.54	4.39 ± 1.5
- Activity	4.71 ± 1.36	4.79 ± 1.44	4.84 ± 1.49	4.55 ± 1.80
- Environment	4.48 ± 1.50	4.55 ± 1.55	4.81 ± 1.67	4.70 ± 2.15

*p<0.05, **p<0.001, ***p<0.0001 *Paucigranulocytic asthma is used as the comparator. Data are presented as mean ± SD or median (range); PC_{20} is expressed as geometric mean (range). ACQ, Asthma Control Questionnaire; AQLQ, Asthma Quality of Life Questionnaire ; FENO, exhaled nitric oxide ; FEV_1, forced expiratory volume in 1s; PC_{20}, concentration required to provoke a fall in FEV_1 of 20% or more; FVC, forced vital capacity. CRP, C-Reactive Protein; FRC: Functional residual capacity; KCO: Carbon monoxide transfer coefficient; TLC: total lung capacity. Paucigranulocytic asthma is used as the comparator.

4.3 Factors associated with eosinophilic versus neutrophilic phenotypes

As induced sputum is time consuming and not widely available, we aimed at determining factors associated with inflammatory phenotypes.

4.3.1 Exhaled nitric oxide

4.3.1.1 Introduction

It has been claimed that exhaled nitric oxide could be regarded as a surrogate marker for sputum eosinophil count in patients with asthma. However the FENO threshold value that identifies a sputum eosinophil count ≥3% in an unselected population of patients with asthma has been poorly studied. Berry *et al.* found a threshold of 8.3ppb in non-smokers predicting a sputum eosinophil count >3% when measured at a flow rate of 250ml/s (49). ERS/ATS guidelines recommend however measuring exhaled nitric oxide at a flow rate of 50ml/sec.

Moreover, both FENO and sputum eosinophil count were found to be strongly reduced by treatment with ICS (18;145). It is well recognised that smoking lowers FENO values in patients with asthma (146), and some studies have found that patients with asthma who smoke are less eosinophilic than non-smoking patients with asthma (147). Atopy was found to be associated with raised FENO irrespective of asthma (148) and is known to favour eosinophilic inflammation. There are, however, no data on how treatment with ICS, smoking and atopy may affect the relationship between FENO and sputum eosinophil count.

4.3.1.2 Results

We performed a retrospective analysis of 295 unselected patients with asthma who underwent both FENO determination and successful sputum induction on the same visit. We determined FENO values that best identified a sputum eosinophil count ≥3%, taking into account important covariates such as the dose of ICS, atopy, smoking status, age and gender. Asthmatics aged 15-84 years were recruited from our asthma clinic in University hospital of Liege between 1st October 2005 and 30th September 2008.

We used Receiver-operating characteristic (ROC) curve and logistic regression analysis to assess the relationship between sputum eosinophil count and FENO. Logistic regression analysis assessed the relationship between the binary outcome (sputum eosinophil count ≥3%) and a set of covariates, individually or in combination. Covariates included FENO (log-transformed), age, gender, smoking, ICS and atopy. Cut-off points on the FENO scale were determined in each case so that there was a ≥50% probability of a sputum eosinophil count ≥3%. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were also calculated for each cut-off point from the original data.

The demographic and functional characteristics of the patients are given in Table 5.

For the whole group there was a significant positive relationship between the FENO level and the percentage of sputum eosinophil count (R_S=0.54, p<0.0001; Fig 5).

Despite this significant correlation, some patients showed marked discordance between the two markers. Patients with low sputum eosinophilia (<25% percentile) and high FENO levels (>75% percentile) were all atopic, non-smokers and receiving low doses of ICS (N=4). By contrast, for patients with high sputum eosinophilia

(>75% percentile) and low levels of FENO (<25% percentile), the only common distinctive feature was high doses of ICS (N=3).

Using the ROC curve method we found that a FENO concentration >41 ppb yielded 65% sensitivity and 79% specificity (AUC=0.78, p<0.0001) for identifying a sputum eosinophil count ≥3% in the whole population (Fig 6).

Table 5. Demographic, functional and inflammatory characteristics of study patients (n=295).

Characteristics	
Gender (M/F)	131/164
Age, yrs	47.3 (14-83)
Height, cm	168.6 ± 9.5
Weight, kg	73.16 ± 15.9
Atopy (Y/N)	208/87
Smoking (Y/N)	58/237
IgE, kU/L	169 (53 -472)
Blood eosinophils, %	3.80 ± 3.4
FEV_1, % predicted	86 ± 19
FEV_1/FVC, %	75 ± 11
PC20M, mg/ml	3.32 (0.025-16)
Reversibility, %	13.1 ± 12.5
Sputum eosinophils, %	1.8 (0.2-9.4)
Sputum neutrophils, %	43.6 (17.8-68.8)
FENO, ppb	31 (15-62)
ACQ	1.84 ± 1.2
AQLQ	4.77 ± 1.3

Data are presented as mean ± SD or median (IQR); PC20M is expressed as geometric mean (range).

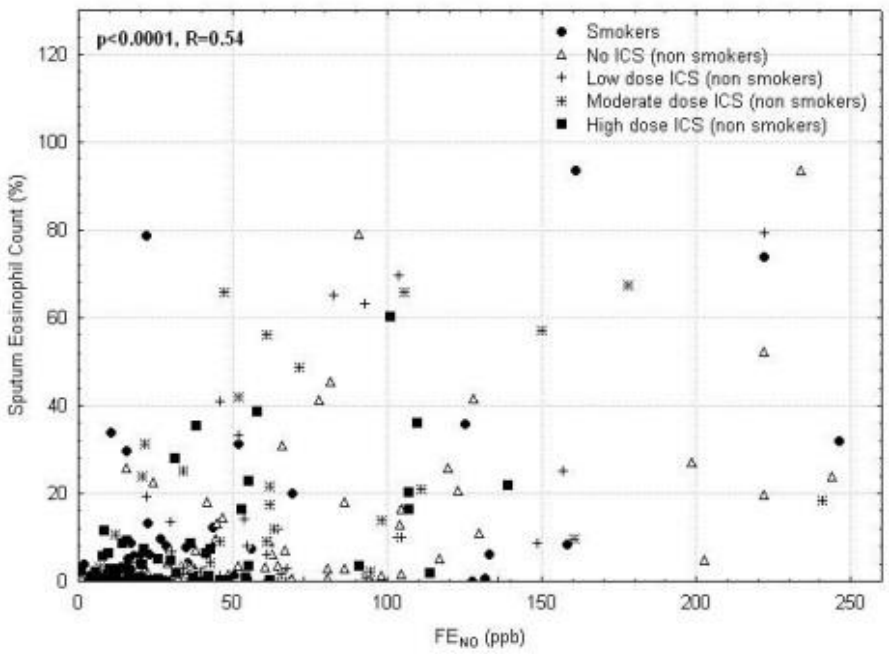

Fig 5. Evaluation of the relationship between sputum eosinophil count and exhaled nitric oxide (FENO) concentration in a cohort of unselected patients with asthma (n=295) by Spearman correlation. There was a highly significant correlation between these two parameters (p<0.0001, R=0.54).

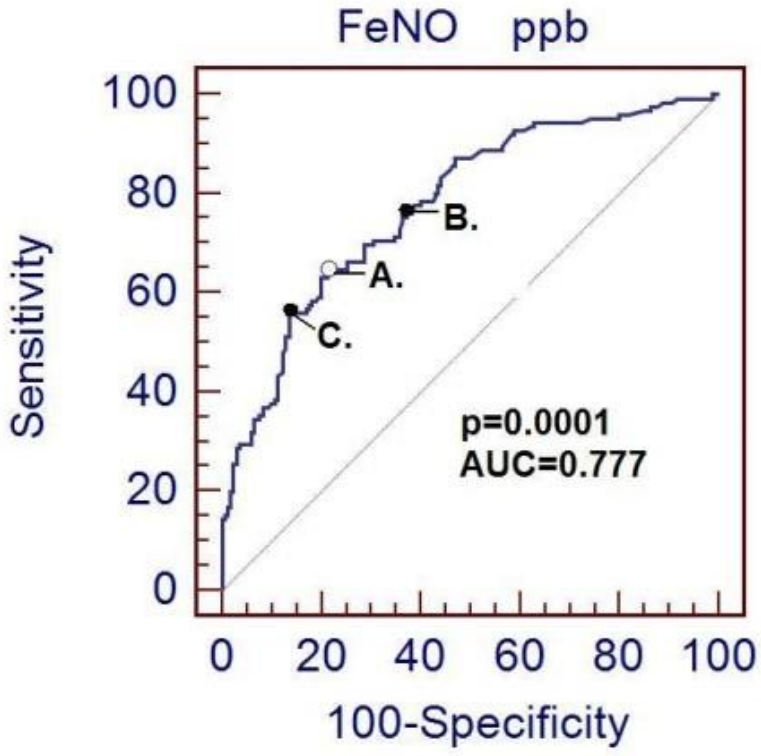

Fig 6. Receiver-operating characteristic curve (ROC) for the whole group to determine the exhaled nitric oxide (FENO)

value which best identified a sputum eosinophil count of ≥3%. The optimum cut-off point was 41 ppb (point A, sensitivity 65% and specificity 79%). FENO levels >25 ppb gave a sensitivity of 78% and a specificity of 60% for identifying a sputum eosinophil count ≥3% (point B, positive predictive value (PPV) 59%, negative predictive value (NPV) 79%). FENO values >50 ppb gave a sensitivity of 56% and a specificity of 86% for identifying a sputum eosinophil count ≥3% (point C, PPV 75%, NPV 73%).

This was confirmed by logistic regression analysis as we found that a threshold of 42 ppb discriminated between eosinophilic and non-eosinophilic asthma with 63% sensitivity and 80% specificity ($p<0.0001$).

Demographic, functional and inflammatory characteristics according to smoking status and the dose of ICS are given in Table 6. When taking the dose of ICS into account in nonsmokers, we distinguished four groups: steroid-naïve patients and patients receiving low doses of ICS (≤500 mg/day beclometasone), moderate doses (500-1000 mg/day beclometasone) and high doses of ICS (>1000 mg/day beclometasone). These groups had significantly different median FENO values, reaching 47 ppb, 46 ppb, 32 ppb and 24 ppb in steroid-naïve patients and those on low, moderate and high ICS doses, respectively ($p<0.001$, Table 6). By contrast, there was no significant difference in sputum eosinophil counts between the four groups (2.8% (IQR 0.8-14.4) in steroid–naïve patients, 1.8% (IQR 0-12.2) in patients on low-dose ICS, 1.0% (IQR 0-10.3) in patients on moderate doses and 1.8% (IQR 0.3-7.4) in the high dose ICS group ($p>0.05$, Table 6).

Table 6. Demographic, functional and inflammatory characteristics of patients with regard to their smoking status and the dose of inhaled corticosteroids.

	Smokers	**Non-smokers**			
		Steroid naïve	**Low ICS**	**Moderate ICS**	**High ICS**
No.	58	70	41	67	59
Gender (M/F)	31/27	26/44	17/24	33/34	24/35
Age, yrs	45,7 (23-81)	42,3 (14-80)	47 (19-80)	47 (16-83)	50,5 (18-82)
Height, cm	170,5±7,8	169±9,5	169,2±11	167±9,7	167±9,2
Weight, kg	73,3±14	73±16	74,3±18	72,6±14	72,9±17,9
Atopy	72%	80%	71%	67%	61%
IgE, KU/l	185 (56-612)	166 (39-358)	210 (60-391)	180 (48-606)	132 (70-430)
Blood eosinophils, %	3,6 ± 3,5	3,87 ± 3,4	3,94 ± 3,2	3,96 ± 3,5	3,26 ± 3,26
FEV1, % pred	81,4±19	90,5±16	93,2±17,7	86,6±20,3	78,1±19
FEV1/FVC, %	72±10,5	78,5±9	77,7±10,5	75±11,4	71,8±12
PC20, mg/ml	4,23(0,025-22)	2,17(0,05-22)	6,6(0,302-22)	3,93(0,2-22)	2,3(0,101-22)
Reversibility,%	15±11	15,3±14,8	12,1±8,5	13,3±14,3	11,1±11,7
Sputum eosinophils, %	1,7 (0,2-8)	2,8 (0,8-14,4)	1,8 (0-12,16)	1(0-10,3)	1,8(0,25-7,35)
Sputum neutrophils, %	43,1(17,6-63,8)	41,7(13-65,8)	32(16,4-49,5)	45(22,7-73,9)	52,5(29,1-75,1)
FENO,ppb	17(12-37)	47(23-87)	46(21-75)	32(13-61)	24(12-45)
ACQ	2,36±1,2	1,57±1,1	1,34±0,9	1,69±1,1	2,16±1,3
AQLQ	4,39±1,28	5,18±1,17	5,15±1,1	4,74±1,37	4,4±1,35

Data are presented as mean ± SD or median (IQR); PC20M is expressed as geometric mean (range).

Using logistic regression we found that the FENO threshold associated with sputum eosinophils ≥3% for patients receiving high doses of ICS was significantly lower (27 ppb) than that of steroid-naïve patients or those receiving low and moderate doses of ICS (48 ppb, p=0.028, Table 7).

Median FENO in atopic subjects was 1.3 times higher (34 ppb) than in non-atopic patients (26 ppb), which is close to that reported by Travers *et al.* (1.2 times, p=0.011) (149). By contrast, the sputum eosinophil count did not differ between atopic and non-atopic asthmatic subjects (median 1.8% vs 2%, p=0.543). Using logistic regression, atopic patients had a significantly greater FENO threshold value (49 ppb) than non-atopic patients (30 ppb, p=0.049) to identify sputum eosinophils ≥3% (Table 7).

Table 7. Simple logistic regression analysis assessing the effect of a high dose of ICS and atopy on the FENO threshold for identifying sputum eosinophilia ≥3%.

Characteristics		FENO cut-off (ppb)	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)	p-value
High dose ICS	Yes	27	62	76	59	78	0.028
	No	48	84	56	72	72	
Atopy	Yes	49	84	56	72	72	0.049
	No	30	64	74	60	77	
Smoking	Yes	28	62	76	59	78	0.066
	No	46	82	58	70	73	
Male	Yes	37	71	66	63	74	0.24
	No	48	84	56	72	72	
Age	≥ 40	43	80	62	69	74	0.87
	< 40	42	79	64	69	75	

The FENO level in smokers was significantly lower than in non-smokers (median 17 ppb for smokers vs 35 ppb for non-smokers, p=0.003). The median FENO level for non-smokers was two times higher than in current smokers, which was higher than 1.18 obtained by Travers *et al.* (149) but in line with the results of Michils *et al.* (150). In contrast, the median (IQR) sputum eosinophil count did not differ significantly between smokers and non-smokers (1.7 (0.2-8) vs (1.8 (0.2-10.7); p=0.765). After logistic regression the FENO level that identified a sputum eosinophil count of ≥3% was lower in smokers than in non-smokers (28 ppb vs 46 ppb, p=0.066, Table 7).

The median FENO value was 1.33 times greater in men than in women (36 ppb vs 27 ppb, p=0.009), which is in keeping with the results of Travers *et al.* (1.24 times)(149). The sputum eosinophil count was greater in men than in women (2.9% vs 1.5%, p=0.042). However, the FENO threshold that best identified a sputum eosinophil count of ≥3% was not significantly different between men and women (Table 7).

Likewise, age did not influence the relationship between FENO and sputum eosinophils.

When combining all variables into the logistic model, FENO (p<0.0001), high-dose ICS (p=0.019) and smoking (p=0.044) were independent predictors of sputum eosinophilia and there was a trend for atopy (p=0.086), but age and gender were not significant (Table 8). The optimal cut-off point for FENO associated with a sputum eosinophil count >3% ranged from 15 ppb for smoking non-atopic patients receiving a high dose of ICS to 58 ppb for non-smoking atopic patients not treated with a high dose of ICS.

Table 8. Cut-off points for FENO for predicting a sputum eosinophil count ≥3% after multiple logistic regression analysis.

Smoking	Atopy	High dose of ICS	FENO cut-off (ppb)	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)
No	No	No	38	74	66	65	75
No	No	Yes	24	59	78	58	79
No	Yes	No	58	88	48	74	70
No	Yes	Yes	35	71	70	64	76
Yes	No	No	23	58	78	58	78
Yes	No	Yes	15	37	94	52	89
Yes	Yes	No	33	68	70	62	76
Yes	Yes	Yes	20	54	85	58	83

When combining all variables into the logistic model, we found that only FENO (p<0.0001), smoking (p=0.044) and ICS (p=0.019) were significant predictors of sputum eosinophil count ≥3 %. When atopy was added, the p-value was not significant although a trend was observed (p=0.086) (For more details on calculation, see on-line supplement).

We compared our FENO values in patients with asthma with those expected based on the equation proposed by Dressel *et al.* (151) in a general population, taking into

account the height, gender, atopy and smoking status. It appeared that our observed values were much higher than expected, which highlights the importance of asthma as a major factor contributing to the rise in FENO (Table 9).

Table 9. Comparison of exhaled nitric oxide (FENO) values measured in patients with asthma with those expected based on the formula proposed by Dressel *et al.* (151) taking into account height, gender, atopy and smoking status*.

	FENO value based on Dressel *et al.* (151)	**Geometric mean FENO value for our population with asthma**	**Optimum cut-off point for predicting a sputum eosinophil count ≥3%**
Males, smokers, atopy	20	50	31
Males, non-smokers, atopy	33	62	51
Males, smokers, no atopy	14	53	22
Males, non-smokers, no atopy	21	48	33
Females, smokers, atopy	16	35	39
Females, non-smokers, atopy	25	47	66
Females, smokers, no atopy	10	14	26
Females, non-smokers, no atopy	16	39	40

$$^{*}FeNO(ppb) = 17.493 \times (1.496\ if\ atopy) \times (0.627\ if\ smoker) \times (1.235\ if\ infection) \times (1.174\ if\ male) \times 1.113 \times \left(\frac{height - 170}{10}\right)$$

4.3.1.3 Discussion

Although the debate is not over, it is generally accepted that FENO has the potential to assess the phenotype of airway inflammation. Like those of Berry *et al.* (49), our data show that FENO can effectively identify sputum eosinophilia in a large cohort of unselected patients with asthma. There is, however, clear variation in the threshold associated with a sputum eosinophil count of ≥3% depending on treatment with ICS, atopy and smoking status.

The technique of induced sputum that allows collection of airway cells has contributed to the emergence of the concept of asthma phenotypes (152). Although introduced in the early 1990s, the technique has not been widely adopted into clinical practice, probably because it is technically demanding and time-consuming.

Given the recognised clinical value of sputum eosinophil count, we wondered whether FENO measured at a flow rate of 50 ml/s could effectively identify a sputum eosinophil count ≥3% in a large cohort of unselected patients with asthma with different disease severity. Overall we found that FENO was a valuable measurement to identify eosinophilic asthma with a threshold value of 41 ppb, having sensitivity and specificity of 65% and 79% respectively. This threshold is twice as high as the level recommended for an asthma diagnosis (20 ppb) but quite close to the level that Smith *et al.* claimed to predict a positive response to ICS (47 ppb)(132). This is also in keeping with the fact that having a sputum eosinophil count of at least 3% is associated with a clinical response to short-term treatment with ICS. Coincidently, our threshold of 41 ppb appears to be the same as the upper limit of the 90% CI found in a normal population (149). There is an emerging consensus that FENO values >50 ppb indicate active eosinophilic airway inflammation (153). Conversely, FENO values <25 ppb are associated with minimal airway eosinophilia. According to our data, FENO <25 ppb has a negative predictive value of 79% for significant airway eosinophilic inflammation and FENO >50 ppb has a positive predictive value of 75% to identify sputum eosinophilia.

It is well established that chronic treatment of asthma with ICS results in a reduction in airway eosinophilia (18;154) together with a fall in FENO (145;155;156). How this treatment may affect the relationship between sputum eosinophilia and FENO remains unclear, however. Our data indicate that FENO is more sensitive than the

sputum eosinophil count to ICS as patients receiving higher doses of ICS had lower FENO values than the other groups, a phenomenon not observed with the sputum eosinophil count. Consequently, the FENO threshold associated with sputum eosinophilia ≥3% will be lower in patients receiving high doses of ICS than in those not treated or receiving low or medium doses of ICS.

In agreement with previous studies (148), the influence of atopy was evident in our study with a 30% increase in FENO in atopic compared with non-atopic patients with asthma. On the other hand, the sputum eosinophil count was similar in both groups. Consequently, the FENO threshold associated with sputum eosinophilia was found to be greater in atopic patients with asthma than in non-atopic patients with asthma. Interestingly, our data show that sputum eosinophilia may be present in subjects with asthma who smoke. Indeed, the median value in asthmatics who smoke was not significantly lower than that in the non-smoking group, which is consistent with the findings of Boulet *et al.* (157). There are no data showing that starting to smoke reduces the sputum eosinophil count in patients with asthma. There are, however, data showing that smoking cessation in asthma does not impact on the sputum eosinophil count (158). In contrast, the FENO levels were clearly lower in patients with asthma who smoke, which confirms the results of a previous study (146). This may be explained by partial irreversible inhibition of nitric oxide synthase by cigarette smoke extract (159;160), or by conversion of nitric oxide to peroxynitrite by superoxide release from neutrophils (161). As tobacco influences FENO and the sputum eosinophil count differently, it therefore changes the FENO cut-off value. As expected, we found higher FENO levels in men than in women. Interestingly, the sputum eosinophil count was lower in women than in men, an observation not previously reported and in keeping with the higher proportion of neutrophilic asthma

in women (13). The reasons for this are unclear at present but might be linked to different immunological mechanisms governing airway inflammation (12). Although gender may influence both the FENO and the sputum eosinophil count, it does not alter the relationship between these two variables. As several parameters were shown to influence the relationship between FENO and sputum eosinophils, a multiple logistic regression approach was used to determine the most useful cut-off point in a patient. It confirmed the importance of high-dose ICS and smoking and, to a lesser extent, atopy. We found that the optimal cut-off points varied from 15 ppb for smoking non-atopic patients receiving a high dose of ICS to 58 ppb for non-smoking atopic patients not treated with a high dose of ICS. The gap between these two extremes is large and ought to be taken into account in the interpretation of airway inflammation. Some of our patients had paucigranulocytic asthma (124) and still exhibited raised FENO levels. This discordance might be explained by inflammation which has not spilled over into the airway lumen, such that induced sputum is negative for eosinophils while this cell type still persists in the airway wall. In these patients, monitoring of FENO could be more informative than measuring sputum eosinophils. While it is a reasonable assumption that our derived FENO cut-off values will be applicable to the general asthma population, further work is required to validate this in practice as we did not divide our dataset into training and testing groups.

We can conclude that FENO is able to identify the presence of sputum eosinophilia in unselected patients with asthma with reasonable accuracy as long as thresholds are adjusted for high doses of ICS, atopy and smoking status.

4.3.2 Blood eosinophils, IgE and FEV_1/FVC

For the 508 patients who underwent a successful sputum induction between October 2005 and June 2011, there was a significant positive relationship between blood eosinophil count, either expressed as percentage or absolute value, and percentage of sputum eosinophil count ($r=0.6$, $p<0.0001$; $r=0.6$, $p<0.0001$; respectively; Fig 7). We constructed ROC curves to determine the concentration of blood eosinophils or neutrophils that best identified a sputum eosinophil count ≥3% or a sputum neutrophil count ≥76% respectively. We found that a blood eosinophil count ≥220/mm^3 yielded 77% sensitivity and 70% specificity (Area under the curve (AUC) = 0.79, $p < 0.0001$, Fig 8) for identifying a sputum eosinophil count ≥3% in the whole population. By constructing a ROC curve we found that a cut-off value of 3% for percentage of blood eosinophils was able to identify the presence of a sputum eosinophil count ≥3% with 75% sensitivity and 73% specificity (AUC=0.81, $p< 0.0001$, Fig 9). The measure of blood eosinophils was as efficient as FENO (cut-off=41 ppb, AUC: 0.79, $p<0.0001$) for identification of sputum eosinophilia ≥3%. The comparison of the AUC for both tests failed to demonstrate a significant difference ($p=0.77$).

We further sought to determine the factors associated with sputum eosinophilic phenotype. The logistic regression analysis was used to assess the relationship between the binary outcome (sputum eosinophil count ≥3%) and a set of covariates (gender, age, height, weight, atopy, smoking status, IgE, blood eosinophil and neutrophil count, FEV_1%, FEV_1/FVC, TLC, FRC, KCO, PC20M, Reversibility, Fibrinogen, CRP, FENO, ACQ, AQLQ, ICS therapy). When combining all variables into the model, percentage of blood eosinophils (logit-transformed; $p<0.0001$), FEV_1/FVC ($p=0.0021$), FENO (log-transformed; $p<0.0001$), and IgE (log-transformed;

p=0.0085) were independent factors associated with the presence of sputum eosinophilic inflammation (Table 10).

Using those variables, we established a formula to compute the probability (π) of a sputum eosinophil count ≥ 3%:

$$\pi = \frac{exp(L)}{1 + exp(L)}$$

where $L = 2.92 + 1.218 \times ln\frac{Blood\ eos\%}{100-Blood\ eos\%} + 0.217 \times ln(IgE) \ - 0.039 \times \frac{FEV_1}{FVC\%} + 0.844 \times ln(FENO)$

π: probability of sputum eosinophil count ≥ 3%.

Blood eos%: Blood eosinophil count in %.

The ability of the equation to predict sputum eosinophilia was tested in an independent population of 138 asthmatics recruited between July 2011 and May 2012 who underwent FENO, spirometry, sputum induction and gave a blood sample. The demographic, functional and inflammatory characteristics of the validation population were similar to those of the study population.

The agreement between predicted and observed value gave a Cohen Kappa's coefficient of 0.59 (p<0.0001, lower limit of confidence interval =0.43). The specificity and sensitivity were 62.7% and 93.7% respectively while the PPV was 67% and the NPV was 93%.

Among the factors shown to contribute to airway eosinophilia, blood eosinophils came first when performing a multiple logistic regression. There are few studies assessing the ability of blood eosinophils to identify airway eosinophilic inflammation. Previous studies showed that peripheral blood eosinophil count was correlated with bronchoalveolar lavage eosinophil count (162) and sputum eosinophil count (163-

165). These studies have, however, investigated a limited number of asthmatics and did not provide any threshold value of blood eosinophils as marker of airway eosinophilia. The American multicentre study found a threshold value of 220/mm³ as the best compromise for predicting sputum eosinophil count ≥2% (140). Another recent study conducted in COPD showed that a cut-off of 2% peripheral blood eosinophils had a sensitivity of 90% and specificity of 60% for identifying a sputum eosinophilia of greater than 3% at exacerbation. In our study, we confirmed the correlation between blood and sputum eosinophilic inflammation in a large cohort of patients. We found the best threshold to be 220/mm³ and 3% and these thresholds were as effective as FENO at predicting uncontrolled airway eosinophilic inflammation. Our findings are in keeping with those recently reported by McGrath *et al.* (140). Compared to blood eosinophils, FENO has however the advantage of giving immediate results and its measurement is more comfortable to the patient.

In this study the performance (threshold, sensitivity, specificity) of FENO to identify sputum eosinophil count is very similar to the one we reported previously (166). The fact that FENO and blood eosinophil counts came out as independent predictors of sputum eosinophilia in the multiple logistic regression suggests that these two markers reflect different mechanisms promoting the recruitment of eosinophils into the airways. Other independent factors to be shown associated with prominent sputum eosinophilia are FEV_1/FVC and total serum IgE level. Previous studies have shown that FEV_1/FVC, an index of airway narrowing, was correlated to sputum eosinophilia (37) and eosinophilic asthma has been recognised to be frequently associated with atopic disease (13;24). In our study, total serum IgE levels were however best predictor of sputum eosinophilia than atopy per se, in line with a recent study (140). From the biological properties of IgE it can be speculated that high tissue

IgE may prime local mast cells and activate them even without intervention of allergens (167). In this view it is interesting to note that airway mast cell activation demonstrated by tryptase release is a phenomenon found to be associated with sputum eosinophils in asthma (168) and COPD (169).

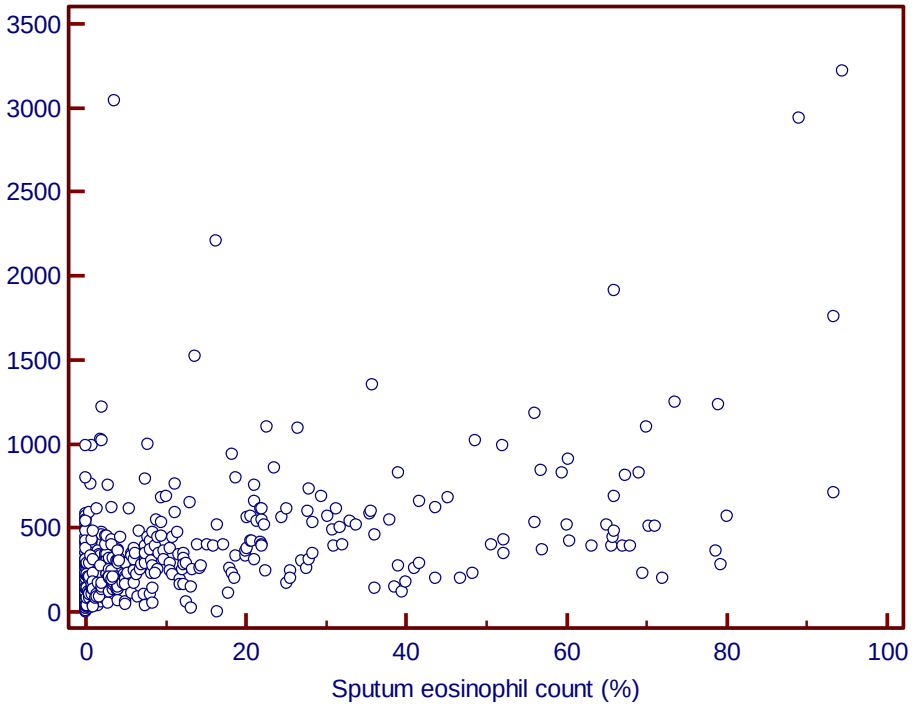

Fig 7. Relationship between sputum eosinophil count and blood eosinophil count in a cohort of unselected patients with asthma (n=508) by Spearman correlation. There is a significant correlation between these two parameters ($p<0.0001$, $Rs= 0.6$). AV: absolute value of blood eosinophils (/mm³).

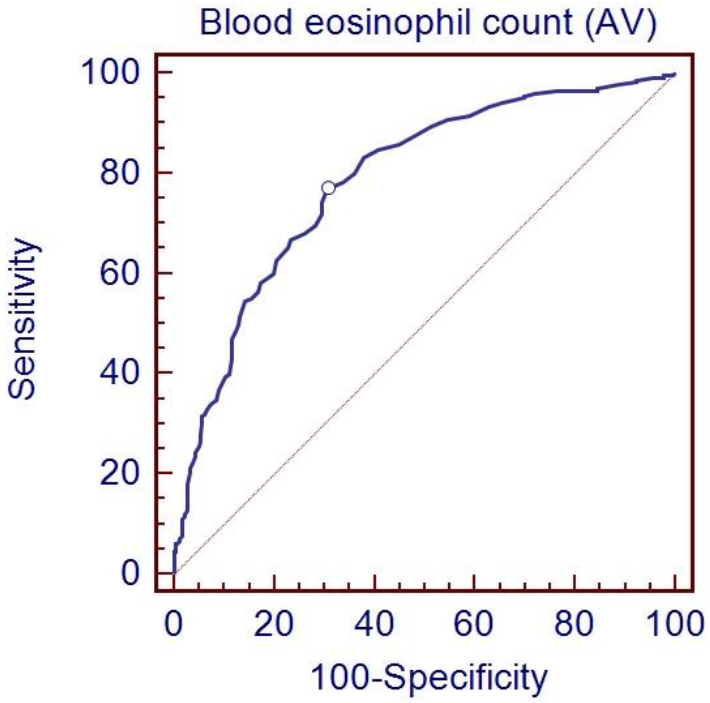

Fig 8. Receiver-operating characteristic (ROC) curve for the whole group to determine the blood eosinophil count value that best identified a sputum eosinophilia ≥3%. The optimum cut-off point was 220/mm³ (Sensitivity 77%, specificity: 70%, AUC: 0.790, p<0.0001).

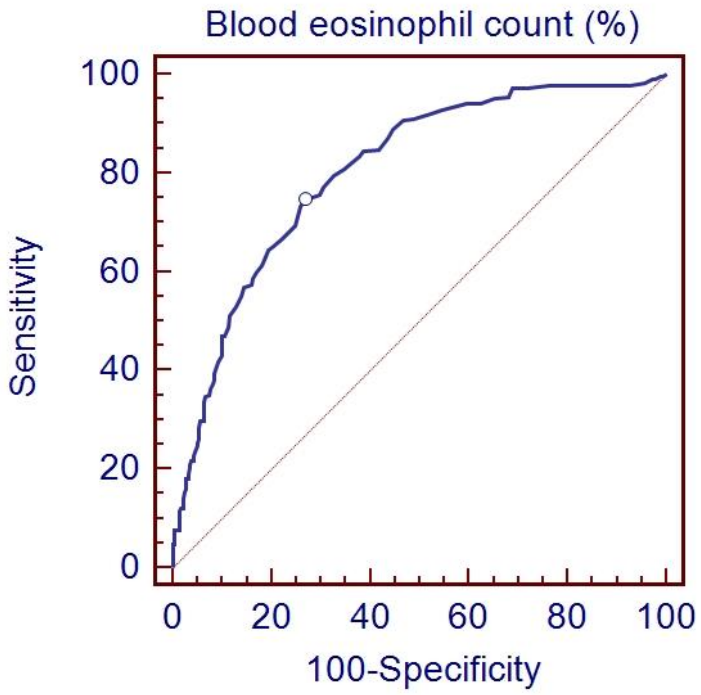

Fig 9. Receiver-operating characteristic (ROC) curve to determine the blood eosinophil count percentage that best identified a sputum eosinophilia ≥3%. The optimum cut-off point was 3% (Sensitivity: 75%, specificity: 73.4%, AUC: 0.81, p<0.0001).

Table 10. Independent predictors of sputum eosinophilia.

Parameter	β	SE	p-value
Logit Blood eosinophils, %	1.218	0.19	P<0.0001
Ln IgE	0.217	0.08	P=0.0085
FEV_1/FVC, %	-0.039	0.01	P=0.0021
FENO	0.844	0.16	P<0.0001

Ln, Natural logarithm; FEV1, Forced expiratory volume in 1 s; FVC: Forced vital capacity; FENO, exhaled nitric oxide; SE: standard error.

4.3.3 Factors associated with neutrophilic phenotype

4.3.3.1 Blood neutrophils, age and FRC

The same approach as for eosinophils was used to predict the presence of sputum neutrophils.

There was a weak correlation between sputum and blood neutrophil count taken in percentage (r=0.19, p=0.0015) but not in absolute value (r=0.19, p=0.11). Despite statistically significant correlation between sputum and percentage blood neutrophils, the strength of the relationship was rather poor.

Using the ROC curve method, we found a cut-off of 4960/mm3 and 66% respectively giving a sensitivity of 49% and 37%, a specificity of 70% and 90%, p=0.03 and p=0.003, AUC=0.59 and AUC=0.63 respectively.
However, when combining all variables into the logistic model, age (p=0.006) and Functional residual capacity (FRC, p=0.001) were independent factors associated with sputum neutrophilia. The multiple logistic regression analysis demonstrated the inability of blood neutrophils to predict uncontrolled sputum neutrophilic inflammation.

The formula to compute the probability (π) of a sputum neutrophil count ≥ 76% was:

$$\pi = \frac{exp(L)}{1 + exp(L)}$$

where $L = -5.32 + 0.032 \times age + 0.022 \times FRC$

π: probability of sputum neutrophil count ≥ 76%.

To test the ability of the equation to predict sputum neutrophilia, we recruited a validation population of 178 asthmatics who underwent spirometry and sputum

induction. The agreement between predicted and observed value of sputum neutrophil counts gave a Cohen Kappa's coefficient of 0.24 ($p<0.0001$, lower limit of confidence interval =0.12). The specificity and sensitivity were 21% and 97% respectively. Patients receiving moderate to high dose ICS had higher sputum neutrophil counts (47.3%) than patients receiving low dose ICS (38.8%, $p=0.017$). Smokers did not have significantly higher proportion of neutrophils in their sputum (Median 48.9%) than ex-smokers (Median 50.6%, $p=0.68$) or never-smokers (Median 44%, $p=0.19$). However, neither smoking status nor the dose of inhaled corticosteroids was able to predict elevated sputum neutrophil count.

The accumulation of airway neutrophils has been reported to be directly associated with the activation of circulating neutrophils in response to the chemokine Interleukin-8 (169-171). Baines *et al.* found a correlation between plasma neutrophil elastase and neutrophilic airway inflammation (172). Such data suggest that airway neutrophilic accumulation could be due to an enhanced neutrophil activation and migration to the airways independently of the number of circulating cells. Moreover, it has been shown that neutrophils can be retained in the pulmonary microvasculature due to their low deformability, resulting in a higher concentration than in the systemic circulation. It is thought that this high concentration of the cells facilitates their effective recruitment to sites of inflammation (173). It seems likely that many could leave the circulation by chemoattraction, entering the lung without necessarily having a detectable effect on circulating levels. From a multiple logistic regression two factors came out as being independently associated with sputum neutrophilia. In keeping to what was found by Thomas *et al.* in healthy subjects (121) and Woodruff *et al.* (41) in asthmatics, age appeared to be a critical factor in our cohort with sputum neutrophilia rising with age. In addition to age we also found that FRC was an

independent factor. This suggests that airway neutrophils may contribute to reduction of inspiratory capacity seen in some asthmatics. Accordingly, two paediatric studies reported that percentage neutrophils in bronchoalveolar lavage directly correlated with air trapping (FRC) in children with cystic fibrosis (174;175). However, neither smoking status nor the dose of inhaled corticosteroids was able to predict the presence of sputum neutrophil count.

4.3.4 Volatile organic compounds (VOCs)

We wanted to determine the ability of VOCs detection in exhaled air to discriminate between paucigranulocytic and eosinophilic/neutrophilic asthma. Our project involved basic experimental and in vivo studies. Chemical identification of the compounds released by eosinophils and neutrophils in vitro gave potential candidates to check in asthmatic patients for asthma phenotyping.

4.3.4.1 In vitro study

We measured VOCs released by neutrophils and eosinophils in vitro. Eosinophils and neutrophils were isolated from 27ml blood of 16 healthy non-smokers by gradient centrifugation using lymphoprep. Eosinophils were isolated from neutrophils by immunomagnetic cell separation (MACS) using anti-CD16 micro beads. CD16 positive cells remained in the column while the other were collected in the falcon tube. The average absolute number of eosinophils and neutrophils upon isolation was 3.5×10^6 and 19.4×10^6 respectively. Cells were incubated in medium RPMI at 37°C and activated with phorbol 12-myristate 13-acetate (100ng/ml). A study published in 2003 effectively showed that eosinophils and neutrophils generated extracellular oxygen radicals in response to activation by PMA and that the best slope of release was obtained with a concentration of 100ng/ml (176).

The viability of the cells was evaluated using trypan blue and mitochondrial activity testing. Headspace air was sampled at time 30', 60' and 90' by introduction of ultra-pure nitrogen in closed flasks at a flow rate of 200 ml/min during 10 min (Fig 10). Each experiment was done 10 times. The air was pushed out onto a carbon tube and the total amount of trapped VOCs (volatome) was analysed by time-of-flight GC-MS. From the 2116 compounds presents in the volatome, those present in at least 8% of

the samples (1123 compounds) were used for further analysis. Leave-one-out method was used as a kind of cross-validation analysis as it is a statistical technique of choice when the number of samples is small. For the leave-one out method, one sample is left out for each statistical analysis and each sample is used once as validation. Discriminant analysis (SPSS statistics19) showed that 2 VOCs were able to distinguish between unactivated eosinophils and unactivated neutrophils with 85% correct classification of original data set and cross-validated observations. Chemical identification of both compounds was 3-methylfuran and benzylalcohol, with higher levels in the neutrophilic culture as compared to eosinophilic culture. Benzylalcohol is very interesting as it was also found to be discriminative in vivo between healthy controls and asthmatics by Ibrahim *et al.* (177). 3-methylfuran has already been detected in human breath with a significant difference between healthy controls and patients with lung cancer (178).

5 VOCs were able to distinguish between both activated culturing types with 100% and 96% correct classification in original and cross-validated sets respectively. Keeping only the 2 most important compounds gave 83% correct classification of the samples. Chemical identification showed that 1-H-indenol and 2-Butoxyethanol discriminated between activated eosinophils and neutrophils.

From this first part of the in vitro study we concluded that analysis of VOCs seems very promising in discriminating between eosinophilic and neutrophilic inflammation but needed further development and *in vivo* confirmation.

We also looked at the VOCs that discriminated between activated and unactivated cells. For eosinophils, p-dichlorobenzene (peak 254) gave 84% correct classification of original and cross-validated sets. Our hypothesis is that when eosinophils are

activated in vitro, they release EPO that is responsible for chlorinated agent production. The main role of eosinophil peroxidase was shown to be the production of oxygen radicals to kill parasites. Eosinophil peroxidase has been shown to be able to use halides or pseudohalides (Cl^-, Br^-, I^-, SCN^-) and H_2O_2 to generate cytotoxic hypohalous acids (179). EPO has also been shown to generate preferentially hypobromous acid and that hypochlorous acid are essentially produced when pH is lower than 6 or if the medium Cl^- concentration increased (179).

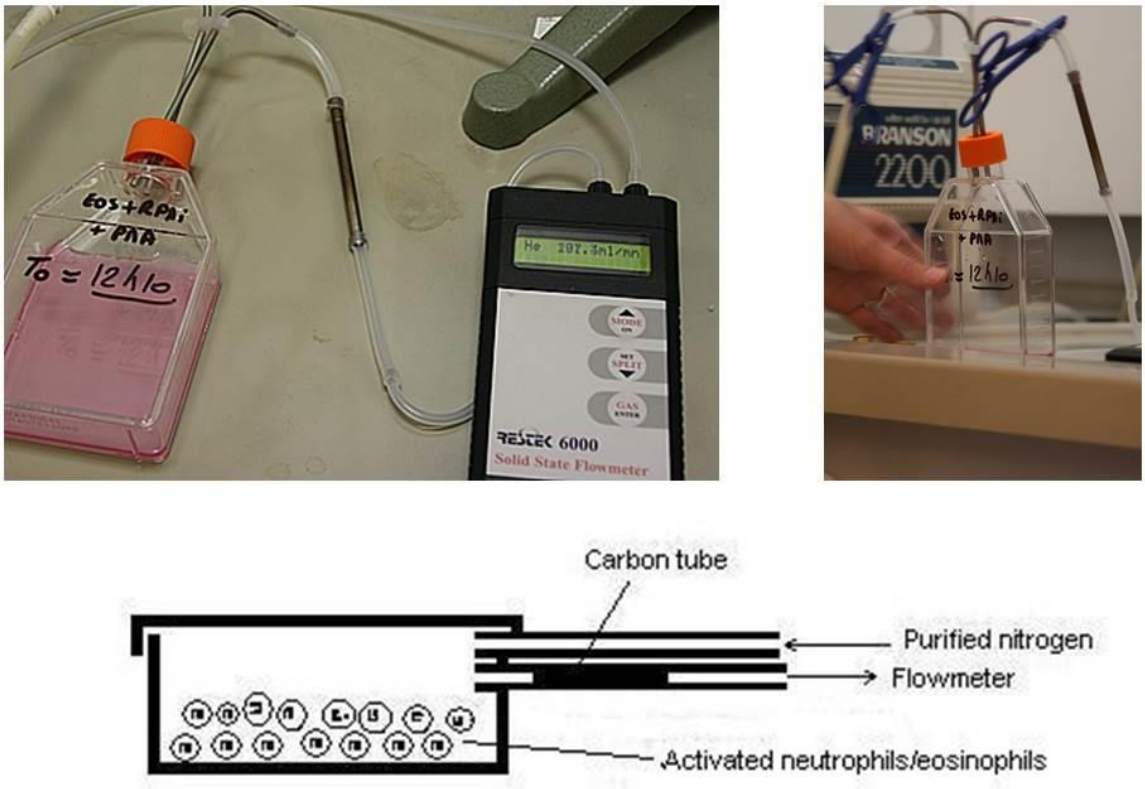

Fig 10. Sampling of the head space of flasks containing activated eosinophils or neutrophils.

We further identified VOCs able to distinguish between activated and unactivated neutrophils. Peak 842, Benzylalcohol and 6,10-dimethyl-5,9 dodecadien-2-one were the three VOCs able to discriminate between unactivated and activated neutrophils. We were unable to identify the component corresponding to peak 842. A possible explanation could be that this compound is not in the NIST Library or that the initial compound structure has been modified during heating of the tube and is no longer recognisable.

4.3.4.2 In vivo VOCs study

276 asthmatics were sampled with 3328 volatile organic compounds detected. From those patients, 122 exhibited eosinophilic asthma, 90 had paucigranulocytic asthma, 50 neutrophilic asthma and 14 mixed granulocytic asthma. Their demographic functional and inflammatory characteristics (Table 11) were similar to those of the prospective asthmatic population studied in chapter 4.5 (Table 18-19).

Table 11. Demographic and functional characteristics of 276 asthmatics recruited for the VOCs study

Characteristics	
N.	276
Age (yrs)	50 ± 15
Gender (% of female)	59
Smokers (%)	18.5
Ex-smokers (%)	36
Non-smokers (%)	45.5
FEV_1 (% pred)	82 (24-133)

Time-of-flight mass spectrometry was used to identify components (peaks) available in the samples (Fig 11). When comparing volatile organic compounds present in the exhaled air of paucigranuloctytic to those present in eosinophilic asthmatics, 3 components (P337, P903 and P923, Fig 12) were shown to be good discriminators. The chemical nature of these compounds cannot be unveiled yet although 2 of them were identified using the NIST Library. P923 remained undetermined. A possible explanation could be that this compound is not in the NIST Library or that the initial compound structure has been modified during heating of the tube and is no longer recognisable.

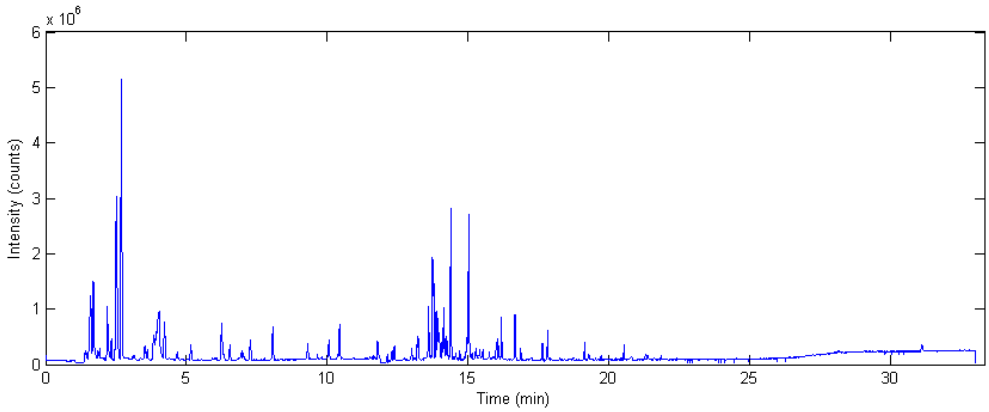

Fig 11. Example of exhaled air sample as measured by thermo-desorption GC-MS.

Column#	Mean decrease in MSE
P337	**0.003138**
P903	**0.002638**
P923	**0.001799**
P2576	0.000812
P1668	0.00073
P2179	0.000555
P2105	0.000528
P2532	0.000528
P3066	0.000419
P2051	0.000419
P1544	0.000406
P640	0.000406
P395	0.000365
P2622	**0.000095**

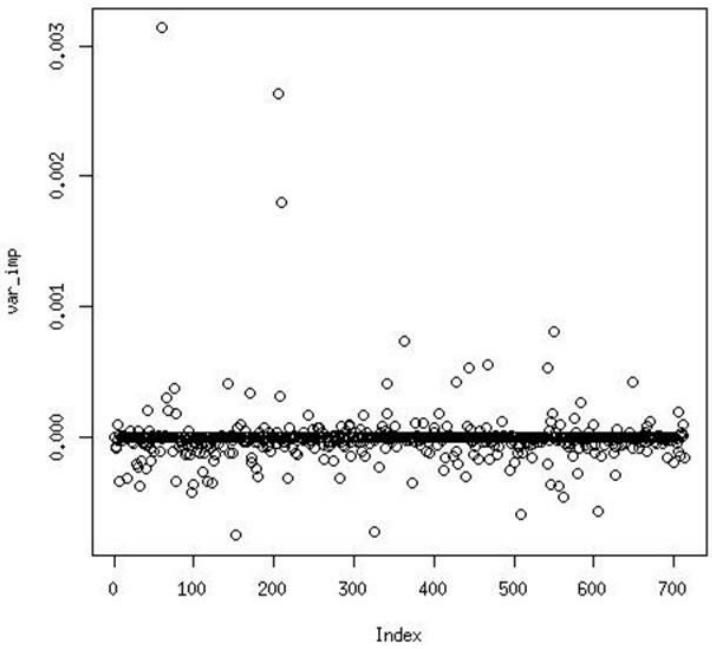

Fig 12. P337, P903 and P923 are good driscriminator between eosinophilic and paucigranulocytic asthma. P# is a consecutive compound number of the original data matrix with the column number.

There have been previous studies using eNose to diagnose asthma vs COPD (63) or using GC-MS VOCs or eNose analysis to discriminate between asthmatics vs healthy

subjects (180;181). The study of Montuschi has also compared the asthma diagnostic performance of FENO, lung function testing and eNose showing that the best performance was obtained by combining eNose and FENO (182). Robroeks *et al.* conducted a study demonstrating that a combination of different exhaled VOCs was able to predict exacerbations in childhood asthma (183). Moreover Ibrahim and colleagues demonstrated on a small population of asthmatics that VOCs profiles were able to diagnose sputum eosinophilia (177). However, our study is the first attempt to characterise VOCs according to sputum eosinophilic, neutrophilic and paucigranulocytic phenotype in a large population of asthmatics. If we accept the definition of asthma being symptoms combined to excessive airway variability, we believe that it is very unlikely that such a complex physiological disorder may be captured by spotting individual biological compounds in the exhaled air. By contrast, because they may reflect some inflammatory pathway we think that VOCs analysis is essentially useful to characterise the inflammatory phenotype of asthma rather than to diagnose asthma itself. This could have treatment implications for patients in whom a quick diagnosis of asthma phenotype could predict the response to treatment.

We further compared the volatile organic compounds presents in the exhaled air of neutrophilic and paucigranulocytic asthmatics to identify discriminative VOCs for the neutrophilic phenotype. We found that P2622 and P2853 were volatile organic compounds able to distinguish asthmatics with increased neutrophil counts as compared to pauci-granulocytic asthma (Fig 13).

Column #	Mean decrease in MSE
P2622	**0.04145**
P2853	**0.002656**
P334	0.000674
P379	0.000102
P2017	0.000082
P32	0.000082
P119	0.000061
P808	0.000061
P338	0.000041
P1004	0.000041
P2545	0.000041
P585	0.00002
P2682	0.00002

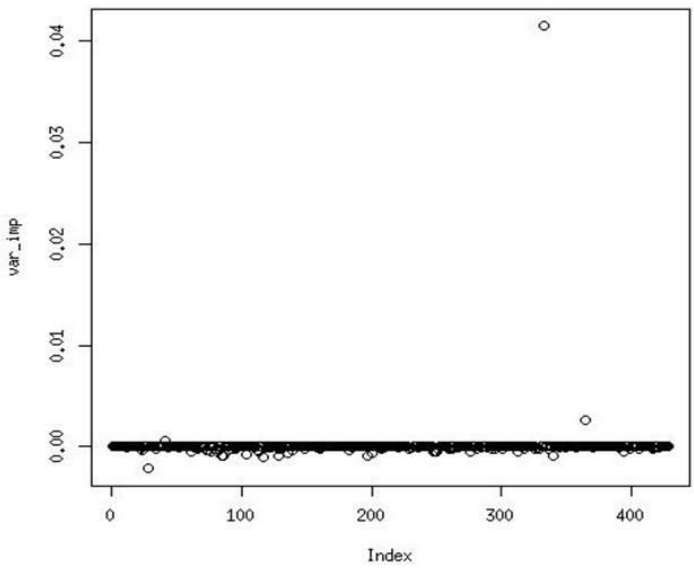

Fig 13. Two peaks are associated with a significant discriminant power. P# is a consecutive compound number of the original data matrix with the column number.

We tested the ability of VOCs to discriminate between eosinophilic and neutrophilic asthma. We showed that Peak 253 and 1913 were able to discriminate between eosinophilic and neutrophilic airway inflammation.

Using canonical discriminant functions, we discriminate between neutrophilic asthma (group 1, blue), eosinophilic asthma (group 2, green) and paucigranulocytic asthma (group 3, yellow) (Fig 14).

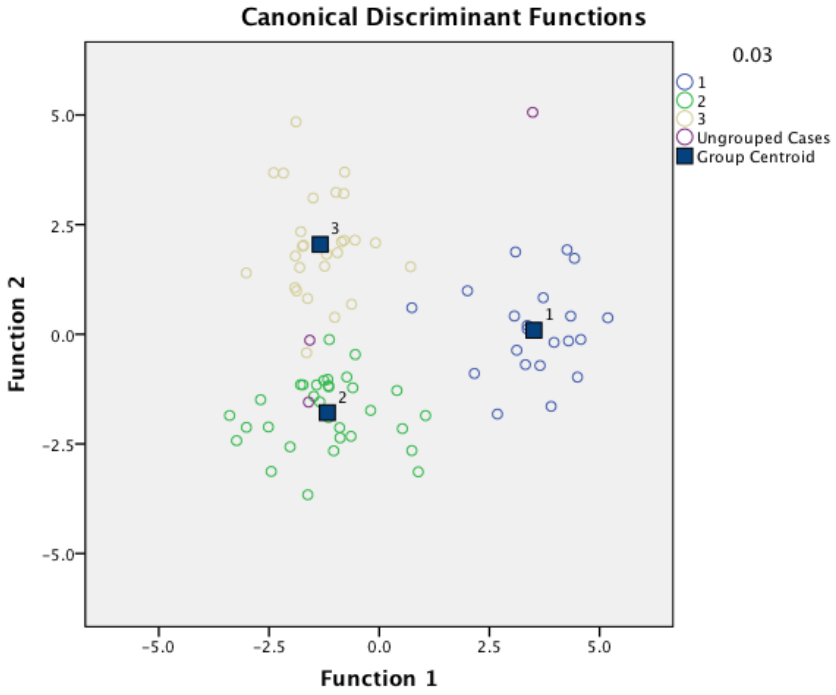

Fig 12. Canonical discriminant functions.

The discriminative capacity of VOCs has of course to be confirmed in a prospective study. We would like to contribute to the development of a small device allowing a quick measurement of airway inflammation that could be used by asthmatics to adapt their treatment and decrease the risk of exacerbations.

Ibrahim *et al.* (177), using gas chromatography and mass spectrometry in a small population of 35 asthmatics, found a model using 15 VOCs that was able to discriminate between asthmatics and healthy controls with 86% accuracy. In this study they also sought to discriminate breath samples from eosinophilic as compared to non-eosinophilic asthmatics and from neutrophilic as compared to non-neutrophilic asthmatics. They found that three principal components had a classification accuracy of 83% for discrimination between patients exhibiting induced sputum eosinophil counts <2% (n=10) as compared to ≥2% (n=8) with a sensitivity of 75% and a

specificity of 90%. When comparing neutrophilic (≥40%, n=8) versus non-neutrophilic asthma (<40%, n=10), one principal component was able to discriminate between both groups with 72% accuracy. The limitation of this study was the low number of patients studied and the choice of the neutrophilic threshold which is well inside the normal range for this cell type (118).

4.4 Exhaled nitric oxide: a marker of airway hyperresponsiveness

4.4.1 Introduction

Some studies have suggested that FENO may be helpful in diagnosing asthma but it remains debated. We conducted a field study assessing the potential of fractional exhaled nitric oxide, measured at a flow rate of 50 ml/s in steroid-naïve patients with suspected asthma sent by respiratory physicians to a routine function laboratory to perform a methacholine challenge.

Asthma diagnosis is usually based on symptoms such as cough, breathlessness, dyspnoea and wheezing together with the demonstration of airflow variability. The airway inflammatory component of the disease is an important feature which is an integral part of the asthma definition (5). Both sputum eosinophil count and FENO have been proposed as a useful diagnostic tool in mild to moderate asthma. There is a need for a simple, quick and reliable test in those patients with suggestive symptoms of asthma.

Early studies have suggested that fractional exhaled nitric oxide, measured at a flow rate of 200ml/s cut-off of 16ppb may help to identify patients with bronchial hyperresponsiveness to histamine or reversibility to β2-agonist among those presenting with chronic respiratory symptoms and normal baseline lung function (73). Airway hyperresponsiveness assessed by methacholine challenge is time consuming and unpleasant to the patient whereas FENO measurement is easy to perform and provides immediate results. The purpose of our study was to see how FENO measured at a flow rate of 50 ml/s may actually reflect the presence of methacholine bronchial hyperresponsiveness assessed by the provocative concentration that causes a 20% fall in FEV_1 (PC20M) in patients referred by chest physicians for

asthma diagnosis to a routine laboratory function. We also sought to establish how different types of respiratory symptoms relate to FENO and PC20M.

We conducted a prospective study on a series of 237 patients recruited from the University Hospital of Liege between 13th March 2009 and 30th December 2009. These patients were addressed by their respiratory physician for a methacholine challenge to detect asthma. Subjects referred to methacholine challenge were those in whom the bronchodilating test failed to demonstrate reversible airways obstruction or those in whom baseline spirometric values were normal giving a low probability for a bronchodilating test to be significant. The patients studied here had either baseline FEV_1≥80% predicted and FEV_1/FVC ratio ≥70% or bronchodilation <12% from baseline and 200ml after 400 µg inhaled salbutamol in case of baseline FEV_1 was <80% predicted or FEV_1/FVC ratio <70%. Patients already receiving inhaled corticosteroids were excluded from the study. The demographic and functional characteristics of the 174 corticosteroid-naïve patients are summarised in Table 12.

Table 12. Demographic, functional and inflammatory characteristics for 174 steroid-naïve patients.

No.	174
Gender (M/F)	72/102
Age, yrs	41 ± 16
Atopy (Y/N)	84/90
Current Smoking (Y/N)	59/115 (34%)
PC20 <16mg/ml (Y/N)	82/92
FEV_1, % predicted	97 ± 13
FVC, % predicted	100 ± 14
FEV_1/VC, %	83 ± 7
FENO50, ppb	17 (4 – 271)

Data are presented as mean ± SD (FEV_1, FVC, FEV_1/VC, age) or as median (range; FE_{NO50}). PC20M is expressed as geometric mean (range).

Symptoms were assessed using a standardised questionnaire that covered symptoms and smoking habits. Symptoms listed were diurnal and nocturnal cough, diurnal and nocturnal wheezing, dyspnoea, chest tightness, chest pain, exercise trigger, humidity trigger, fumes trigger, dust trigger, pollen trigger, emotional trigger, rhinitis, urticaria and pyrosis. Patients underwent FENO and a methacholine challenge as described in the method section. The ROC curve was constructed to determine the value of FENO that best identified a bronchial hyperresponsiveness in the whole population. Logistic regression analysis was used to assess the relationship between the binary outcome (PC20M ≤16 mg/ml) and a set of covariates, individually or in combination. Covariates included FENO (log transformed), age, gender, FEV_1, smoking and atopy.

4.4.2 Asthma diagnosis

Among the 174 patients referred for a methacholine challenge, 82 had a PC20M ≤16 mg/ml and were thus considered as being asthmatics. The demographic and functional characteristics of patients according to their level of bronchial responsiveness towards methacholine are given in Table 13.

Patients with positive methacholine challenge had lower baseline FEV_1 (95% predicted vs 102% predicted, $p < 0.001$) and lower FEV_1/FVC ($p < 0.05$) even if the average value clearly remained within the normal range. FENO was significantly higher in patients with positive methacholine challenge than in their negative counterparts (19 ppb vs. 15 ppb, $p<0.05$). When combining all variables into the logistic model, FENO ($p=0.0011$) and FEV_1 ($p<0.0001$) were independent predictors of bronchial hyperresponsiveness to methacholine whereas age ($p=0.12$), gender ($p=0.56$), smoking status ($p=0.56$) and atopy ($p=0.65$) were not significant (Table 14).

Table 13. Demographic, functional and inflammatory characteristics for patients with and without asthma.

	PC20M ≤ 16mg/ml	**PC20M > 16mg/ml**
N.	82	92
Gender (M/F)	33/49	39/53
Age, yrs	38 ± 18 *	44 ± 15
Atopy (Y/N)	43/39 (52%)	41/51 (45%)
Smoking (Y/N, %)	25/57 (30%)	34/58 (37%)
PC20, mg/ml	2.44 (0.02 – 16)	
DRS FEV_1 (%/µmol)	0.0033 (0.0006 – 0.4337) ***	0.0004 (0.0001 – 0.0007)
FEV_1, % predicted	95 ± 14 **	102 ± 12
FVC, % predicted	99 ± 14	102 ± 13
FEV_1/VC, %	82 ± 7 *	84 ± 6
FENO, (ppb)	19 (4 - 271)*	15 (4 - 120)

Data are presented as mean ± SD or as median (range). PC20M is expressed as geometric mean (range).

Table 14. Relationship between bronchial hyperresponsiveness to methacholine and a set of covariates including FENO, smoking status, age, FEV_1, atopy and gender.

Analysis of Maximum Likelihood Estimates		
Parameter	**Coefficient ± SE**	**P-value**
Intercept	3.82 ± 1.47	0.0091
LnFENO	0.82 ± 0.25	0.0011
Smoking	-0.22 ± 0.37	0.56
Age	-0.02 ± 0.01	0.12
FEV_1	-0.06 ± 0.01	<0.0001
LnFENO *Atopy	0.05 ± 0.11	0.65
Gender	-0.19 ± 0.33	0.56

Lower baseline FEV_1 values and higher FENO values were associated with PC20M≤16 mg/ml. We constructed a ROC curve to establish the ability of FENO to identify bronchial hyperresponsiveness assessed by methacholine challenge (Fig 15).

We found that FENO significantly predicted PC20 ≤16 mg⁄ml with a cut-off value of 34 ppb. However, this FENO cut-point offers much greater specificity (95%) and positive predictive value (PPV) (88%) than sensitivity (35%) and negative predictive value (NPV) (62%). In patients with a negative methacholine challenge the upper limit of the 95% CI of FENO was 35 ppb. When referring to FENO normal values as defined by Travers *et al.* (149), we found that 22 patients (13%) had FENO values greater than the 95% confidence interval. The ability of FENO to identify airway hyperresponsiveness was high in those patients. Indeed 20 of the 22 patients with FENO values out of range according to Travers had bronchial hyperresponsiveness whereas this was only the case in 62 of the 152 patients in whom FENO was within the normal range according to Travers *et al.* (Odds ratio 14.5, p<0.0001).

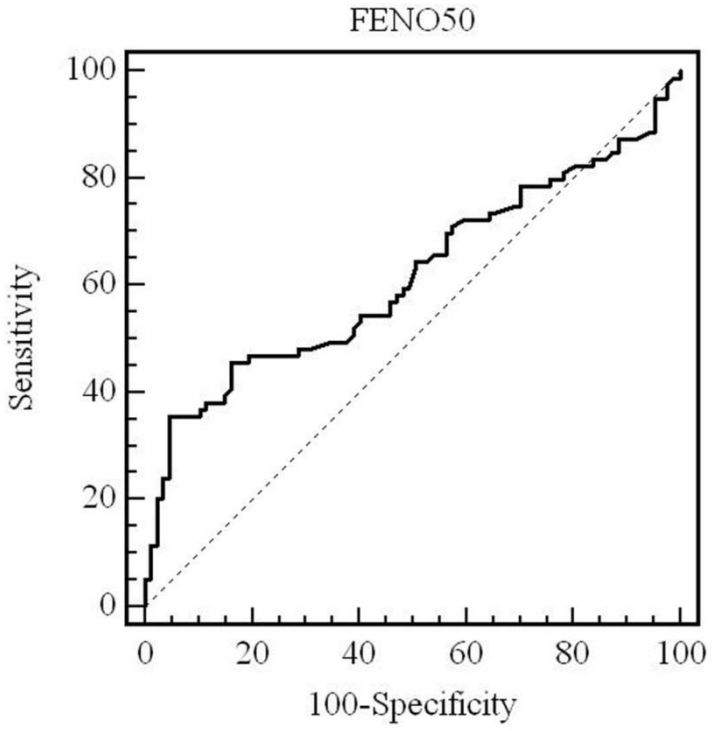

Fig 15. Receiver-operating characteristic curve (ROC) for the whole group determining FENO value which best identified the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in 1 s, PC20M ≤16

mg⁄ml. Cut-off point: 34ppb (Specificity: 95.4%, Sensitivity: 35.4%, positive predictive value: 88%, negative predictive value: 62%, p = 0.0033. AUC = 0.62).

We constructed a ROC curve to identify which FEV_1 cut-off was best related to the prediction of the presence of a bronchial hyperresponsiveness (Fig 16). We found that FEV_1 significantly predicted PC20M ≤16 mg⁄ml with a cut-off value of 101%. The sensitivity and specificity of this threshold was 71% and 57% respectively (p=0.0001, AUC=0.67).

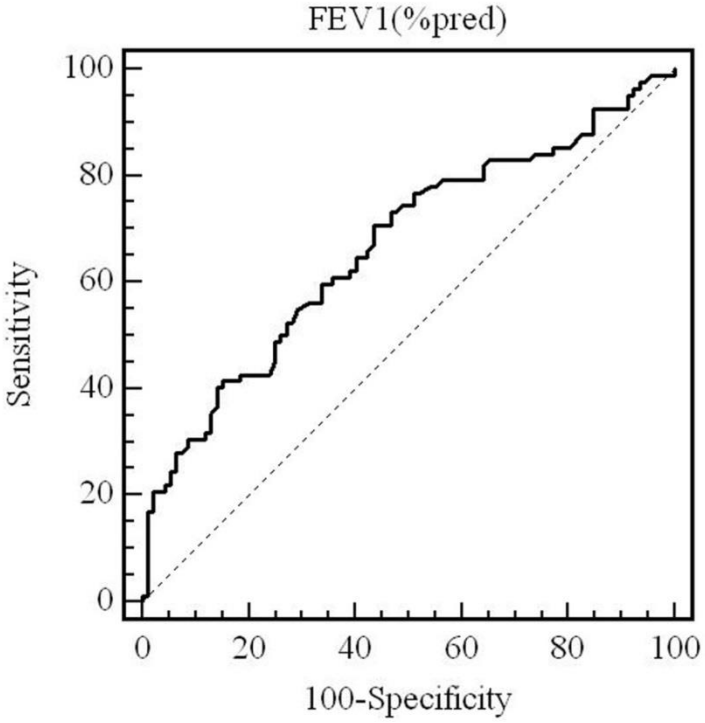

Fig 16. Receiver-operating characteristic curve (ROC) for the whole group determining % predicted FEV_1 value which best identified the provocative concentration of methacholine causing a 20% fall in FEV_1, PC20M≤16mg⁄ml. Cut-off point: 101% (Specificity: 57%, Sensitivity: 71%, positive predictive value: 59%, negative predictive value: 68%, p=0.0001. AUC=0.67).

When combining FENO and FEV_1 values to predict the presence of a bronchial hyperresponsiveness to methacholine, we found that the presence of both FENO>34ppb and FEV_1≤101% predicted gave a high specificity (98.9%) but a poor sensitivity (24.4%) for identifying patients with positive methacholine challenge (Table 15).

Table 15. Combination of FENO and FEV_1 to predict the presence of a bronchial hyperresponsiveness to methacholine challenge according to FENO and FEV_1 cut-off value defined by the ROC curve.

FENO	FEV_1	PCM20 ≤ 16 (n=82)		PCM20 > 16 (n=92)		Frequency ratio
		n	% (A)	N	% (B)	A/B
>34	≤101	20	24.4	1	1.1	22.4
>34	>101	11	13.4	2	2.2	6.2
≤34	≤101	39	47.6	40	43.5	1.1
≤34	>101	12	14.6	49	53.3	0.3

When FENO value >34ppb is associated with FEV_1 ≤ 101% predicted, 24.4% of patients have bronchial hyperresponsiveness to methacholine and are thus true positives while there are only 1.1% of false positives. When FENO value is ≤ 34ppb and FEV_1 >101%, 53.3% of the patients did not have bronchial hyperresponsiveness to methacholine and thus were true negatives while 14.6% were false negative. The combination of FENO and FEV_1 gave a high specificity (98.9%) but a poor sensitivity (24.4%) for identifying patients with a positive bronchial hyperresponsiveness to methacholine. The Table also shows that the presence of a FENO >34ppb is more frequently associated with FEV_1 ≤101% in patients with bronchial hyperresponsiveness than in patients without asthma (ratio=22.4). This ratio decreases if either FENO or FEV_1 cut-off is not reached. A FENO value ≤34ppb associated with FEV_1 >101% is however more frequently encountered in patients with negative methacholine challenge (ratio= 1/0.3 = 3.3).

4.4.3 Relationship between FENO and methacholine responsiveness

On the whole population the dose-response slope (DRS) for methacholine weakly correlated with FENO (r=0.18; p=0.03). Among those patients positive to methacholine there was, however, no relationship between the magnitude of bronchial hyperresponsiveness (PC20M) and the level of FENO (r= -0.06, p=0.6, Fig 17).

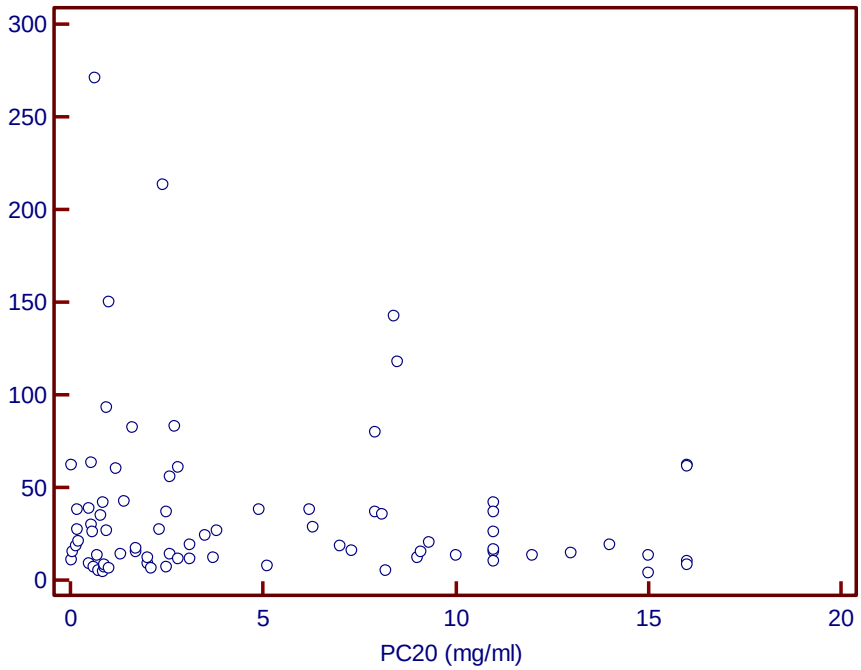

Fig 17. Spearman test for patients exhibiting a 20% fall in FEV_1 for a provocative concentration of methacholine ≤16 mg⁄ml. We did not find any correlation between FENO and provocative concentration of methacholine causing a 20% fall in FEV1 (PC20M) in those patients considered as asthmatics (r= -0.06, p=0.59).

Table 16 shows FENO according to the presence of respiratory symptoms in our population. Diurnal and nocturnal wheezing were associated with raised levels of FENO ($p<0.001$ and $p<0.05$, respectively).

Table 16. FENO according to the presence of respiratory symptoms in a population of patients referred for asthma diagnosis.

Symptoms	**FENO (ppb)**	
	Presence of symptom	**Absence of symptom**
Diurnal cough	16 (4 – 271) N= 122	19 (4 - 213) N= 52
Nocturnal cough	14 (4 – 213) N= 62	19 (4 – 271) N= 112
Diurnal wheezing	20 (7 – 271)** N= 82	14 (4 – 213) N= 92
Nocturnal wheezing	20 (5 – 213) * N= 65	15 (4 – 271) N= 119
Dyspnoea	17 (5 – 213) N= 111	15 (4 – 271) N= 63
Chest tightness	16 (5 – 271) N= 115	18 (4 – 142) N= 59
Chest pain	14 (4 – 80) N= 46	18 (4 – 271) N= 128
Exercise trigger	15 (5 – 213) N= 99	19 (4 – 271) N= 75
Humidity trigger	15 (4 – 213) N= 64	18 (4 – 271) N= 110
Fumes trigger	17 (4 – 150) N= 87	18 (4 – 271) N= 87
Dust trigger	19 (5 – 142) N= 78	16 (4 – 271) N= 96
Pollen trigger	19 (5 – 118) N= 50	16 (4 – 271) N= 124
Emotional trigger	15 (5 – 213) N= 77	18 (4 – 271) N= 97
Rhinitis	17 (4 – 142) N= 95	17 (4 – 271) N= 79
Urticaria	19 (5 – 213) N= 48	16 (4 – 271) N= 126
Pyrosis	15 (4 – 142) N= 91	19 (5 – 271) N= 83

*p<0.05, **p<0.001.

Table 17 shows the proportion of symptoms according to the results of methacholine challenge. Patients reporting dyspnoea, diurnal and nocturnal wheezing and chest tightness were more likely to have a positive methacholine challenge.

Table 17. Proportion of symptoms according to the results of the methacholine challenge in a population referred for asthma diagnosis.

Symptoms	PC20M < 16mg/ml	PC20M > 16mg/ml
Diurnal cough (Y/N, %)	54/28 (66%)	68/24 (74%)
Nocturnal cough (Y/N, %)	30/52 (37%)	32/60 (35%)
Diurnal wheezing (Y/N, %)	47/35 (57%)*	35/57 (38%)
Nocturnal wheezing (Y/N, %)	46/36 (56%)**	19/73 (21%)
Dyspnoea (Y/N, %)	60/22 (73%)**	41/51 (45%)
Chest tightness (Y/N, %)	60/22 (73%)**	37/55 (40%)
Chest pain (Y/N, %)	20/62 (24%)	26/66 (28%)
Exercise trigger (Y/N, %)	53/29 (65%)	46/46 (50%)
Humidity trigger (Y/N, %)	33/49 (40%)	31/61 (34%)
Fumes trigger (Y/N, %)	40/42 (49%)	47/45 (51%)
Dust trigger (Y/N, %)	42/40 (51%)	36/56 (39%)
Pollen trigger (Y/N, %)	28/54 (34%)	22/70 (24%)
Emotional trigger (Y/N, %)	37/45 (45%)	40/52 (43%)
Rhinitis (Y/N, %)	49/33 (60%)	46/46 (50%)
Urticaria (Y/N, %)	25/57 (30%)	23/69 (25%)
Pyrosis (Y/N, %)	39/43 (48%)	52/40 (57%)

*****p<0.05, ******p<0.0001.

4.4.4 Discussion

Our results shows that FENO>34 ppb has a high positive predictive value to identify bronchial hyperresponsiveness to methacholine in patients who had respiratory symptoms suggestive for asthma and in whom the respiratory physician had no argument for airway flow variability either because baseline calibre was considered to be normal or because bronchodilation to inhaled β2-agonist was weak (184). However, the sensitivity of 34 ppb cut-off is poor and FENO values below this threshold clearly do not rule out bronchial hyperresponsiveness. Furthermore, we

found that, among a list of respiratory symptoms, wheezing was the symptom that was the most convincingly associated with raised FENO. Airway hyperresponsiveness and airway inflammation are acknowledged to be key but largely independent features of asthma (2;76). Many asthmatics are routinely diagnosed based on the association between chronic respiratory symptoms and the demonstration of airway variability. Reversibility to inhaled β2-agonist and methacholine/histamine bronchial challenge are the most common ways used to confirm suspected asthma. In those patients with normal baseline lung function it was shown that a bronchodilation test and peak expiratory flow rate variability perform rather weakly to ascertain the diagnosis (70;71). FENO has been advocated as a useful tool to make asthma diagnosis in steroid-naïve patients with respiratory symptoms (70;73). The population selected in our study is slightly different from those described in previous studies in that only patients in whom asthma diagnosis remains uncertain after reversibility testing and／or baseline spirometry were sent to our routine function laboratory for a methacholine challenge. Furthermore, it is of interest to note that the proportion of atopic patients was rather low (50%) and the proportion of active smokers rather high (35%) for a population of

mild to moderate steroid-naive asthmatics. Dupont (73) and Smith (70) excluded smokers and the series of Smith *et al.* (70) included 76% of atopic subjects whereas their proportion was not mentioned in the study of Dupont *et al.* The relatively weak proportion of atopy and the presence of smokers certainly explain why the average FENO value in our series is clearly lower than that reported in patients attending an asthma clinic (150;166). Our results show that bronchial NO may predict methacholine hyperresponsiveness reflected by PC20M ≤16 mg／ml with FENO cut-off >34 ppb yielding 95% specificity and 88% positive predictive value. Our data show

that 20% with confirmed asthma had FENO value >34 ppb. In contrast to the specificity, sensitivity of 34 ppb threshold is poor and a value below this threshold clearly does not exclude the presence of bronchial hyperresponsiveness. It is important to realise that FENO and PC20M values are largely independent variables. Indeed the correlation between DRS (dose-response slope) for methacholine and FENO is weak for the whole population and we did not find any significant relationship between FENO and PC20M in those patients diagnosed as asthmatics. This contrasts with what we have recently found concerning the relationship between FENO and sputum eosinophils in a large heterogeneous series of asthmatics encountered in daily practice (166).

The relationship between FENO and airway hyperresponsiveness is controversial and conflicting results have been published (52;69;70;185-188). Compared with our patients, the studies showing a more convincing relationship between FENO and bronchial hyperresponsiveness included a significantly higher proportion of atopic patients. We found, however, by a multiple regression analysis that atopy, in contrast to FENO, was not an independent predictor of bronchial hyperresponsiveness. On the other hand, Smith *et al.* (70) used hypertonic saline, an indirect stimulus, to measure bronchial responsiveness. It is recognised that airway inflammation is better related to indirect than to direct bronchial hyperresponsiveness (189). Although FENO and PC20M reflect different dimensions in asthma, it does not exclude functional relationship between the two variables. It is admitted that part of the bronchial hyperresponsiveness in asthma is linked to an airway eosinophilic inflammation that can be attenuated by corticosteroids (190).

Furthermore, nitric oxide may itself contribute to bronchial hyperresponsiveness by increasing airway oedema as it is a potent vasodilator responsible for plasma

exudation from bronchial vessels (191) and the transformation of NO in peroxynitrite was shown to induce airway hyperresponsiveness in guinea pigs (192). This may explain the good specificity of FENO to detect methacholine responsiveness even if it is not perfect, as increased FENO may be observed in other pathological conditions such as eosinophilic bronchitis (74) where bronchial hyperresponsiveness is absent. It is also interesting to note that FENO values outside the normal range as defined by Travers *et al.* (149), whilst being rather rare in our series (13%), carry a high odds ratio (14.5) in favor of bronchial hyperresponsiveness. This observation highlights the fact that consistent airway inflammation may be a determinant factor of bronchial hyperresponsiveness. The multiple logistic regression analysis confirmed the effect of baseline airway caliber as a strong independent predictor of the presence of bronchial hyperresponsiveness to methacholine. Some studies have shown a correlation between FEV_1 and bronchial hyperresponsiveness (187;193;194). This suggests that airway geometric factors are involved in the mechanisms of bronchial hyperresponsiveness in asthma. Beyond geometry there is also solid argument to support the role of bronchial smooth muscle dysfunction in determining hyperresponsiveness to a direct constricting agent (195). Although atopy was shown to correlate with bronchial hyperresponsiveness in epidemiological and clinical studies (196), our data suggest that its influence may be mediated by an increase in airway inflammation as atopic patients clearly exhibited higher FENO than non-atopic (19ppb vs. 15ppb, $p < 0.01$). We therefore believe that it is not atopy per se that matters in determining bronchial hyperresponsiveness but rather the fact that atopy may favour airway inflammation in the event sensitised patients are exposed to a relevant allergen. It is important to emphasise that smoking status did not impact on bronchial hyperresponsiveness to methacholine in our study. Smoking has been

shown to induce airway hyperresponsiveness in the general population (197). Our data show that smoking may be less critical when considering selected patients based on the presence of chronic respiratory symptoms. There are only limited data on the precise relationship between the type of symptoms and airway inflammation. In our study diurnal and nocturnal wheezing was associated with proximal airway inflammation as reflected by raised levels of FENO. Leuppi *et al.* reported the same observation in a population of children (198). Although it is admitted that asthma may sometimes be revealed by isolated coughing (199), our data show that coughing is generally poorly related to methacholine hyperresponsiveness and to FENO. As compared with FENO, bronchial hyperresponsiveness to methacholine is associated with a broader spectrum of symptoms including not only wheezing but also dyspnoea and chest tightness that are likely to better reflect airflow limitation than wheezing alone.

The purpose of our study was not to look at asthma phenotypes but rather to validate an inflammometer as a diagnostic tool for the currently accepted definition of asthma according to GINA. A key issue is to know whether or not those patients with chronic respiratory symptoms and high FENO are better responsive to inhaled corticoids irrespective of their level of bronchial hyperresponsiveness and their ‘asthma’ label. This has been suggested by a pilot monocentric study (200) but has yet to be confirmed in a study conducted on a larger scale.

4.4.5 Conclusion

We conclude that FENO measurement may be useful to the clinician in diagnosing asthma in patients with chronic respiratory symptoms in whom the bronchodilating test failed to demonstrate reversibility or was not indicated. However, the poor

sensitivity of FENO to detect bronchial hyperresponsiveness should prompt the clinician to ask for a methacholine challenge when asthma is suspected based on clinical history in case of FENO<34 ppb (Fig 18). According to our data, application of the algorithm proposed in Fig 18 could reduce methacholine challenges performed in a routine pulmonary function laboratory by 20%. This is significant as methacholine challenge is time consuming and uncomfortable to the patients.

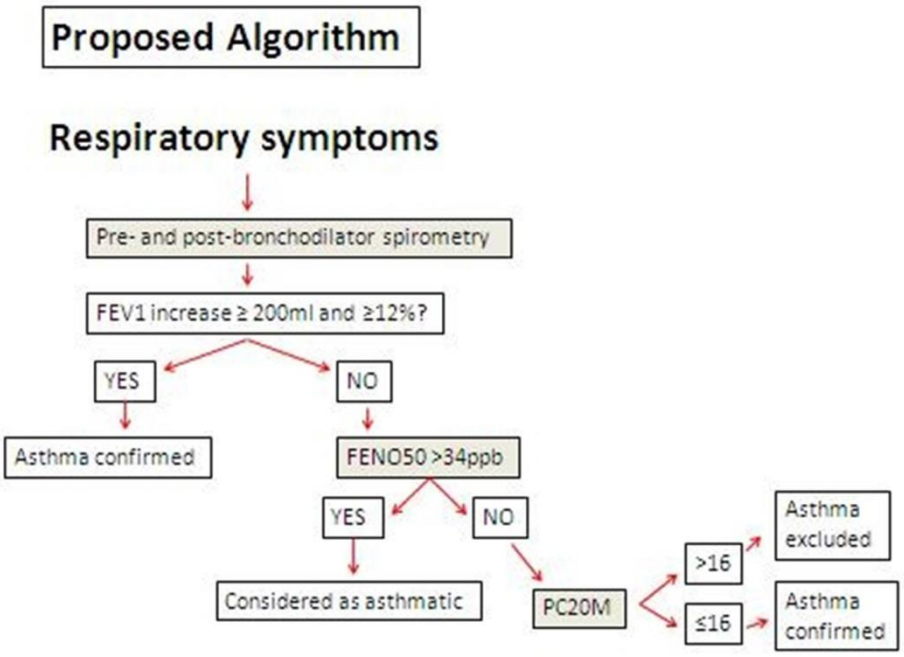

Fig 18. Proposed algorithm for asthma diagnosis. Values <34 ppb should prompt the clinician to ask for a methacholine challenge when asthma is suspected based on clinical history. The application of the proposed algorithm could reduce by 20% the methacholine challenges performed in a routine lung function laboratory.

4.5 Value of measuring blood and sputum eosinophil cell counts

It is now accepted that eosinophilic asthma, defined as a sputum eosinophil count of 2–3% or higher, represents slightly less than half of the asthmatic population (13;43;124;128). In asthma, sputum eosinophil count correlates with blood eosinophil count (128;163-165) and blood eosinophil count is considered as a good surrogate marker for sputum eosinophil count (over 2–3% with a cut-off of 220 cells per mm^3 or 3%) (128;140). However, the sensitivity and specificity of blood eosinophils to predict a sputum eosinophil proportion of ≥3% are <80%, and some patients show discordance between local and systemic inflammation.

In this study, we sought to determine whether looking at blood and sputum eosinophilic inflammation might help in our understanding of asthma severity. We first retrospectively investigated the demographic, functional and symptomatic features of 508 asthmatics recruited from the University Asthma Clinic of Liege between the 1st October 2005 and 27th June 2011 and classified them according to systemic and local eosinophilic inflammation, then prospectively validated the relationship between asthma control and eosinophilic inflammation in a new cohort of 250 patients well-matched to our retrospective cohort with respect to demographic, functional and treatment characteristics.

The patients came from the routine practice to University Hospital, Liege, and were recruited by two clinicians involved in asthma. Inclusion criteria were any patients with asthma aged ≥18 years who agreed to undergo detailed investigation at the asthma clinic. The visits were not part of an asthma trial. All the patients who had a successful sputum induction were included in the study. Their demographic and functional characteristics are summarised in Tables 18 and 19.

Table 18. Demographic, control and treatment characteristics for the whole population.

Characteristics	Retrospective population	Prospective population
N.	508	250
Gender (M/F)	201/307	99/151
Age, yrs	52 (19-88)	50 (16-85)
Age of onset		
<12	24%	22%
12-40	37%	36%
≥40	39%	42%
Height, cm	167 ± 9	168 ± 9
Weight, kg	74 ± 16	73 ± 17
Atopy (Y/N, %)	296/212 (58%)	148/102 (59%)
Current Smoker (n)	101 (20%)	55 (22%)
(pack-yr)	22 (0.5-60)	25 (2-60)
Ex-smokers (n)	99 (19%)	38 (15%)
(pack-yr)	15 (0.5-90)	17 (0.5-63)
Bronchiectasis (%)	19% (n=174)	17%
Gastro-oesophageal reflux (%)	79%	71%*
Nasal polyposis	22% (n=273)	27%
Sinusitis	42% (n=273)	41%
Rhinitis	58%	56%
Exacerbations (/patient/yr)	0.68 ± 1.50 (n=428)	0.86 ± 2.02 (n=237)
LABA, Y (%)	319 (63%)	141 (56%)
LTRA, Y (%)	93 (18%)	50 (20%)
Theophylline, Y (%)	16 (3%)	11 (4%)
ICS therapy, Y (%)		
Steroid naïve	153 (30.5%)	82 (33%)
Low dose ICS	73 (14%)	35 (14%)
Moderate dose ICS	138 (27.5%)	63 (25%)
High dose ICS	144 (28%)	70 (28%)
Oral corticosteroids, Y (%)	32 (6%)	25 (10%)

Data are presented as mean ± SD or median (range). Y: yes. Exacerbations were evaluated during the year prior to the visit. *p<0.05.

Exacerbation in the previous year was defined by a course of oral corticoids for ≥3 days for a case of worsening asthma. Nasal polyps and sinusitis was diagnosed by an ear, nose and throat physician, either by endoscopy or sinus computed tomography. Gastro-oesophageal reflux was diagnosed either by symptoms of pyrosis at history taking or by the presence of oesophagitis demonstrated by gastroscopy.

Table 19. Functional and inflammatory characteristics for the whole population

Characteristics	Retrospective population	Prospective population
FEV_1, % predicted	84 ± 19	82 ± 21
FEV1/FVC, %	73 ± 11	71 ± 15*
PC20M, mg/ml	3.20 (0.025-16)	2.92 (0.05-16)
Reversibility, %	11 ± 14	9 ± 10
ACQ	2.01 ± 1.38	2.00 ± 1.25
AQLQ	4.61 ± 1.35	4.46 ± 1.39
Blood eosinophil count, /mm³	230 (0-3220)	188 (0-1133)*
FENO50	27 (0-247)	25 (4-348)
Sputum eosinophils, %	2 (0-94)	2.8 (0-90)
Sputum neutrophils, %	45 (0-100)	49 (0-100)

Data are presented as mean ± SD or median (range). PC20M is expressed as geometric mean (range). *p<0.05.

To validate the results of the retrospective analysis of the link between asthma control and eosinophilic inflammation we conducted a prospective study. Power calculations indicated a required total sample size of 250 subjects to confirm a change in ACQ score ≥0.5 between non-eosinophilic asthmatics and patients exhibiting diffuse eosinophilic inflammation, with a power of 80%. A new cohort of 250 consecutive patients was thus recruited from routine practice between 30th June 2011 and 12th January 2013. None of these patients had been included in the retrospective cohort. Their demographic, functional and treatment characteristics were similar to the retrospective population (Tables 18 and 19). Exacerbation rate for the prospective population was measured through a telephone call over a period of 12 months following the visit to the asthma clinic, during which treatment was initiated or adjusted according to asthma control, lung function and inflammatory markers at the discretion of the clinician. 13 patients were lost to follow-up, and in the 17 patients in whom the observation period was <1 year, we calculated the annualised exacerbation rate. We used blood eosinophil count and sputum eosinophil percentage to subdivide our asthmatic population into four groups. The chosen blood

(≥400 cells per mm3) (10) and sputum (≥3%) threshold values were considered as the limit of abnormality by our routine laboratory. The demographic, functional and inflammatory characteristics of the retrospective cohort of asthmatics, classified according to their blood and sputum eosinophil counts are described in Table 20-22.

Table 20. Retrospective cohort: demographic and treatment characteristics of asthmatics (n=508) according to blood and sputum eosinophil count.

	Blood eos <400/µl Sputum eos <3%	**Blood eos<400/µl Sputum eos ≥3%**	**Blood eos≥400/µl Sputum eos <3%**	**Blood eos ≥400/µl Sputum eos ≥3%**
N	249 (49%)	128 (25%)	34 (7%)	97 (19%)
Gender (M/F)	78/171	54/74*	16/18	53/44***
Age, yrs	52 (21-86)	53 (21-88)	51 (21-85)	51 (19-86)
Age of onset				
<12	22.6%	27.6%	26.5%	23.3%
12-40	34.5%	36.2%	32.3%	45.6%
≥ 40	42.9%	36.2%	41.2%	31.1%
Height, cm	166±9	168±9	169±9	169±9
Weight, kg	73±16	74±15	76±17	75±17
BMI (kg/m²)	26.3±5	26.4±5	26.3±4.8	26.4±5.3
Atopy (Y/N, %)	126/123 (51%)	82/46 (64%)*	22/12 (65%)	66/31 (68%)**
Current smoker	54 (22%)	29 (23%)	6 (18%)	12 (12%)*
Bronchiectasis (%) (n=174)	19%	13%	17%	26%
Gastro-oesophageal reflux (%)	77%	81%	86%	77%
Nasal Polyposis	9%	25%***	37%***	43%***
Sinusitis (n=273)	34%	38%	42%	61%***
Rhinitis	53%	59%	72%	65%
Exacerbations (/patient/yr)	0.42 ± 0.9	0.93 ± 2.72*	0.59 ± 0.98	1.5 ± 2.5***
ICS therapy				
Steroid naïve	82 (33%)	31 (24%)	9 (26.5%)	31 (32%)
Low dose	30 (12%)	20 (16%)	5 (15%)	18 (18%)
Moderate	70 (28%)	37 (29%)	11 (32%)	20 (21%)
High dose	67 (27%)	40 (31%)*	9 (26.5%)	28 (29%)

*p<0.05, **p<0.01, ***p<0.001. Data are presented as mean ± SD or median (range). Column of blood eosinophil count <400/µl and sputum eosinophil count <3% is used as the comparator.

Table 21. Retrospective cohort. Functional characteristics, asthma control and quality of life of asthmatics (n=508) according to blood and sputum eosinophil count.

	Blood eos <400/µl Sputum eos <3%	**Blood eos<400/µl Sputum eos ≥3%**	**Blood eos≥400/µl Sputum eos <3%**	**Blood eos ≥400/µl Sputum eos ≥3%**
FEV_1, % predicted	87±19	83±20*	84±23	75±19***
FEV_1/FVC, %	75±10	72±9*	77±10	71±10***
TLC (% predicted)	101±16	101±20	92±21	103±15
FRC (% pred)	109±29	108±20	97±30	101±18
KCO (% pred)	89±19	92±20	99±14**	94±26*
PC20, mg/ml	3.99 (0.05-16)	2.32 (0.025-16)*	4.53 (0.05-16)	1.49 (0.05-16)**
Reversibility, %	8 ± 9	13 ± 14*	9 ± 12	17 ± 16***
ACQ	1.88±1.39	1.87±1.19	1.98±1.5	2.54±1.45***
<0.75 (n)	58 (23%)	26 (20.5%)	7 (21%)	10 (10.5%)
0.75-1.5 (n)	45 (18%)	30 (23.5%)	10 (29%)	15 (15.5%)
>1.5 (n)	146 (59%)	72 (56%)	17 (50%)	72 (74%)*
AQLQ	4.67±1.36	4.84±1.3	4.47±1.26	4.3±1.4*

*p<0.05, **p<0.01, ***p<0.001. Data are presented as mean ± SD or median (range); PC20 is expressed as geometric mean (range). Column of blood eosinophil count <400/µl and sputum eosinophil count <3% is used as the comparator.

Table 22. Retrospective cohort. Inflammatory characteristics of asthmatics (n=508) according to blood and sputum eosinophil count.

	Blood eos <400/µl Sputum eos <3%	**Blood eos<400/µl Sputum eos ≥3%**	**Blood eos≥400/µl Sputum eos <3%**	**Blood eos ≥400/µl Sputum eos ≥3%**
IgE, kU/l	87 (1-7338)	211 (3-6785)***	180 (13-2329)*	225 (1-17183)***
Blood eosinophils, %	1.7 (0-5.4)	3.2 (0-7)***	6 (0.3 – 15)***	8 (0.4 – 30)***
Blood eosinophils, /mm³	140 (0-380)	250 (0-390)***	490 (400-1220)***	590 (400-3220)***
Blood neutrophils, %	59 (27-82)	57 (34-91)	57 (41-76)	52 (32-67)***
Blood neutrophils, /mm³	4180 (76-11080)	4370 (2290-15410)	5040 (1760-10010)	3965 (1820-8670)
Sputum eosinophils, %	0.3 (0-2.9)	9 (3-79)***	0.6 (0-2.8)	26 (3.2-94)***
Sputum eosinophils, /mm³	2.7 (0 – 1020)	70 (6-5226)***	4.8 (0-1796)	287 (5-33375)***
Sputum neutrophils, %	58 (0-100)	39 (0-90)***	57 (0.2-99)	30 (0.2 -91)***
Sputum neutrophils, /mm³	422 (0 – 73440)	334 (1-9588)	560 (14 -160974)	259 (1-15441)
Fibrinogen (g/l)	3.2 (1.9-10)	3 (2-6)	3.3 (2.6-5)	3.4 (2.2-7)
CRP (mg/l)	1.7 (0.2-10)	2 (0.2-14)	1.4 (0.5-4)	1.6 (0.2-13)
FENO, ppb	17 (0-192)	37 (2-222)***	32 (5-93)**	77 (11-247)***

*p<0.05, **p<0.01, ***p<0.001. Data are presented as mean ± SD or median (range); Column of blood eosinophil count <400/µl and sputum eosinophil count <3% is used as the comparator.

The patients without evidence of eosinophilic inflammation represented the largest group, accounting for 49% of the cohort. Those patients with selective airway eosinophilic inflammation came second, accounting for 25% of the patients, whereas those exhibiting the reverse pattern were much less numerous, only representing 7%. Patients combining systemic and airway eosinophilic inflammation account for 19% of the patients. The inhaled corticoids treatment regimen was similar between subgroups (Table 20). Compared with non-eosinophilic asthmatics, the characteristics of patients exhibiting isolated sputum eosinophilia were a higher proportion of males and atopic subjects, higher total serum IgE levels, lower FEV_1 and FEV_1/forced vital capacity ratio, and higher bronchial hyperresponsiveness and reversibility to β2-agonists (Fig 19, and Tables 21 and 22). As for patients with high levels of blood eosinophils without sputum eosinophilia, they had a higher total serum IgE compared with noneosinophilic asthmatics (Fig 19 and Table 22). The presence of both local and systemic eosinophilic inflammation was strikingly more frequent in males, and associated with the greatest lung function impairment, and the lowest asthma control (mean±SEM ACQ score 2.54±1.45 versus 1.88±1.39; mean difference 0.66, 95% CI -0.99– -0.32 (p=0.0001)) (Fig 19 and 20) and quality of life (Table 21).

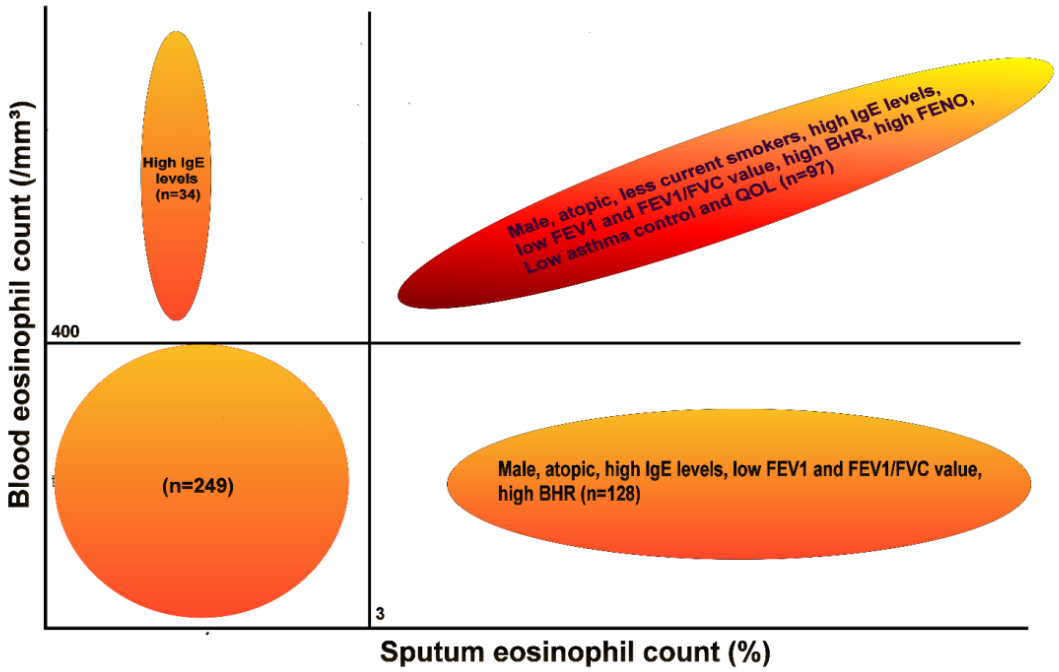

Fig 19. Demographic, functional and inflammatory characteristics of patients according to their level of local and systemic eosinophil count.

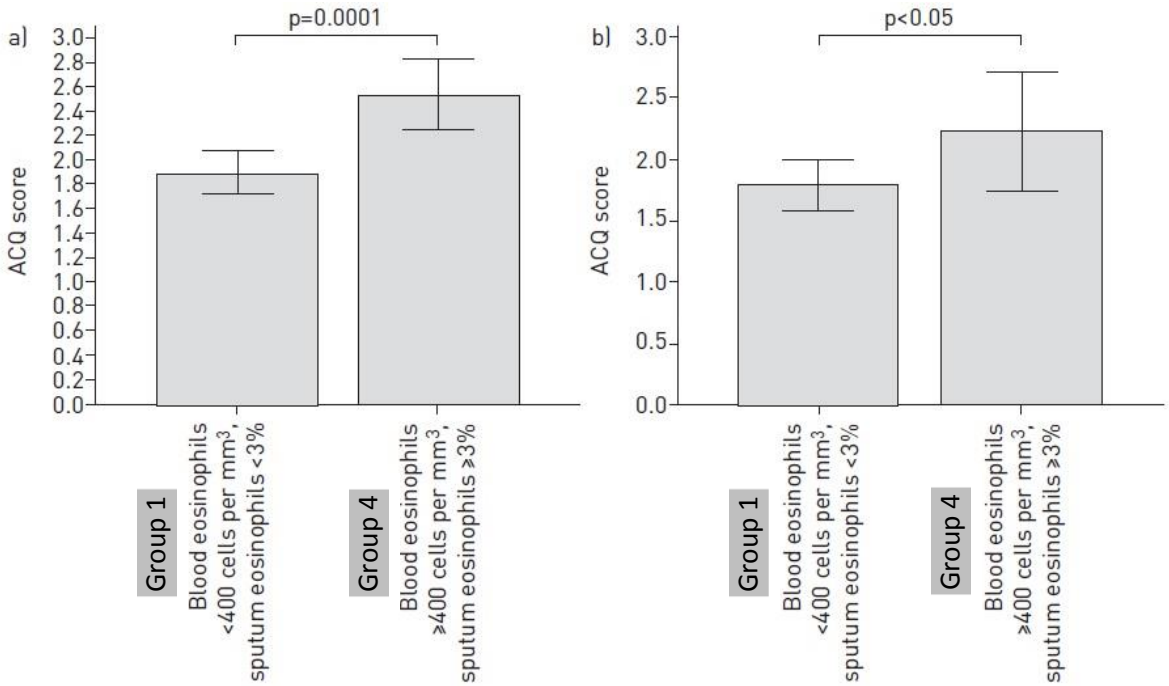

Fig 20. Asthma control questionnaire (ACQ) according to the level of blood and sputum eosinophil count**.** The presence of both local and systemic eosinophilic inflammation was associated with lower asthma control. Group 1: blood eosinophil count <400/µl and sputum eosinophil count <3%. Group 4: blood eosinophil count ≥400/µl and sputum eosinophil count ≥3%. Panel a: retrospective population (n=508). Panel b: prospective population (n=250).

They also had the highest FENO while those with eosinophilia in only one compartment had similar, intermediate FENO. Non-eosinophilic asthmatics had the lowest FENO, comparable to that found in healthy subjects (Fig 21 and Table 22).

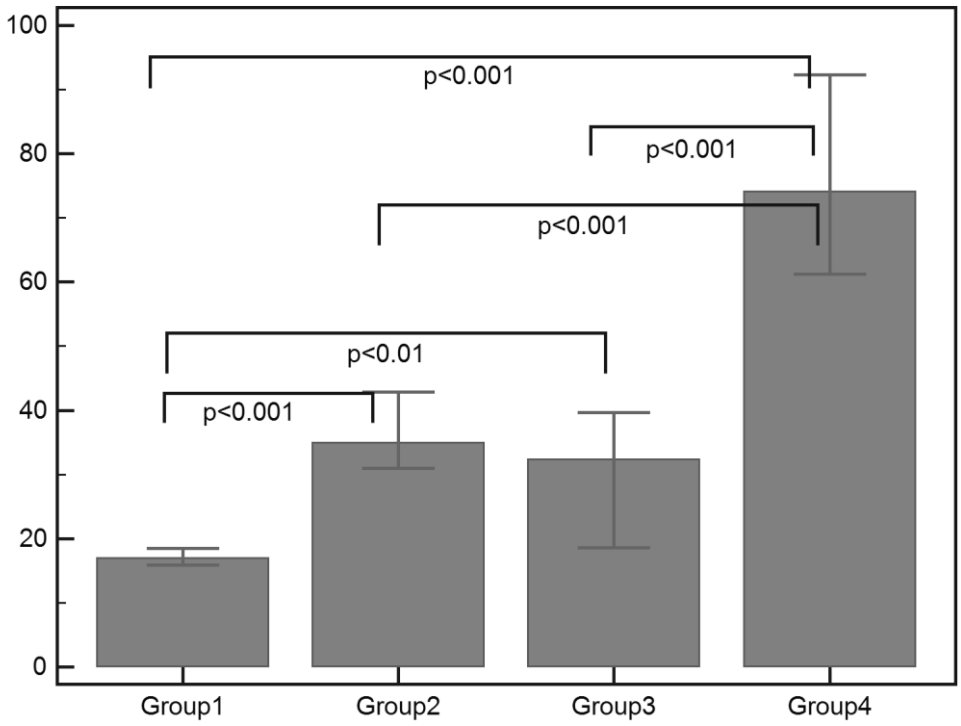

Fig 21. FENO levels in patients classified according to the presence and/or absence of blood and sputum eosinophilia**.** Patients exhibiting both local and systemic inflammation had the highest level of FENO in their exhaled air (n=508). Presence of uncontrolled eosinophilic inflammation either at local or systemic level was associated with intermediate levels of FENO. The group of asthmatics showing no increase in eosinophilic inflammation had the lowest level of FENO. Group 1: blood eosinophil count <400/µl and sputum eosinophil count <3%; Group 2: blood eosinophil count <400/µl and sputum eosinophil count ≥3%; Group 3: blood eosinophil count ≥400/µl and sputum eosinophil count <3%; Group 4: blood eosinophil count ≥400/µl and sputum eosinophil count ≥3%.

Patients with increased sputum eosinophilia reported greater numbers of severe exacerbations in the previous year, and this was particularly the case for those displaying concordant systemic and airway eosinophilia (Table 20). Likewise, the proportion of sinusitis and nasal polyps was clearly higher in eosinophilic asthmatics however there was no difference regarding gastro-oesophageal reflux (Table 20).

In patients treated with high doses of inhaled corticosteroids (n=144), the proportions of the different subgroups were similar to those found in the whole cohort, and the subgroup exhibiting both bronchial and systemic eosinophilic inflammation had the highest exacerbation rate and the poorest lung function (Table 23).

Table 23. Demographic, functional and inflammatory characteristics of patients receiving high doses corticosteroids in the retrospective cohort (n=144).

	Blood eos <400/µl Sputum eos <3%	**Blood eos<400/µl Sputum eos ≥3%**	**Blood eos≥400/µl Sputum eos <3%**	**Blood eos ≥400/µl Sputum eos ≥3%**
N	67 (47%)	40 (28%)	9 (6%)	28 (19%)
Gender (M/F)	20/47	16/24	5/4	15/13
Age, yrs	52 (21 – 86)	59 (26 – 88)	62 (44 – 85)	52 (29 – 86)
Age of onset,%				
<12	25	35	0	30
12-40	33	30	50	40
≥ 40	42	35	50	30
Exacerbations (/patient/yr)	0.76 ± 1.06	0.95 ± 1.29	1.25 ± 1.17	3.52 ± 5.00***
$FEV_{1,}$ % pred	77 ± 17	77 ± 20	71 ± 28	65 ± 18**
FEV_1/FVC, %	73 ± 11	68 ± 13	70 ± 11	64 ± 9**
ACQ	2.57 ± 1.96	1.97 ± 1.25	3.19 ± 1.79	3.18 ± 1.38
AQLQ	4.11 ± 1.39	4.52 ± 1.40	3.59 ± 1.47	3.50 ± 1.31
FENO50	14 (4-114)	30 (8 – 119)***	10 (5 – 58)	55 (11 – 139)***
Blood eosinophil count, /mm³	160 (10 – 350)	225 (0 -390)***	465 (400 – 1030)***	615 (400 – 3220)***
Sputum eosinophil count, %	0.25 (0 – 2.6)	8.4 (3 – 52.2)***	0.4 (0 – 2.7)	22.3 (3.6 – 94.4)***

*p<0.05, **p<0.01,***p<0.001. Column of blood eosinophil count <400/µl and sputum eosinophil count <3% is used as the comparator.

In order to validate the relationship between poor asthma control and comprehensive eosinophilic inflammation, we prospectively recruited a population of 250 asthmatics whose demographic, functional and inflammatory characteristics were similar to our retrospective cohort (Tables 20 and 21). In this population, we found similar proportions of patients in the different subgroups (47% were patients without evidence of eosinophilic inflammation, 32% exhibited isolated sputum eosinophilia, 4% had isolated systemic eosinophilia and 17% combined systemic and airway eosinophilia) and confirmed that patients with both systemic and airway eosinophilic inflammation had poorer asthma control compared with non-eosinophilic asthma (mean±SEM ACQ score 2.23±1.40 versus 1.79±1.12; mean difference 0.44, 95%CI 0.007–0.89 ($p<0.05$)) (Fig 16). In the prospective cohort, patients exhibiting diffuse eosinophilic inflammation had a higher number of exacerbations in the year prior to the visit to the asthma clinic (mean±SD 1.11±1.65, n=41) than non-eosinophilic patients (mean±SD 0.77±2.55, n=111) ($p<0.05$). Interestingly, for the whole cohort, the exacerbation rate over the 12months following the asthma clinic visit decreased compared with that occurring in the previous year (mean 0.86±2.02 (95% CI 0.61–1.12) versus mean 0.50±1.18 (95% CI 0.35–0.65) after) ($p=0.015$). Moreover, in the year following the visit to the asthma clinic, the number of exacerbations was significantly higher in patients advised to take high-dose ICS (mean±SD 1.06±1.63, n=93) than in those advised to take low (mean±SD 0.17±0.62, n=58) or moderate (mean±SD 0.15±0.40, n=55) doses of ICS or not to take ICS at all (0±0, n=31) ($p<0.0001$).

When pooling both the retrospective and the prospective cohort (n=758), there was a weak but significant relationship between ACQ and sputum eosinophil count ($r=0.16$, $p<0.0001$) but not between blood eosinophils and ACQ ($r=0.02$, $p=0.57$) (Fig 22).

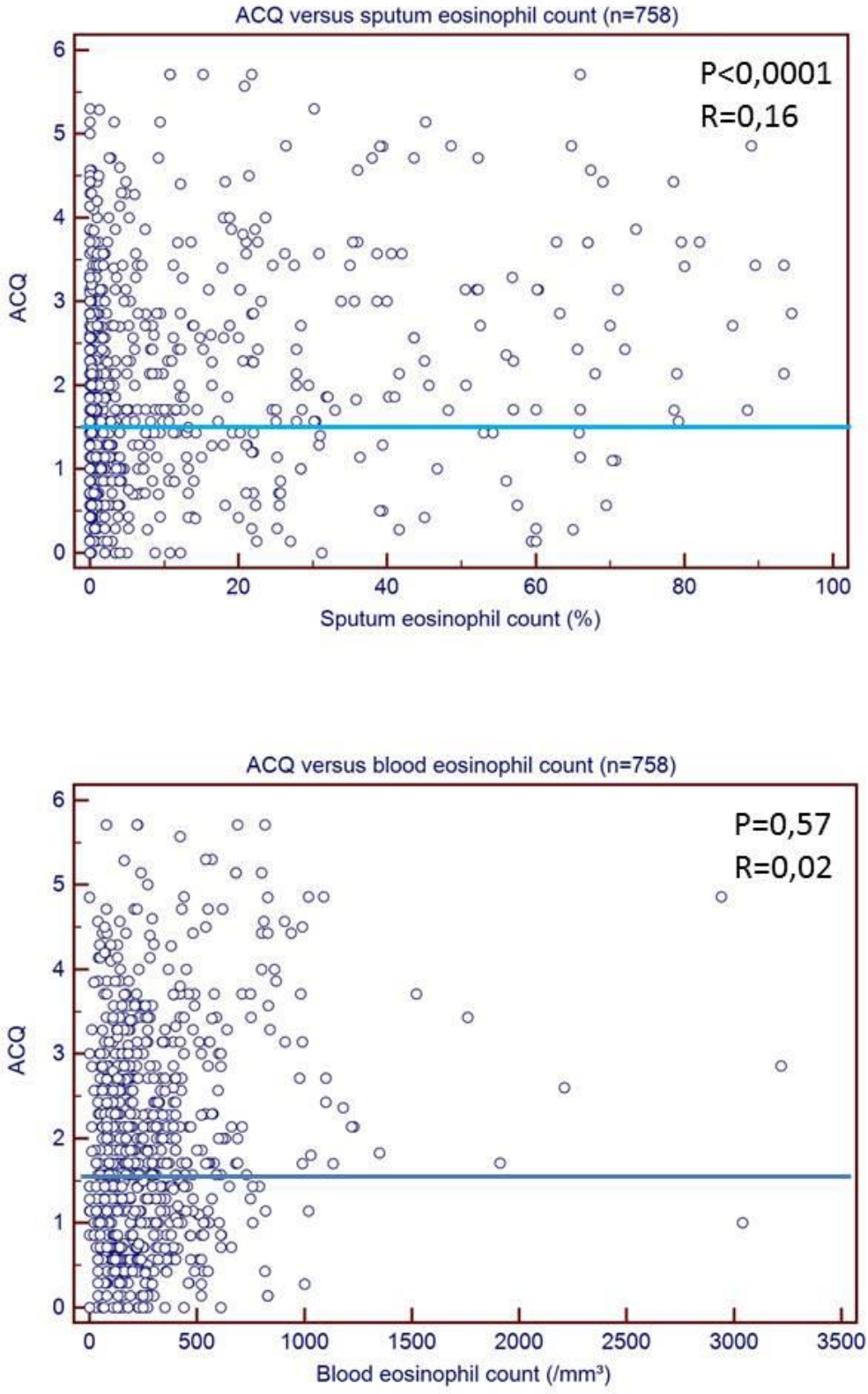

Fig 22. Correlation between ACQ and sputum eosinophil count (upper figure) and blood eosinophil count (lower figure) when combining the prospective and retrospective cohort (n=758). Asthma is considered uncontrolled if ACQ >1.5 (blue line).

The original finding of this study is that patients exhibiting both local and systemic eosinophilic inflammation had more severe asthma reflected by lower baseline lung function, higher bronchial responsiveness to methacholine, poorer asthma control and quality of life, and a greater number of exacerbations in the previous year. This suggests that the global magnitude of eosinophilic inflammation is a significant factor in disease severity. Another new finding of this study is that it provides figures on the proportion of asthmatics classified according to the site of eosinophilic inflammation. Overall, asthmatics without any sign of eosinophilic inflammation account for almost half of the patients while one-quarter to one-third had selective airway eosinophilia. Patients with systemic and airway eosinophilic inflammation represent one-fifth of the patients, while those with isolated systemic eosinophilic inflammation are rather rare. These proportions are found irrespective of asthma treatment received including high doses of inhaled corticoids. We believe our new classification based on eosinophilic inflammation is pertinent to the clinician and is in line with the need to phenotype severe asthmatics as advocated by the recent ERS/ATS guidelines (93).

As mentioned, the link between eosinophils and asthma severity has been extensively debated. The first attempts to investigate this relationship were based on sampling BAL or biopsies during bronchoscopy. The invasive nature of the procedure has obviously limited the number of subjects studied, which may have led to contrasting results because of interindividual patient variability. Being less invasive, the technique of induced sputum has considerably widened the series of patients investigated and has been key to the emergence of recognition of several inflammatory asthma phenotypes. Previous studies found higher levels of sputum eosinophil counts in uncontrolled asthmatics (50;142). Another study showed that patients with high blood eosinophilia (>250 cells per mm^3) had lower FEV_1 values and

worse asthma control than those with normal blood eosinophil counts (201). Recently, Volbeda *et al.* (85) have shown that patients with uncontrolled asthma exhibit higher eosinophil numbers in peripheral blood and a trend for a higher eosinophil count in induced sputum, but they did not provide detailed analysis on the discordance between blood and airway eosinophilia. Here, in our retrospective cohort, we found that patients exhibiting eosinophilic inflammation both in blood and sputum had more severe asthma and poorer asthma control than noneosinophilic asthmatics, a finding that was validated in our prospective cohort. Our data show that isolated sputum eosinophilic inflammation is associated with impaired airway calibre and increased airway hyperresponsiveness compared with non-eosinophilic asthmatics, while those combining both systemic and eosinophilic had further functional impairment, which is likely to partly contribute to worse asthma control and quality of life. Three-quarters of this group had an ACQ score >1.5 while only 10% had well-controlled asthma with an ACQ score <0.75. However, having an eosinophilic trait, whichever the compartment, is associated with a slightly raised proportion of atopy and higher serum IgE levels.

It is important to note that patients in the group with blood and sputum eosinophilia had much higher sputum eosinophil counts compared with those with sputum eosinophilia alone. Beyond the categorical analysis, the extent of eosinophilic airway infiltration has to be taken into consideration. Our finding reinforces the role of airway eosinophilia in the loss of asthma control previously suggested on a smaller sample of patients (40). The intensity of blood eosinophilic inflammation in patients exhibiting diffuse eosinophilic inflammation was comparable to that of asthmatics with isolated blood eosinophilia, a group that was less severe. Moreover, there was no correlation between ACQ and blood eosinophil counts. This suggests that circulating eosinophils

by themselves are not direct actors of asthma severity but are important in providing a pool of cells that can be attracted in the airways.

Approximately 70% of patients exhibiting diffuse eosinophilic inflammation were receiving maintenance treatment with inhaled corticoids. While we could speculate that raising the dose of ICS in those receiving low to moderate doses would have resulted in a reduction of eosinophilic inflammation, it is noteworthy that 30% of the ICS treated patients were already receiving high doses. This highlights the relative resistance to corticoids in some patients and the need for complementary treatment to target eosinophils. Among the patients receiving high doses of inhaled corticoids, 53% exhibited either blood or sputum eosinophilia that could make them potential candidates for treatment with anti-interleukin-5 (10;11). A recent study has shown that omalizumab is more efficient in reducing exacerbation when patients express a Th2 profile including high blood eosinophil counts or FENO levels (98). Of course, as our study was a field study, we cannot rule out poor compliance in some patients, making the interpretation of corticoid resistance in these asthmatics difficult.

Here, we deliberately chose to classify our patients according to pre-specified criteria, which were blood and sputum eosinophils. Our approach was therefore different from that used in hierarchical unsupervised cluster analysis. We do not deny the great interest of this type of cluster analysis in the emergence of clinical asthma phenotypes, but we believe that classifying asthmatics according to their eosinophilic profile is useful because sputum and blood eosinophils are good biomarkers to target treatment and predict response to corticoids or biologicals directed towards Th2 cytokines (202;203). Furthermore, it has recently been advocated that sputum eosinophils may be a valuable outcome in asthma drug trials (202). How our asthma

groups may fit with those found by others after cluster analysis remains to be determined (6;204;205). There are some similarities coming out of both approaches regarding the link between eosinophilia and gender. While females are the dominant sex among adult asthmatics, our data show that this is mainly accounted for by the non-eosinophilic group even if females remained the predominant sex in those with isolated airway eosinophilic asthma. It is noteworthy that the proportion of males was significantly higher in those patients with an eosinophilic trait compared with their non-eosinophilic counterparts and, in our study, it is striking that males become the predominant sex in the case of diffuse eosinophilic inflammation. This is likely to reflect the different hormonal status and its effect on eosinophil biology. Few studies have evaluated the relationship between eosinophils and sex hormones. It seems that β-oestradiol significantly enhances eosinophil adhesion to human mucosal microvascular endothelial cells and their degranulation (206;207).

By contrast, our data do not point to any particular link between obesity and non-eosinophilic asthma, as BMI was similar in all our groups of patients.

Of interest is the fact that classic systemic inflammatory markers such as fibrinogen and C-reactive protein do not link at all with the severity of eosinophilic inflammation, a phenomenon that contrasts with what is usually seen in airway diseases exhibiting neutrophilic inflammation (208;209).

Approximately one-third of asthmatics exhibit dissociation between airway and systemic inflammation. The reasons why there may be discordant eosinophilic inflammation between blood and sputum remains unclear. In case of intense airway eosinophilic inflammation without blood eosinophilia, we could speculate about a massive local attraction due to the release of chemotactic agents without heavy stimulation of bone marrow. In patients with high blood eosinophil count and low

sputum eosinophils, there could be a lack of transendothelial migration of eosinophils due to altered receptor expression or receptor down-regulation. An alternative explanation would be that the airway eosinophilic inflammation is masked by macrophage phagocytosis of eosinophils (134). This could explain the intermittent eosinophilic phenotype (140) and the intermediate FENO we found in this subgroup, as FENO is a good surrogate marker for sputum eosinophilia (166).

However simply looking at cell counts does not provide a complete picture of the cell role in pathophysiology (210). Eosinophil activation by primary lysis results in the spilling of toxic protein-releasing free eosinophil granules in the bronchial tissue (85;211). Lysis of tissue-dwelling eosinophils may partly explain lack of sputum eosinophilia in patients who nevertheless suffer from eosinophilic asthma (212). Asthma-relevant stimuli, including allergens and microbial factors, produce prompt eosinophil lysis. Primary lysis of eosinophils in the airways is likely to be an essential contributor to the intensity of airway eosinophilic inflammation and thereby asthma control. Therefore the relationship with asthma control could have been stronger if we had looked at eosinophil activation.

Even if eosinophilic inflammation appears to be important in driving asthma severity, it has to be kept in mind that half of asthmatics did not display any sign of eosinophilic inflammation, among whom 59% remained poorly controlled. Of course, for those already receiving inhaled corticoids, it could be argued that some of them may have been eosinophilic prior to initiation of ICS treatment. The current literature suggests, however, that initiating or increasing the dose of inhaled corticoids in those non-eosinophilic patients is probably useless for improving asthma control (87). This highlights the need to develop and test new drugs in this asthma phenotype. Clarithromycin was shown to slightly improve quality of life in refractory neutrophilic

asthmatics and a recent study suggests that azithromycin may reduce exacerbation in patients with low blood eosinophil counts (25).

The main limitation of our study is the single time-point measurement that does not take into account the possible fluctuation of the patients from one group to another over time. Indeed, some patients were shown to be intermittently eosinophilic in their sputum, which may cause bias when dealing with a single measurement. However, we believe that looking at the relationship between ACQ score and sputum eosinophils based on one single evaluation remains valid, as ACQ score reflects disease control on a short time period (1 week), a period over which sputum eosinophil count was found to be reproducible (213).

Exacerbation rate, which was clearly higher in the group with combined eosinophilic inflammation, must be considered cautiously as it was based on history taking, and it is not always easy to disentangle what was justified by genuine asthma worsening or by a flare-up of sinusitis with coughing. Nevertheless, the fact that the prospective cohort confirmed the proportion of eosinophilic subgroups and the exacerbation rates found in the retrospective cohort is quite reassuring of the validity of our classification. Of interest also is the fact that exacerbation rate decreased on average by 42% in the year after the visit to our asthma clinic, suggesting that what the clinician decided based on a comprehensive investigation has had an impact on long-term asthma control. Reassuring is the fact those patients for whom the clinician did not feel the need to prescribe an ICS after their asthma clinic visit did not report any exacerbation in the following year. This suggests that using mere cell count to assess inflammation in clinical practice may still carry some value and help to phenotype asthma patients (203). Naturally, this is an observational finding that does

not obey the tight criteria of randomised controlled trials, and our finding has to be confirmed in randomised trials comparing different management strategies.

Our proportion of eosinophilic asthma is greater than that found by McGrath *et al.* (140), as they only found eosinophilic asthma in 17–36% of patients. However, their study recruited patients highly selectively, as is usually the case in clinical drug trials, thereby excluding quite a proportion of patients seen in clinical practice (i.e. smokers, those poorly reversible to β2-agonists and those with co-morbidity), which was not the case in our field study. Of course, we can also speculate that compliance to ICS was better in patients described by McGrath *et al.* (140), which may have also resulted in lower proportions of eosinophilic asthma. It is worth noting that our proportion of non-eosinophilic versus eosinophilic asthma based on sputum analysis is rather similar to what was reported in another field study (13).

In conclusion, concomitant systemic and bronchial eosinophilic inflammation contribute to poor asthma control. As there was dissociation between systemic and airway eosinophilic inflammation in 30% of patients, assessment of inflammation in both airways and peripheral blood provides additional information about asthma status. We believe our classification may be valid to start intervention studies using drugs mainly targeting either the airways or the blood compartment according to the patient's profile.

4.6 Severe asthma (SA) subphenotypes

4.6.1 Introduction

After detailed assessment, 350 severe asthmatics that fulfilled the American Thoracic Society definition of refractory asthma (214) were recruited between March 2009 and January 2014 from 9 Belgian academic centres.

The Belgian Registry is a secured web database and admits password-protected anonymised data, after fully-informed written consent. Individual centre data can be downloaded locally by registered users.

The definition of severe asthma requires one major criterion: either treatment with continuous or near continuous (>50% of year) oral corticosteroids or a requirement for combination high-dose ICS (Beclomethasone or Budesonide > 1000 µg/d, Fluticasone > 500 µg/d) and long-acting β-2 agonists (LABA). The major criterion has to be associated with at least two minor criteria: need for additional daily controller medication in addition to ICS-LABA combinations (Leucotrienes antagonists (LTRA), theophylline), persistent airway obstruction (FEV_1 <80% pred, PEF variability > 20%), asthma symptoms needing short-acting β2 agonist on a daily or near-daily basis, one or more urgent care visits for asthma per year, three or more oral corticosteroids bursts per year, prompt deterioration with < 25% reduction in oral or ICS use, or near-fatal asthma event in the past.

The prerequisite for inclusion was age ≥18years, asthma follow-up by a respiratory physician for at least 12 months, education on the disease provided to the patient, and compliance thought to be satisfactory.

Exacerbation in the previous year was defined by a course of oral corticosteroids for at least 3 days in case of asthma worsening. Nasal polyps and sinusitis was diagnosed by Ear Nose and Throat specialists either by endoscopy or Sinus CT scanner. Gastro oesophageal reflux was diagnosed either by symptoms of heartburn at history taking or the presence of oesophagitis demonstrated by gastroscopy.

4.6.2 Criteria for inflammatory phenotyping

Patients underwent FENO measurement and sputum was only induced at Liege CHU with a success rate of 77%. In a subanalysis we identified patients with type2-high and type2-low inflammation according to the following criteria: sputum eosinophil count < or ≥3% (11), exhaled nitric oxide < or ≥27ppb (166) and blood eosinophil count < or ≥188/mm^3.

4.6.3 Results

We recruited 350 SA as defined by the ATS (2000) criteria from 9 Belgian centres. From those patients, 333 are still defined as severe asthmatics according to ERS/ATS guidelines on severe asthma (2014) (93). The demographic, functional, clinical and inflammatory characteristics of the 350 severe asthmatics are summarised in Table 24.

Table 24. Patients characteristics of the Belgian SA cohort

Patient characteristics	
N.	350
Female (%)	57%
Age	55 ± 0.8
Age at onset	
<12 years	32%
12 – 40 years	36%
>40 years	31%
Height, m	167 ± 0.5
Weight, kg	75 ± 0.9
BMI	26 (16 – 43)
Smoking status	
Never	200 (57%)
Ex-smoker	108 (31%)
(pack-years median IQR)	(15 (11-24))
Current smokers	40 (12%)
(pack-years median IQR)	(11 (10-15))
Atopy, %	70
Current House environment (%)	
Country side	39
Suburban area	29
City	31
Unknown	1
Pre-BD FEV_1, % pred	68 ± 1.2
FVC, % pred	89 ± 1.1
FEV_1/FVC, %	63 ± 0.7
FEV_1Reversibility (% from baseline)	11 ± 0.8
FRC (%) (n=271)	120 ± 2
RV (%) (n=311)	140 ± 2.8
TLC (%) (n=305)	102 ± 1.1
DLCO (%) (n=273)	78 ± 1.2
KCO (%) (n=273)	97 ± 1.3
Airway inflammatory indices	
$FENO_{50}$ (ppb) (n=271)	26 (4 – 250)
Sputum eosinophil count (%) (n=86)	7 (0 – 92)
Sputum neutrophil count (%) (n=86)	51 (0 – 99)
Sputum inflammatory subphenotype (n=86)	
Paucigranulocytic	17%
Eosinophilic (≥3%)	55%
Neutrophilic (≥76%)	22%
Mixed granulocytic	6%
Serum IgE (kU/l) (n=295)	207 (2 – 10000)
Blood eosinophils (%) (n=272)	3 (0 – 50)
Blood eosinophils (/mm^3) (n=272)	240 (0 – 3144)
ACT (n=207)	13 ± 0.4
ACQ (n=213)	2.57 ± 0.09

AQLQ (n=244)	4.14 ± 0.09
ICS dose (BDP µg equivalent/d)	2000 (190 - 6000)
LABA	91
Anti-histamines, %	26
LTRA, %	65
Anti-cholinergics, %	28
Anti-IgE, %	27
Theophylline, %	22
Maintenance oral corticosteroids, %	24
Specific immunotherapy, %	0.6
Comorbidities (%)	
Rhinosinusitis % (Y/N/Ukn)	49% (167/151/32)
Gastrooesophageal reflux (Y/N/Ukn)	36% (124/205/21)
Nasal polyps (Y/N/Ukn)	19% (167/151/32)
Overweight (Y/N/Ukn)	47% (162/173/15)
Psychopathology (Y/N/Ukn)	19% (65/266/19)
Catamenial asthma (Y/N/Ukn)	0.9% (3/340/7)
Aspirin sensitive asthma (Y/N/Ukn)	8% (28/315/7)
Occupational asthma (Y/N/Ukn)	4% (15/328/7)
Churg Strauss syndrom (Y/N/Ukn)	3% (10/333/7)
ABPA (Y/N/Ukn)	3% (11/332/7)
Bronchiectasis (Y/N/Ukn)	16% (54/289/7)
Emphysema (Y/N/Ukn)	7% (24/319/7)
Treatment of comorbidities	
Proton pump inhibitors	39%
Anti-depressive/ anxiolytics	17%/14%
Intranasal steroids	39%
Oral steroids courses during previous yr	2.03 (0 – 7)
Number of hospitalisations during previous yr	0.95 (0 – 7) (n=113)
Number of hospitalization during the last three years	1.7 (0 – 8) (n=103)

4.6.3.1 Demographics and treatment characteristics

Female was the predominant gender (57%) and mean age was 55 yrs. 31% of the severe asthmatics had late-onset asthma (starting after the age of 40). BMI was slightly increased (26kg/m²) and one-quarter of the patients had a BMI >30. 31% were ex-smokers (64% with at least 10 pack-yrs) while 12% were current smokers. The prevalence of atopy was 70%.

One third of Belgian SA lived in cities, one third in suburban areas while 39% lived in the country-side.

In addition to high doses of ICS+LABA, 65% of the patients received anti-leukotrienes. 24% of severe asthmatics were treated with chronic systemic corticosteroids on a daily basis, 26% with anti-histamines and 27% with anti-IgE. Theophylline was administered to 22% of the patients and 0.6% received specific immunotherapy.

Educational level was low in the majority of patients with 6% leaving school after primary school, 21% after lower secondary school and 25% after higher secondary school. Only 16% of severe asthmatics were graduated from non-university post-secondary school and 6% from university (Fig 23A). Data are unknown in one-quarter of the patients. 36% of SA were employed while 21% were retired (Fig 23B).

Co-morbidities were highly prevalent and included chronic rhinosinusitis (49%), nasal polyposis (19%), oesophageal reflux (36%), being overweight and obese (47%) and depression (19%). Bronchiectasis diagnosed based on classical CT criteria were reported in 16% and aspirin-sensitive asthma in 8% of SA while occupational asthma (4%), Churg Strauss syndrome (3%), ABPA (3%) and catamenial asthma (0.9%) were less frequent. Emphysema was present in 24 SA (7%), with 42% of those patients being current smokers, 42% ex-smokers and 16% non-smokers.

The number of oral steroid courses during the previous year was 2.03. The number of hospitalisations during the previous year and the last three years was 0.95 and 1.7 respectively (Table 25). Patients with a history of hospitalisation due to asthma did not have predominant sputum inflammation feature or more severe airway obstruction.

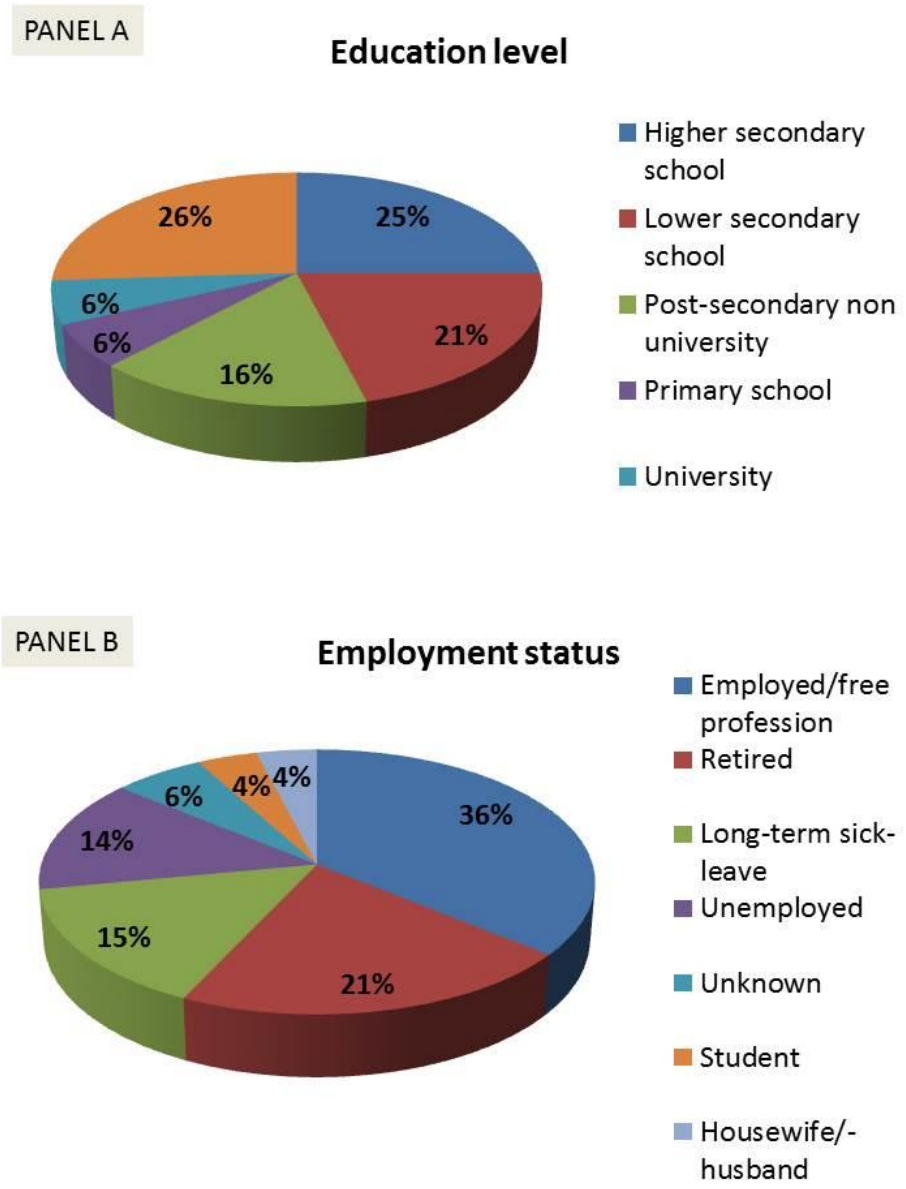

Fig 23. Panel A. Education level in severe asthma in Belgium. Panel B. Employment status in severe asthmatics in Belgium.

Table 25. Number of hospitalisations and steroid bursts in Belgian severe asthmatics during the previous year.

Number during the last year	% of patients with hospitalisation (n=106)	% of patients with systemic corticosteroid courses (n=344)
0	40 (n=42)	26 (n=90)
1	35 (n=37)	15 (n=53)
2	19 (n=20)	16 (n=56)
3	5 (n=5)	14 (n=48)
>3	2 (n=2)	28 (n=97)

4.6.3.2 Lung function

Despite impaired flow rates (mean FEV_1, 68%pred; FEV_1/FVC ratio, 63%), only 65% of SA had post-bronchodilator FEV_1/FVC ratio <70%. We found that 60% of SA had FEV_1<80% and FEV_1/FVC<70%. The mean reversibility was still 11% while patients were on long-acting β2 agonists. 36% of SA exhibited ≥12% FEV_1 reversibility to 400µg Salbutamol and 16% of SA had a reversibility ≥20%.

Severe asthma was associated with significant air trapping. Despite normal total lung capacity (102%pred) there were signs of air trapping suggested by raised FRC (120% of predicted values) and RV (140% of predicted). DLCO was slightly impaired (78%) but KCO was well preserved (97% pred).

4.6.3.3 Inflammatory characteristics

The median FENO value was 26ppb (4 - 250ppb). The fraction of patients with FENO≥50ppb was 22% (Fig 24).

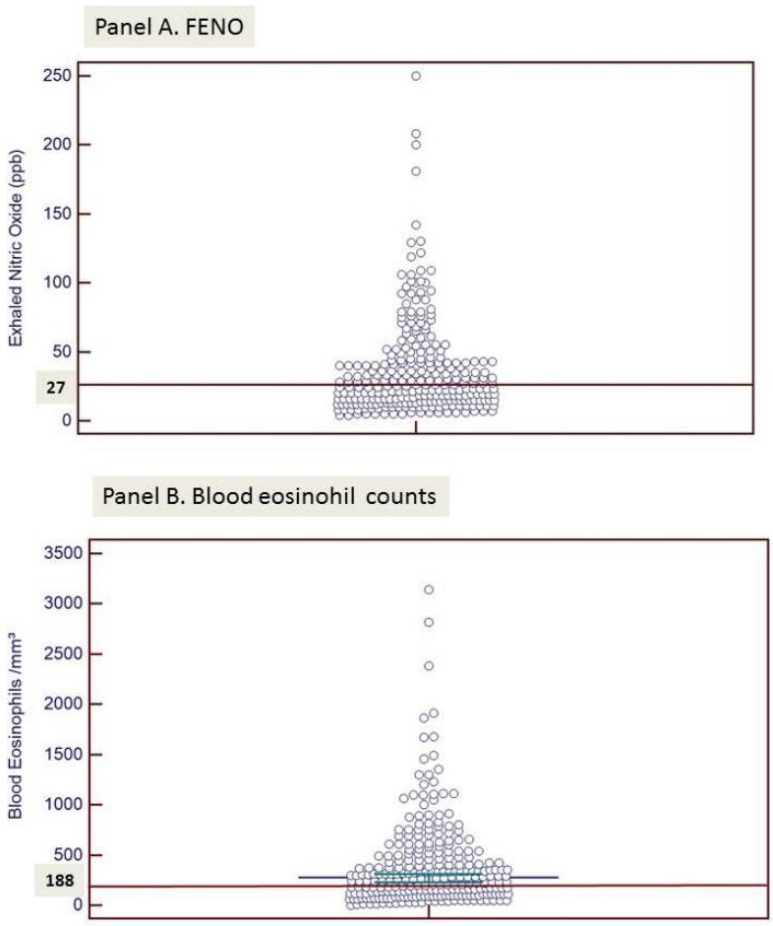

Fig 24. Panel A. Distribution of FENO in the population of severe asthmatics. 49% of SA had FENO levels higher than 27ppb suggestive of persistent sputum eosinophilic inflammation. Panel B. Distribution of blood eosinophils in severe asthmatics. 58% had blood eosinophil count ≥188/mm³.

The median blood eosinophil count was 240/mm³ (Fig 24). We found elevated blood eosinophil counts (>220/mm³) in 53% of SA. Importantly we demonstrated a significant correlation between blood eosinophil count (/mm³) and sputum eosinophil

count (%) (p-value <0.0001; r=0.53). The median sputum eosinophil count and sputum neutrophil count was 7% and 51% respectively.

By constructing a ROC curve, we found that the blood eosinophil count was able to identify sputum eosinophil count ≥3% with the best cut-off point of 188/mm³ providing a 72% sensitivity and 73% specificity (n=80, AUC=0.745, Fig 25).

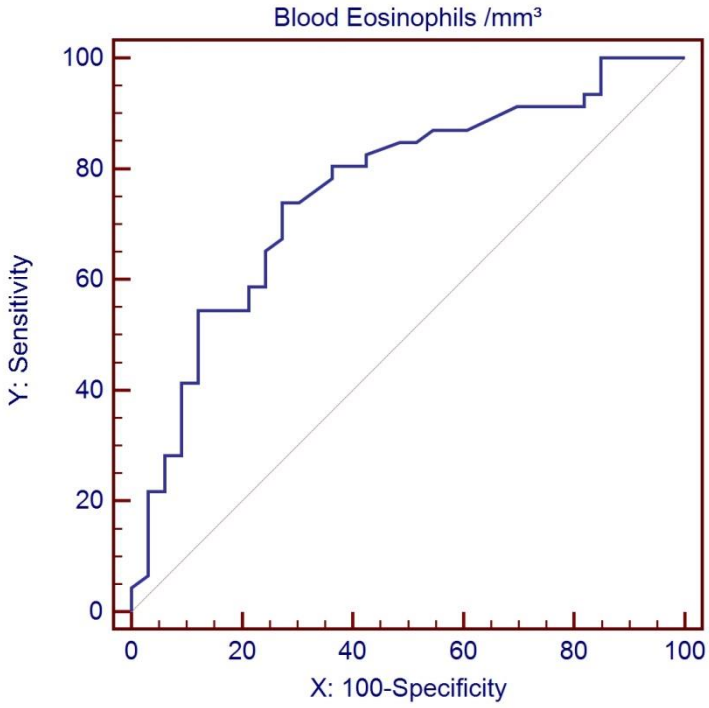

Fig 25. ROC curve showing the best cut-off of blood eosinophil count to identify sputum eosinophil count ≥3% in SRA. Sensitivity 72.3%, specificity 72.7%, Cut-off: 188/mm³, n=80.

We found a modest but significant correlation between FENO and sputum eosinophil count (r=0.37, p<0.001) and between FENO and blood eosinophils (r=0.29,

p<0.0001). By constructing a ROC curve, we found that the best cut-off of FENO was 28ppb to identify sputum eosinophil counts ≥3% in severe asthmatics.

We assessed the proportion of patients exhibiting concordant and discordant blood and sputum eosinophilia. Blood eosinophilia ≥400/mm³ (10) and sputum eosinophil count ≥3% was found in 23% of the patients while exhibiting elevated sputum eosinophil count without increased blood eosinophil count was found in 35%. Normal blood and sputum eosinophil count was common (38%) while isolated elevation of blood eosinophil count was rare (4%). If we chose the threshold value of ≥300/mm³ as recommended by some authors (11), diffuse eosinophilic inflammation was found in 36% of the patients, isolated sputum eosinophilic inflammation was found in 24%, normal blood and sputum eosinophil count was common (33%), while isolated blood eosinophilia was rare (7%).

4.6.3.4 Asthma control and quality of life

Asthma was uncontrolled in 77% of severe asthmatics as defined by an ACQ score >1.5 and in 83% as defined by ACT <20.

We found however that 8% had well-controlled severe asthma (ACQ <0.75) and that 71% of those patients were women with a high proportion being employed (65%). None of these patients were current smokers (88% non-smokers, 12% former smokers). BMI was slightly lower (24kg/m²) than in the general SA population and atopy was more frequent (77%). Quality of life assessed by AQLQ was also better in this sub-population. They had a better lung function (FEV_1 mean: 92%±1.2, FEV_1/FVC: 70±0.9) and fewer signs of air trapping (RV:120±2.5, FRC: 113±2). Only

12% were treated with oral corticosteroids. They exhibited lower FENO levels (17(5-76)) and lower blood eosinophil counts (120 (0-1200)).

4.6.3.5 Relationship between lung function, inflammation, asthma control and quality of life

Average AQLQ was 4.14 (1.2-7). We found a positive correlation between FEV_1 and AQLQ ($r=0.21$, $p<0.0001$) and an inverse correlation between FEV_1 and ACQ ($r=-0.49$, $p<0.0001$). We did not find any significant correlation between blood eosinophils or sputum eosinophil count and AQLQ ($r=-0.01$, $p=0.8$; $r=-0.06$, $p=0.58$ respectively) or between blood eosinophils or sputum eosinophils and ACQ ($r=-0.02$, $p=0.8$; $r=0.06$, $p=0.57$). We did not find any significant correlation between BMI and ACQ or AQLQ.

4.6.3.6 Type 2-high versus type 2-low criteria for targeted therapy

The median total serum IgE level was 207kU/l and 58% of SA with atopic status exhibited IgE levels between 76 and 700kU/l, the range to possibly consider treatment with omalizumab in Belgium (215). In non-atopic SA, 48% had IgE levels between 76 and 700kU/l. Detailed data on sensitisation were available in the Liege cohort (n=111) where 45% of SA were atopic to house dust mite, 39% to cat, 31% to dog, 29% to grass pollen, 23% to birch pollen, 21% to moulds and 12% to horse. According to Belgian's reimbursement criteria, treatment with omalizumab could be proposed in 27% of SA in Liege.

In the DREAM study (11), asthmatics had a history of 2 or more exacerbations requiring systemic corticosteroids in the previous year. Additionally, they had evidence of eosinophilic inflammation as shown by one or more criteria: a sputum

eosinophil count of ≥3%, FENO ≥50ppb, blood eosinophils ≥300/mm³ or prompt deterioration of asthma control after a 25% or less reduction in regular maintenance ICS or OCS. According to these criteria (11), 106 patients (30%) of this cohort could be eligible for anti-IL5 therapy.

We further classified our SA into inflammatory phenotypes according to evidence of either sputum eosinophil count ≥3% or the presence of both FENO ≥27ppb and blood eosinophil count ≥188/mm³. We found that 57% of SA fulfilled those criteria (Fig 26). Eosinophilic SA was more frequently associated with chronic rhinosinusitis (65%) and nasal polyps (24%) in case of late-onset asthma compared to 52% and 18% in early onset asthma.

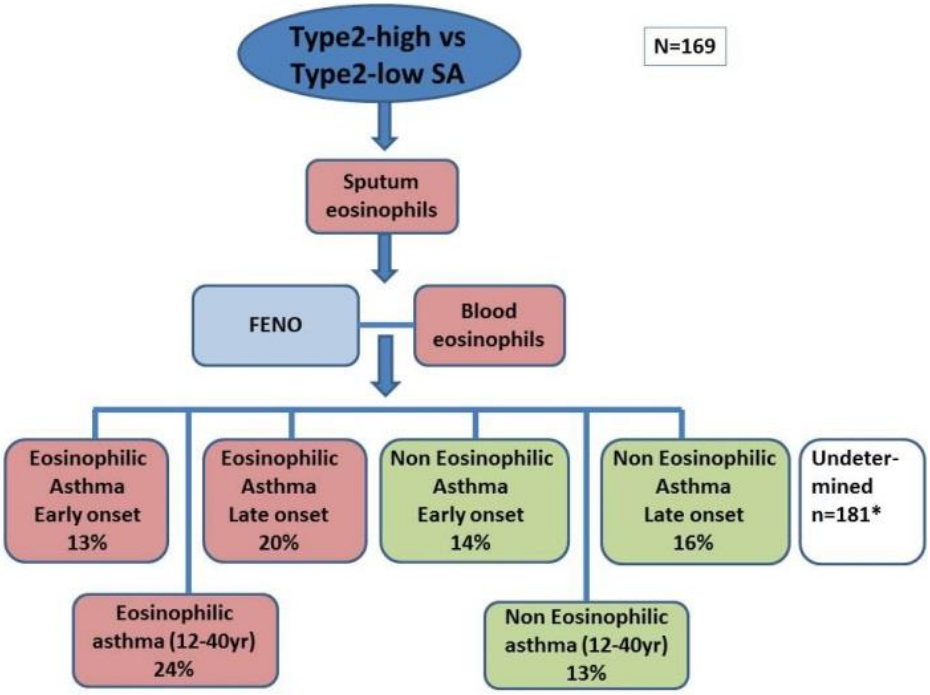

Fig 26. Classification of SA in Type 2-high versus Type 2-low phenotype according to sputum eosinophil count (≥ or <3%), FENO levels (≥ or <27 ppb), and blood eosinophil count (≥ or <188/mm³). SA were classified as Th2-high phenotype if induced sputum showed ≥3% eosinophils or FENO levels ≥27ppb and blood eosinophil count ≥188/mm³ while Type 2-low was chosen if induced sputum eosinophil count was <3% or FENO and Blood eosinophil count were <27ppb and <188/mm³ respectively. 169 patients were classified in eosinophilic and non-eosinophilic asthma according to this

definition and further characterised as early (<12yrs), intermediate (12-40yrs) or late onset (>40 yr). *181 patients were unclassified due to the lack of information on induced sputum, FENO or blood eosinophil count or discordant FENO and blood eosinophils information.

4.6.3.7 Characteristics of patients according to Belgian region

It should be noted that the 350 severe asthmatics were recruited from 9 Belgian centres including CHU Liege (32%), Ghent University Hospital (28%), CHU Mont-Godinne (15%), Erasme (8%), Cliniques universitaires Saint-Luc (7%), CHU Vesale (6%), CHU St Pierre (3%), Brugman (1%) and UZ Leuven (<1%) (Fig 27).

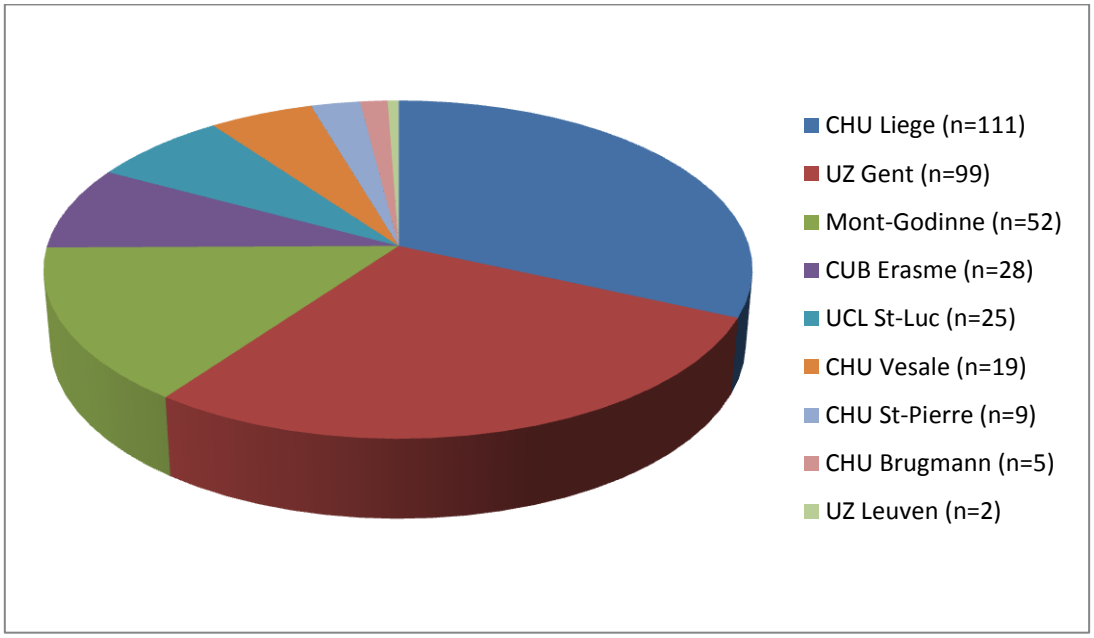

Fig 27. Proportion of patients recruited by the different Belgian centres.

The three Belgian regions are Flanders (Dutch-speaking part), Wallonia (French- and German- speaking part) and Brussels (Dutch- and French- speaking part); patients' characteristics were compared according to the region.

We found higher numbers of patients with smoking history in Wallonia and Brussels than in Flanders (Table 26), the highest tobacco history was observed in Wallonia. Despite similar FEV_1/FVC ratios, FEV_1 was clearly more altered in patients from

Wallonia and Brussels than those from Flanders. Reversibility to β2-agonists and signs of air trapping were significantly higher in Brussels and Wallonia than in Flanders. Asthma control and quality of life was better in patients from Flanders than in Wallonia and Brussels.

We also found discrepancies concerning the treatment prescribed in the different regions. In Flanders, there were fewer patients prescribed with leukotriene receptor antagonists but higher frequency with LAMA and oral corticosteroids. Anti-IgE and Theophylline use was significantly higher in Brussels.

Gastro-oesophageal reflux was frequent in SA patients from Wallonia. Overweight was encountered significantly less in Brussels. We found less psychopathology and less emphysema in Flanders. The number of hospitalisations during the last three years was highest in Wallonia.

Table 26. Patient characteristics of the SA classified according to the region of recruitment.

	Wallonia	**Flanders**	**Brussels**
N.	182	101	67
Female (%)	59	53	58
Age	54±1	57±1.5	56±2
Age at onset			
<12 years	34%	33%	34%
12 – 40 years	36%	36%	35%
>40 years	30%	31%	31%
Height, m	167 ± 0.7	168 ± 0.9	166 ± 1.2
Weight, kg	76 ± 1.2	76 ± 1.4	72 ± 2.1
BMI	26 (17 – 43)	26 (18 – 40)	25 (16-39)
Never smoker	50%	75%***	61%
Ex-smoker (pack-years	39%	22%**	27 %
median IQR)	(15 (6 – 25))	(10 (8 – 20))	(10 (5 – 19))[§]
Current smokers	17%	3%**	12%[§]
(pack-years median IQR)	(15 (9 - 32))	(5)*	(19 (10 – 30))
Atopy, %	70	63	78
House environment			
Country side	28%	27%	35%
Suburban area	24%	51%***	11%[§###]
City	48%	22%***	54%[###]
Pre-BD FEV_1, % pred	62 ± 1.4	82 ± 2.3***	64 ± 2.1[###]

FVC, % pred	81 ± 1.3	105 ± 1.9***	85 ± 2$^{###}$
FEV_1/FVC, %	62 ± 0.8	65 ± 1.3	63 ± 1.5
FEV_1 Reversibility (% from baseline)	11 ± 0.9	6 ± 1***	16 ± 3.5$^{###}$
FRC (%)	120 ± 2.8	115 ± 3.1	128 ± 6.1$^{#}$
RV (%)	142 ± 4	127 ± 4.2*	153 ± 7$^{##}$
TLC (%)	107 ± 1.5	102 ± 1.8	104 ± 3.7
DLCO (%)	76 ± 1.7	85 ± 1.5**	71 ± 3.7$^{§§###}$
KCO (%)	98 ± 1.7	101 ± 1.8	85 ± 4$^{##}$
FENO, ppb	27 (4-250)	21 (6-200)	29 (5-208)
Serum IgE (kU/l)	216 (2-10000)	160 (4-5421)	260 (2-5767)
Blood eosinophils (%)	3 (0-38)	3 (0-50)	4 (0-21)
Blood eosinophils (/mm^3)	232 (0-3144)	250 (0-1460)	260 (0-1677)
ACT	13 ± 0.4	13 ± 1.9	14 ± 0.9
ACQ	2.82 ± 0.1	1.54 ± 0.2***	2.72 ± 0.2$^{###}$
AQLQ	3.84 ± 0.1	5.16 ± 0.2***	4.15 ± 0.2$^{###}$
ICS dose (BDP µg equivalent/d)	2000 (200-6000)	2000 (770-6000)	2000 (190-4000)$^{##}$
Anti-histamines, %	28	33	14$^{#}$
LTRA, %	71	49**	71$^{#}$
LAMA, %	27	39	19$^{#}$
Anti-IgE, %	24	18	46$^{§§§###}$
Theophylline, %	15	22	29§
Maintenance oral corticosteroids, %	20	34*	19
Specific immunotherapy, %	1.3	0	0
Rhinosinusitis,%	53	42	47
GERD,%	49	19***	28§§§
Nasal polyps,%	20	17	17
Overweight,%	53	50	27$^{§§§##}$
Psychopathology,%	25	7***	20$^{#}$
Catamenial,%	2	0	0
Aspirin sensitive, %	6	12	8
Occupational, %	4	3	6
Churg Strauss, %	2	6	0
ABPA,%	4	2	3
Bronchiectasis,%	17	15	13
Emphysema,%	12	1**	3
Oral steroids courses during previous yr	2.1/patient/yr	1.9/patient/yr	1.8/patient/yr
Number of hospitalisation during previous yr	0.38/patient/yr	0.21/patient/yr	0.21/patient/yr
Number of hospitalisation during the last three years	0.67/patient/yr	0.21/patient/yr*	0.41/patient/yr

*for comparisons between Wallonia and Flanders. $^{#}$for comparisons between Flanders and Brussels. §for comparisons between Wallonia and Brussels. GERD: gastro-oesophageal reflux.

4.6.4 Discussion

Our data confirm that the majority of severe asthmatics are female and atopic (216). Moreover we have shown that asthma started after the age of 12 in two-thirds of SRA, and that SRA exhibited increased airway granulocytic inflammation compared to a general population of asthmatics (128). Dominant co-morbidities were chronic rhinosinusitis, overweight and gastrooesophageal reflux. Type2-high and Type2-low SA were diagnosed based on induced sputum, blood eosinophil count, IgE and FENO levels. A trait suggesting type2-high was identified in the majority of SA despite treatment with high doses of ICS (and oral corticoids in a subgroup).

4.6.4.1 Demographic and general clinical data

Our data confirm the usual female preponderance (110;111;217;218). In comparison with a general population of asthmatics (219), SRA was rather similar in age (55 vs 52). Late-onset asthma, starting after the age of 40, was observed in 31% of patients. The proportion of current smokers was higher than that observed in the UK registry (111) (12% vs 6%) while 31% of our population recognised past smoking history (similar to the UK registry). Thomson's study (220) showed similar data with 9% of British severe asthmatics being current smokers, while 28% were ex-smokers and 62% never-smokers. In this study, current smokers had poorer asthma control and more unscheduled health care visits and rescue courses of oral steroids than ex-smokers or never-smokers. Their inflammatory profiles in sputum and blood also differed.

BMI was slightly increased but remained in the non-obese range in the majority of patients (only one-quarter had BMI > 30) and was similar to that observed in the

general population of asthmatics (219), in the UK registry (111), ENFUMOSA (107) and BIOAIR studies (108).

Severe asthmatics in Belgium have a rather low level of education with only 22% having graduated from post-secondary school education. Our interpretation is that asthmatics with low level of education are more prone to be exposed to noxious particles, massive allergen amount in their daily life because being less cautious in taking care of their health. Less than 40% of severe asthmatics are still professionally active. This was similar to what was observed in the UK where 53.4% were not working (111); the ENFUMOSA study (221) concluded that fewer severe asthma patients than mild asthma patients were currently employed.

The patients with SA that were entered in the registry were more severe in Wallonia and Brussels. The more frequent smoking history (222) and co-morbidities (223) observed in Wallonia may contribute to severity and hospital admissions observed in this region.

4.6.4.2 Treatment and co-morbidities

In addition to high doses ICS-LABA, SRA in Belgium received LTRA and omalizumab more frequently than in the UK (111) while theophylline and oral corticosteroids were less commonly encountered than in the UK registry and ENFUMOSA. The higher number of anti-IgE treated patients in our registry is probably due to the inclusion of a high number of Belgian severe refractory asthmatics in the PERSIST study (224). For the management of co-morbidities, intranasal corticosteroids and proton pump inhibitors (PPI) were more frequently prescribed in Belgium than in the UK (111).

The number of oral steroids courses during the year preceding inclusion in the SRA was lower than that observed in the UK register (111) while the number of hospitalisations was higher.

The dominant co-morbidities encountered in the Belgian severe asthma population were rhinosinusitis, overweight and gastrooesophageal reflux. The proportions were similar those previously reported (111), except for chronic rhinosinusitis that was more frequently encountered in Belgium. The treatment of rhinosinusitis by nasal corticosteroids is in accordance with the high proportion of rhinitis observed in a large population of asthmatics in Belgium (225). We found a similar proportion of occupational asthma and aspirin-sensitive asthma in our cohort as compared to the UK cohort (111).

4.6.4.3 Lung function

Overall there was a moderate obstructive airway pattern. The obstruction level was similar to ENFUMOSA (107) and the UK register (111), but lower than in the SARP study (110). Surprisingly, several patients with normal FEV_1 were included in the registry due to high respiratory symptoms despite high-dose ICS. They are patients who still report symptoms and bronchodilator use despite good airway function. Although we cannot exclude that these patients exhibit persistent BHR, they might have ‘discordant disease’ (6) as their indices of airway inflammation were low (median FENO 22ppb, median sputum eosinophils 2.4%). 59% of these SA had obesity and late-onset disease (>40yrs) with characteristics similar to the ‘obese non-eosinophilic’ asthma reported by Haldar *et al.* As in the UK registry (111) and ENFUMOSA study (107), the coefficient transfer was well-preserved in our population, which can be considered as a sign that our asthmatics are very different

from COPD patients even if the majority of our patients displayed fixed airway obstruction. The normal KCO suggests a preserved alveolocapillary membrane. We confirmed that severe asthma is associated with significant increase in air trapping with normal total lung capacity suggesting involvement of small airways (89;110;205).

4.6.4.4 Inflammatory subphenotypes

The frequency of atopy remained high despite two-thirds of SRA experiencing the onset of asthma after the age of 12. Atopy was similar to that observed in SARP study (110) but higher than in the BIOAIR study (108), ENFUMOSA (107) and the UK registry (111). SARP data and ours are based on skin prick tests and/or specific IgE level while in the UK registry it was based on the history, which may have led to underestimation.

The median FENO value is in the lower part of the grey zone (25-50ppb (153)) in severe asthma. This is probably due to higher doses of inhaled corticosteroids in this sub-population. This suggests the persistence of an airway inflammatory process since we were able to show that in patients receiving high-dose ICS the FENO threshold predicting sputum eosinophil count ≥3% was shown to be 27ppb (166). 49% of severe asthmatics exhibited FENO levels >27ppb suggesting an eosinophilic phenotype. As compared to the UK register (111), SARP (110) and BIOAIR (108), FENO levels were lower in our register.

Our median blood eosinophil count was slightly lower than that observed in the UK register (111). The blood eosinophil count threshold that best predicts the presence of uncontrolled airway eosinophilia in SA was found to be 188/mm^3. According to this threshold, 58% exhibited eosinophilic asthma.

Asthma was uncontrolled in 77% of severe asthmatics according to ACQ. The 8% well-controlled severe asthmatics were non-smoking patients with a higher rate of atopy, receiving high doses of corticosteroids and exhibiting less severe airway obstruction and fewer respiratory symptoms. They had less intense eosinophilic inflammation.

4.6.4.5 Correlation between clinical and inflammatory data

We found a correlation between asthma control and airway calibre, a fact that has already been observed in asthma in general (226;227). There was a weak correlation between quality of life and airway calibre. A similar correlation between FEV_1 and AQLQ has already been demonstrated in a large population of persistent asthmatics (228). We did not find any significant correlation between systemic eosinophilic inflammation and asthma control or quality of life. In a previous study conducted in a large population of unselected asthmatics (219), we did not find any significant correlation between ACQ and blood eosinophil count while there was a weak correlation between ACQ and sputum eosinophil count. The fact that we did not find any correlation between blood eosinophil count or sputum eosinophil count and AQLQ is in line with the DREAM study, as anti-IL5 therapy was able to decrease sputum and blood eosinophil count but was not associated with an improvement in AQLQ (229) in severe eosinophilic asthmatics compared with placebo.

BMI was correlated neither with ACQ nor with AQLQ in our severe asthma population. Median BMI in our Belgian severe asthma population is rather similar to BMI found in a general population of asthmatics (128;219). Lavoie *et al.* previously failed to find any association with asthma severity, which is consistent with the

present findings (230). The impact of BMI is probably lower in severe asthmatics as there are several other factors influencing asthma control in this population.

4.6.4.6 Therapeutic implications

We found elevated levels of IgE in our population of severe asthmatics whatever the atopic status. For the management of severe asthma in Belgium, the only biological treatment already commercially available is anti-IgE, reimbursed in case of sensitisation to perennial allergen and a level of IgE between 76 and 700kU/l. The median IgE was higher in our register than that observed in the UK register (111), ENFUMOSA (107) and SARP (110).

The majority of patients have residual eosinophilic inflammation both at systemic and airway level and are therefore potential candidates for anti-IL5 treatment. In the DREAM study (11), asthmatics had a history of two or more exacerbations requiring systemic corticosteroids in the previous year. Additionally, they had evidence of eosinophilic inflammation as shown by one or more criteria: a sputum eosinophil count of ≥3%, FENO of 50ppb or more, blood eosinophils of 300/mm^3 or more, or prompt deterioration of asthma control after a 25% or less reduction in regular maintenance ICS or OCS. We identified 30% of potential candidates for anti-IL5 therapy according to DREAM criteria (11). However taking into account the adapted threshold (for FENO and blood eosinophil count) to identify eosinophilic inflammation in asthmatics treated with high doses ICS, 57% of patients had eosinophilic asthma.

Although the mechanism of action of bronchial thermoplasty is not currently completely understood, this bronchoscopy technique seems to act on smooth muscle mass. Thermoplasty could emerge as a therapeutic option in SA who exhibit high

bronchodilatation after salbutamol (16% of SA in our registry). The role of macrolides in non-eosinophilic severe asthma (25) remains to be clarified in larger studies.

For the recruitment of Belgian SRA, we followed 2000 ATS criteria (214) because the collection of data started in 2009. Our study limitations are the limited number of patients with detailed allergic characteristics and induced sputum analysis and the recruitment at tertiary care University hospitals with potential selection bias.

4.6.5 Conclusion

In this Belgian cohort of patients with SA, we show that atopic background and eosinophilic inflammation represent the predominant features. Findings are consistent with other European and American registries, and will help to identify candidates for upcoming targeted therapeutic approaches. Further studies are also needed to clarify whether particular endotypes could be identified in this heterogeneous group of asthma patients with severe disease that is refractory to current therapies.

Chapter 5. General discussion and perspectives

The different phenotypes of bronchial asthma described in the literature confirm the heterogeneous nature of the disease. This heterogeneity is reflected by various clinical characteristics according to asthma subtypes defined by either inflammation, demographic and functional parameters. The classification of asthmatics in different inflammatory phenotypes has therapeutic implications. The eosinophilic phenotype has been shown to be corticosensitive while non-eosinophilic asthma is not improved by corticosteroids in short term studies. In neutrophilic and paucigranulocytic asthma, other treatment such as long-acting β2-agonists, long-acting anti-muscarinic agents or macrolides could be good candidates for targeted treatment. We are happy to see that the new GINA guidelines recognise the utility of induced sputum in specialised centres to guide treatment in patients who are uncontrolled despite high doses of inhaled corticosteroids associated to other controller therapy.

We have confirmed on a large asthmatic cohort the heterogeneity of airway inflammation and provided proportions of each inflammatory phenotype as encountered in clinical practice at a university hospital. The most frequently observed inflammatory subtypes were eosinophilic and paucigranulocytic phenotypes.
This classification remains interesting as it shows that eosinophilic asthma is more prone to exacerbations.

Applying Simpson classification we have shown that factors associated with eosinophilic inflammation differ from those associated with neutrophilic phenotype. Eosinophilic asthma is associated with blood eosinophils, elevated FENO levels, high

blood IgE levels and a low FEV_1/FVC ratio, while neutrophilic asthma was associated with higher FRC and age. However asthma control was rather similar whatever the sputum inflammatory phenotype. The link between neutrophils or eosinophils and asthma severity remains controversial. Without denying the interest of the Simpson classification, we have proposed a new way to classify asthma. It seems interesting to us to analyse eosinophilic inflammation more in depth by confronting systemic and airway eosinophilia. We have identified a subgroup of asthmatics including a majority of males, exhibiting both blood and airway eosinophilic inflammation more prone to exacerbations, expressing lower asthma control and poorer lung function. Interestingly, this group had more often nasal polyposis. Our new classification allows identification of a subgroup of asthmatics who are poorly controlled and at risk of recurrent exacerbations. We think our classification has greater clinical impact than just looking at airway inflammation, and reveals the relationship between eosinophilic inflammation and asthma severity. Looking at both inflammatory sites is valid to start intervention studies as the novel biotherapies such anti-IL5 have been developed based on inflammatory processes. We believe our new classification is timely as we have now available treatments targeting either airway (ICS) or systemic (OCS, anti-IL5) eosinophilic inflammation. ICS were shown to be very potent in decreasing airway eosinophilia while their effect on blood eosinophil count was limited. Anti-IL5 therapy is particularly efficient in those patients with high blood eosinophils by reducing exacerbations (15;16;231-235).

We have also described the Belgian severe asthma population and their inflammatory characteristics. What is striking is that the proportion of paucigranulocytic asthma is very low, supporting the role of uncontrolled airway

inflammation as a key factor in determining asthma severity. Another finding of our study is that residual eosinophilic inflammation is still seen in many severe patients. This highlights the need for add-on therapy to inhaled corticosteroids to combat eosinophilic inflammation. One-fifth of the patients had heavy airway neutrophilic inflammation. Our Belgian registry will serve as a pool of patients that can be included in clinical studies on new targeted biotherapies or in studies designed to understand the pathophysiology of severe asthma.

We have been able to show that FENO and blood eosinophil thresholds that predict airway eosinophilic inflammation may be influenced by the dose of inhaled corticosteroids and smoking history for the former. Consequently our data show that FENO and blood eosinophil levels that predict airway eosinophilic inflammation are lower in severe asthmatics than in mild to moderate patients.

While FENO and blood eosinophils may suggest the presence of airway eosinophilic inflammation, we currently lack of markers for identifying neutrophilic inflammation. Our work has identified potential markers for airway neutrophilic inflammation. The usefulness of biomarkers (VOCs) identified in exhaled air has to be confirmed in a prospective validation study. In the future we would like to investigate if FENO could provide complementary information to exhaled VOCs in a combined model that could be even more powerful.

The future for asthma management may be that patients use small devices to detect VOCs such as the glucometers used for diabetes, to receive immediate information on inflammatory subtypes of the disease in order to adapt their treatment to reach asthma control with reduction of future risk. In chronic obstructive pulmonary disease (COPD) several phenotypes have also been identified (236), and it has been shown

that a small proportion of these COPD patients exhibit eosinophilic inflammation during and even outside the exacerbation. Perhaps we will be able to go one step further by discarding the labels ‘asthma’ and ‘COPD’ in order to focus on eosinophilic versus non-eosinophilic airway disease, and choosing and adjusting treatment accordingly.

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ANNEXES

Publications