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PHENOTYPING OF SEVERE ASTHMA IN A CLINICAL CONTEXT

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PHENOTYPING OF SEVERE ASTHMA IN A CLINICAL CONTEXT

Thesis for Doctoral Degree (Ph.D.)

By

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”Я єсть народ, якого Правди сила

ніким звойована ще не була.”

(Павло Тичина)

To all people of Ukraine and my family. Together against war

POPULAR SCIENCE SUMMARY OF THE THESIS

What is asthma?

Imagine a situation when you can't get enough air, despite being desperate to breathe and you fear for your life. If you have ever been very high up a mountain or found yourself in a closed space without access to fresh air, then you know what I mean. Then you also can imagine how a patient with asthma feels during an attack. Fortunately, most asthmatics have mild or moderate disease and can be treated with modern inhalers which provide good control of their disease. This thesis is based on a research project that focused on a smaller group of patients (about 4% of all asthmatics) with the most severe asthma.

Why is it important to have research on severe asthma?

Firstly, these patients experience continuous breathing problems as well as suffering from asthma attacks several times every day and even at night. This is despite treatment with the whole arsenal of existing asthma medications at maximal doses. As asthma is a chronic inflammatory disease, they also often need regular treatment with high doses of oral corticosteroids to try to reduce this inflammation. Treatment with oral corticosteroids is associated with many serious side effects and as a result, patients may also develop diabetes, osteoporosis, and high blood pressure. The body can also stop producing its own hormones (specific proteins that regulate key functions in human body) as a side effect. Aside from such steroid-related complications, these patients are also constantly living with the fear of not being able to breathe which in turn leads to anxiety. In addition, many of these patients are unable to work.

Secondly, there are still about 150 people per year in Sweden that die due to asthma despite the availability of good modern medicines and the disease is considered to be well treated. Thirdly, asthma is a common lung disease, the symptoms of which can start at different ages and then remain for life. In Sweden it is estimated that asthma affects approximately 10-12% of the adult population and represents a high cost to society (>100 billion SEK per year). Finally, although the number of patients with severe asthma represents a relatively small proportion of the asthma population, the societal costs account for an unproportionally large share (> 50%) of the total costs attributed to asthma (Canonica et al., 2020).

The overall purpose of this research project was to try to increase our understanding of clinical aspects of severe asthma and its therapy and by doing so, improve the management and care of these patients. Over the past few years, researchers have managed to discover new medicines, so called biologics, that can target and block the effects of individual proteins in the human body. These new medications have been shown to be highly effective when a specific, targeted treatment is given to the right patient from selected subgroups of severe asthmatics with certain disease characteristics. However, if given to the wrong patient such medications may be useless and potentially have side-effect. The specific focus of this research is to study which patients

show the best response to treatment with biologics, and further, to test a new method that can help measure response to therapy.

In this thesis I report the compiled results of three different investigations. In the first study, an analysis was made of information collected from the national registries of eleven countries across Europe. The results of this analysis revealed differences between European countries in terms of investigations, diagnosis, and management of asthma. The definition of severe asthma in current guidelines was also found to be in discordance with the characteristics of severe asthmatics in the real-world, and to differ between countries. In conclusion, the areas addressed in the first investigation could be an important stepping-stone towards future discussions aimed at standardizing severe asthma care and treatment throughout Europe.

In the second investigation, we measured levels of male hormones and cortisol (the main stress hormone in human body) in the urine of severe asthmatics and compared these measurements to healthy controls and mild asthmatics. Results showed that patients with severe asthma had decreased levels of both cortisol and male hormones. This decrease was more pronounced in women compared to men and was strongly related to oral corticosteroid treatment. This relative deficiency in male hormones as a side effect of corticosteroids was not known before. As this decrease in hormone levels is associated with more severe asthma, poorer asthma control and poorer quality of life, this could partly explain why female asthma is more severe. In conclusion, low levels of male hormones could be one contributing cause to sex differences in asthma severity. Since this study demonstrated that treatment with corticosteroids suppresses both cortisol and male hormones, we suggest that both of these should be monitored as part of routine clinical practice in all patients with severe asthma treated with oral corticosteroids.

In the third investigation, we modified a new strategy for measuring response to treatment. The method, a quantitative algorithm, was originally developed in a European collaboration and here we test the algorithm in a clinical research study that includes 77 patients treated with a new biological medicine called mepolizumab for at least one year. Initially, when response to the biologic was assessed in a traditional way, we saw that some patients showed marked improvements while others did not respond so well. However, when we used the modified quantitative algorithm, which included patient-reported information such as an asthma control test and quality of life test, we could refine the assessment of response to treatment. In addition, this method has been shown to be more individualized, and can identify non-responders who need to change their treatment much earlier, which helps clinicians to use the new expensive biological treatments in a more cost-efficient way.

In summary, this thesis has generated results that not only increase our general understanding of patients with severe asthma in a clinical context, but also identified changes that need to be made regarding their management and care across Europe, which has important clinical implications.

ABSTRACT

Severe asthma is a chronic heterogeneous inflammatory disease characterized by several clinical phenotypes and molecular endotypes. Although it affects a relatively small proportion of the asthma population (approximately 4%-10%), with an even smaller proportion of these having severe uncontrolled eosinophilic asthma, it accounts for > 50% of the costs attributed to the disease. Despite the availability of modern medicines and improvements in certain outcomes, severe asthma is still a cause of mortality. Although it is known that severe asthma is driven by type 2 inflammation in most cases, and we now have the possibility to use specific biological therapies targeting this particular type of inflammation, many patients are still sub-optimally controlled due to the heterogenous nature of this disease with its multiple sub-phenotypes. There is, therefore, an unmet need to characterize and classify these patients with a view to improve therapy and reduce costs on a global scale. Furthermore, selection of the most appropriate biologic and the best clinical outcomes and biomarkers with which to monitor response to therapy, are still issues of debate and the subject of ongoing research.

Three clinical severe asthma studies are included in this thesis, the overall aim of which was to provide an increased understanding of the clinical features and treatment effects associated with the different sub-phenotypes of severe asthma. A specific focus was also to validate different clinical outcomes and assess their importance for the management of asthma.

The three studies address these aims in different ways including an epidemiological investigation (**Paper I**), a pharmacological assessment of the drugs used to treat asthma as well as their side effects and relationship with asthma severity (**Paper II**) and a clinical intervention applied in a "real-life" setting, including a preliminary meta-analysis with the objective to develop a new method for assessment of response to therapy (**Paper III**).

Several important observations were made in **Paper I**. The results of this study revealed differences in clinical characteristics, lifestyle factors and treatment patterns among severe asthmatics in Europe, confirming the heterogeneity of this disease. Moreover, the severe asthma definition in current guidelines did not correspond to the characteristics of real-world severe asthmatics, and the definitions also differed between countries. Finally, **Paper I** emphasized the importance of harmonizing severe asthma registries throughout Europe, and the need for long-term follow-up of this group of patients.

In **Paper II** was done analysis of data from 478 well characterized asthmatics and 98 healthy controls in the U-BIOPRED study. **Paper II** shows that severe asthmatics have significant suppression of androgens and cortisol compared to patients with mild-to-moderate asthma and healthy control according to extensive analysis of urinary endogenous and exogenous steroids. This suppression is more pronounced in women compared to men. Moreover, the data show that this adrenal suppression is depended on the level of treatment with exogenous corticosteroids. Thus, our results provide support to the hypothesis that this relative deficiency in androgen levels during steroid treatment that is disproportional greater in women compared to men may partly explain gender differences in the severity of asthma and prevalence.

Finally, our study supports that reduction of high dose inhaled corticosteroids (ICS), and especially the taper of oral corticosteroids (OCS) should be a clinical goal in order to reduce the side effects of corticosteroids.

In **Paper III**, we modified a quantitative algorithm that was originally developed in a European collaboration to assess response to therapy and evaluate efficacy, and then tested this strategy in patients with severe asthma undergoing treatment with the biologic mepolizumab. The method was able to quantify response to an expensive biological treatment and identify four groups with different degrees of response to mepolizumab: super response, substantial response, sufficient response, and non-response. The super responder group had the greatest improvement in lung function, asthma quality of life questionnaire, asthma control questionnaire and the highest reductions in exacerbations and OCS use, whereas the non-responders lost asthma control, discontinued mepolizumab treatment and switched to other biologics. This new, quantitative algorithm was shown to provide a more individualized assessment of treatment response and identified non-responders in need of revised treatment. Further, this method can be implemented in clinical practice for greater precision in early clinical decision-making regarding the use of biological therapy.

In conclusion, the three clinical studies included in the thesis have contributed to an increased understanding of the clinical phenotypes of severe asthma. The experiences accumulated during this work allow for some general implications. For example, longitudinal, prospective studies carried out in a real-world setting are important for evaluation of response to treatment with new drugs since the differing responses of well-characterized and phenotyped patients can reveal clinical sub-phenotypes and their relationship to underlying molecular mechanisms.

The utility of different clinical outcomes could be validated and their importance for asthma management assessed. Clinical studies also provide an opportunity to investigate requirements for improved management and care of severe asthmatics. Patient-centred research contributes to a better understanding of patient needs, and thereby facilitates refined assessment of clinical response to treatment.

LIST OF SCIENTIFIC PAPERS

I. van Bragt JJMH, Adcock IM, Bel EHD, Braunstahl GJ, Ten Brinke A, Busby J, Canonica GW, Cao H, Chung KF, Csoma Z, Dahlén B, Davin E, Hansen S, Heffler E, Horvath I, Korn S, Kots M, Kuna P, Kwon N, Louis R, Plaza V, Porsbjerg C, Ramos-Barbon D, Richards LB, Škrgat S, Sont JK, Vijverberg SJH, Weersink EJM, **Yasinska V**, Wagers SS, Djukanovic R, Maitland-van der Zee AH; (2020). **Characteristics and treatment regimens across ERS SHARP severe asthma registries.** The European Respiratory Journal, 55(1), 1901163-. https://doi.org/10.1183/13993003.01163-2019

II. **Yasinska V**, Gómez C, Kolmert J, Ericsson M, Pohanka A, James A, Andersson LI, Sparreman-Mikus M, Sousa AR, Riley JH, Bates S, Bakke PS, Kermani NZ, Caruso M, Chanez P, Fowler SJ, Geiser T, Howarth PH, Horváth I, Krug N, Montuschi P, Sanak M, Behndig A, Shaw DE, Knowles RG, Dahlén B, Maitland-van der Zee AH, Sterk PJ, Djukanovic R, Adcock IM, Chung KF, Wheelock CE, Dahlén S-E, Wikström Jonsson E. (2023). **Low levels of endogenous anabolic androgenic steroids in females with severe asthma taking corticosteroids**. ERJ Open Research, 9(5), 269-. https://doi.org/10.1183/23120541.00269-2023

III. **Yasinska V**, Kolmert J, Andersson LI, Sparreman-Mikus M, Rydell N, [possible additional co-authors], Janson C, Malinovschi A, Mjösberg J, Wheelock CE, Dahlén B, Dahlén S-E. **A quantitative score algorithm to refine response evaluation in mepolizumab treated severe asthmatics.** *(manuscript, originalarbete)*

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II. Janson, C., Bjermer, L., Lehtimäki, L., Kankaanranta, H., Karjalainen, J., Altraja, A., **Yasinska, V.,** Aarli, B., Rådinger, M., Hellgren, J., Lofdahl, M., Howarth, P. H., and Porsbjerg, C. (2022). Eosinophilic airway diseases: basic science, clinical manifestations and future challenges. European Clinical Respiratory Journal, 9(1), 2040707–2040707. https://doi.org/10.1080/20018525.2022.2040707

III. Eger, K., Dora, P., Alison, B., Florence, S., Svetlana, S., Arnaud, B., Isabelle, V., Eleftherios, Z., Konstantinos, K., Dimosthenis, P., Zsuzsanna, C., Enrico, H., Canonica, G., Ineta, G., Kristina, B., Jolita, P., Anneke, T., Hashimoto, S.,

Wjm, S., **Yasinska, V.,** … Paroczai, D. (2022). The effect of the COVID-19 pandemic on severe asthma care in Europe - will care change for good? EUROPEAN RESPIRATORY JOURNAL, 60. https://doi.org/10.1183/13993003.congress-2022.192

IV. Khaleva, E., Rattu, A., Brightling, C., Bush, A., Bossios, A., Bourdin, A., Chung, K. F., Chaudhuri, R., Coleman, C., Dahlén, S.-E., Djukanovic, R., Deschildre, A., Fleming, L., Fowler, S. J., Gupta, A., Hamelmann, E., Hashimoto, S., Hedlin, G., Koppelman, G. H., **Yasinska V.,** … Roberts, G. (2023). Development of Core Outcome Measures sets for paediatric and adult Severe Asthma (COMSA). The European Respiratory Journal, 61(4), 2200606-. https://doi.org/10.1183/13993003.00606-2022

V. Khaleva, E., Rattu, A., Brightling, C., Bush, A., Bourdin, A., Bossios, A., Chung, K. F., Chaudhuri, R., Coleman, C., Djukanovic, R., Dahlén, S.-E., Exley, A., Fleming, L., Fowler, S. J., Gupta, A., Hamelmann, E., Koppelman, G. H., Melén, E., Mahler, V., … **Yasinska V.,** Roberts, G. (2023). Definitions of non-response and response to biological therapy for severe asthma: a systematic review. ERJ Open Research, 9(3), 444–2022. https://doi.org/10.1183/23120541.00444-2022

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Contents

List of abbreviations

AA	Arachidonic acid
AAB-17G	5-Alpha-Androstane-3α,17β-diol 17β-D-glucuronide
ABB-3G	5β-Androstane-3α,17β-diol-3α-glucuronide
ACT	Asthma control test
ACQ	Asthma control questionnaire
AHR	Airway hyperreactivity
AQLQ	Asthma quality of life questionnaire
ATS	American Thoracic Socety
BAB-3G	5β-Androstane-3α,17β-diol-3α-glucuronide
BAB-17G	5β-Androstane-3α,17β-diol 17β-D-glucuronide
B-eos	Blood eosinophil counts
BIOAIR	BIOmarkers in Severe Chronic AIRway Disease
BIOCROSS	BIOmarkers in CROSS-sectional study
BIOCTEROID	Biology of steroids
BMI	Body Mass Index
CLA	Clinical Lung and Allergy research unit
COM	Core Outcome Measures
COMSA	Core Outcome Measures sets for paediatric and adult Severe Asthma
COPD	Chronic obstructive lung disease
COX	Cyclooxygenase
CRC	Clinical Research Collaboration
CT	Computer tomography
CXCL	Chemokine ligand
CysLT	Cysteinyl leukotriene
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulphated
DHT-G	Dihydrotestosterone-glucuronide
DCs	Dendritic cells
EAAS	Endogenous androgenic anabolic steroid

EDN	Eosinophil derived neurotoxin
EFPIA	European Federation of Pharmaceutical and Industries and Associations
EGPA	Eosinophilic granulomatous with polyangiitis
ERS	European Respiratory Society
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
F_ENO	Fractional exhaled nitric oxide
G	Glucuronide (excreted metabolite of endogenous steroid)
GINA	Global Initiative for Asthma
HADS	Hospital anxiety and depression scale
HC	Healthy controls
HPA	Human Protein Atlas
HPA-axis	hypothalamus-pituitary-adrenal axis
HPG-axis	hypothalamic-pituitary-gonadal axis
HRT	Hormone replacement therapy
KI	Karolinska Institutet
KUH	Karolinska University Hospital
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IMI	Innovative Medicines Initiative
iNOS	Inducible NO synthase
LABA	Long-acting β-agonists
LAMA	Long-acting muscarinic antagonists
LOD	Limit of detection
LOX	Lipoxygenase
LT	Leukotriene
M-COMSA	Modified Core Outcome Measures sets for paediatric and adult Severe Asthma

MMA	Mild-to-moderate asthma
MMP-3	Matrix Metalloproteinase 3
NICE	National Institute of Clinical Excellence
NIH	National Institutes of Health
NORDSTAR	A pan-Nordic multi-party research collaboration platform
NSAN	Nordic Severe Asthma Network
OCS	Oral corticosteroids
PEFR	Peak expiratory flow rate
PG	Prostaglandin
PROs	Patient-reported outcomes
RASP	Severe Asthma Research Programme
RCTs	Randomized controlled trials
SA	Severe asthma
SABAs	Short-acting β-agonists
SAMAs	Short-acting muscarinic antagonists
SHARP	Severe Heterogeneous Asthma Research collaboration, Patient-centered
TLSP	Thymic stromal lymphopoetin
TNF	Tumour necrosis factor
3TR	Taxonomy, Treatment, Target and Remission
TXs	Thromboxanes
U-BIOPRED	Unbiased Biomarkers for the Predictions of Respiratory Disease outcomes
UHPLC-HRMS	Ultra High-Performance Liquid Chromatography-High Resolution Mass Spectrometry
WADA	World Anti-Doping Agency

1 INTRODUCTION

Asthma is one of the most common chronic inflammatory airway disorders and affects between 300 and 400 million people worldwide (Reddel et al., 2022). The global prevalence of asthma symptoms in adults is 3-10% and varies between different countries (Porsbjerg et al., 2023). Prevalence has risen over the past few decades and is highest in developed countries. However, it is presumably underestimated in low and middle-income countries due to difficulties regarding access to healthcare, diagnosis, and appropriate treatment (Cruz et al., 2017; Vos et al., 2012). In Sweden, asthma affects 8-12% of the adult population and represents a significant cost to society, justifying its designation as public health disease (Jansson et al., 2007; Lundback et al., 2016).

Patients with asthma have symptoms that are among the top five reasons for primary care visits among both adults and children. The most common asthma symptoms are shortness of breath, cough, and increased sputum. These symptoms are nonspecific and can occur in many other respiratory diseases including a chronic obstructive lung disease (COPD), interstitial lung disease, pulmonary fibrosis, bronchiectasis, and respiratory tract infections. However, there is currently no single test that can quickly and reliably provide a correct asthma diagnosis.

Many patients have a typical variant of asthma, where the historical occurrence of an asthma attack in combination with a positive reversibility test provides a good basis for an asthma diagnosis. Such patients are often well-controlled on the available inhaled therapies. However, other patients do not have typical symptoms and respond less well to treatment because asthma is a heterogeneous disease characterized by several clinical phenotypes and molecular endotypes (Wenzel, 2012). Although patients may have similar symptoms and the same diagnosis of asthma, treatment must be individualized because targeted treatments can have remarkable effects in specific preselected subgroups of patients but may be worthless, and side-effect prone in others.

If the diagnosis of asthma is difficult to determine, then the diagnosis of severe asthma is even more of a challenge and can feel like an impossible task.

Some patients with severe asthma develop their condition as the result of a long disease duration. These patients have poor asthma control and poor quality of life, and their spirometry curves have a COPD-like picture with non-reversible airflow limitation. Others have late-onset asthma, developing as they get older alongside one or more other comorbidities, which can mask the asthma and make diagnostics complicated. This type av asthma is usually already more difficult from the beginning. These patients experience worsening events (exacerbations) despite taking a combination of several different asthma medications at maximal doses. Moreover, they have also often had different periods of symptomatic treatment, often without objective effects. They have often met several different doctors, conducted a multitude of examinations that include lung function measurements, x-rays, computed tomography, exclusion of alternative diseases, yet often without achieving a correct diagnosis.

Taken together, there is a great need for better characterization and classification of patients with severe asthma, which can provide better understanding of clinical sub-phenotypes of these patients and in combination with new predictive biomarkers can be used to explore phenotype-differences with a view to improve therapy and predict response.

This thesis includes three clinically important studies that investigate certain aspects mentioned above and which can increase understanding of when and how to adjust the treatment. The first study explores differences between different registries in Europe and suggests that harmonization of these may increase the understanding of clinical phenotypes, and that long-term follow-up of these patients can improve understanding of response to treatment, which can consequently lead to improved care for severe asthmatics across Europe (**Paper I**). In addition, this thesis includes a mechanistic analysis of asthma with different severity and how these are affected by treatment (**Paper II**) and a study of response to therapy and how assessment of response can be improved (**Paper III).**

2 BACKGROUND

2.1 ASTHMA

The first attempt to distinguish asthma from other conditions described by the Greek term "shortness of breath" was made by Henry Hyde Salter as early as 1860 in his dissertation "On Asthma: Its Pathology and Treatment"(Salter, 1869). He carefully separated asthma from other obstructive diseases of the airways by identifying the contraction of smooth muscle as the main cause of airway obstruction" (Cockcroft et al., 1977). More recently, experimental studies of asthma have been able to demonstrate the contractility of smooth muscle and airway obstruction. Moreover, reversible airflow obstruction is known to be one of the pathognomonic signs for an asthma diagnosis. This can be demonstrated by worsening airflow obstruction in the setting of an airway provocation, or improved airflow after bronchodilator administration or anti-inflammatory treatment.

Although asthma as a disease has been known for almost 200 years, and much research has been carried out in the field, we still have an incomplete understanding of why some develop asthma, and some do not, and why asthma does not develop in the same way in different individuals. However, cumulative environmental exposures including allergens, cigarette smoke, respiratory tract infections, and air pollution have all been identified as underlying causes leading to progressive disease. Differences regarding the effects of genes and the environment result in a broad spectrum of heterogeneity and asthma severity (Carr & Bleecker, 2016). The heterogeneity is present not only in the clinical expressions of the disease (phenotypes) with various symptoms, but also in the underlying molecular mechanisms (endotypes). In-depth studies of phenotype and endotype and their interaction are important from a clinical perspective as a basis for understanding mechanisms and choosing the right treatment.

Recurrent asthma symptoms are cough, shortness of breath, chest tightness, and wheezing reflecting episodes of reversible airflow obstruction, which may diminish or disappear spontaneously or with treatment. In some patients, the symptoms may however become persistent over time and lead to chronic progressive airflow limitation with potentially irreversible changes in lung structure and function. Asthma is most common during childhood but can affect people of all ages. Although some children may "grow out" of asthma and become healthy after adolescence, for some patients this disease may be a lifelong burden (To et al., 2012). Some patients get the disease later in life, in studies this is often called late-onset asthma with disease inception over the age of forty. This type of asthma is usually more difficult already from the beginning (Hekking et al., 2014).

2.1.1 ASTHMA PATHOBIOLOGY

Asthma is characterized by chronic airway inflammation in which many cells of the innate and adaptive immune systems act together with epithelial cells to cause airway hyperreactivity (AHR) to various triggers, mucus overproduction, airway wall remodelling and, above all,

airway narrowing due to bronchial smooth muscle constriction (Lambrecht & Hammad, 2015). Asthma is classically described as being allergic, with elevated T-helper cells of type 2. These cells release type 2 cytokines (interleukin-4 (IL-4), IL-5, and IL-13) and recruit eosinophils to the site of inflammation. This type of inflammation is called type 2 inflammation.

Transcriptomic profiles of bronchoscopic samples from patients with severe asthma have identified profiles of molecular phenotypes consistent with high type 2 immunity and low type 2 immunity asthma, as well as other patterns (Kuo et al., 2017). Eosinophilic, type 2 high airway inflammation occurs in about 50% of adults with asthma (Chung & Adcock, 2015). Allergy is present in most children with asthma (Akar-Ghibril et al., 2020) and it is the most common cause of severe asthma in childhood (Del Giacco et al., 2017). Allergic sensitization is present in about 50% of adults (Papi et al., 2017), but the majority of adults with severe asthma have late-onset disease and non-allergic eosinophilic inflammation is common cause of severe asthma in adult.

The pathogenesis of eosinophilic allergic asthma begins with sensitization to various allergens and stimulation of dendritic cells (DCs) in the presence of epithelial derived thymic stromal lymphopoetin (TSLP) (Figure 1 below). The DCs interact with naïve T-cells, which switch to adaptive T helper 2 cells and begin to produce IL-5, IL-4, and IL-13. Interleukin-5 recruits and activates eosinophils. Recruitment of eosinophils to the lung mucosa is mediated via C-C motif chemokine receptor 3 which is activated by eotaxin and other eosinophil chemoattractants. Mast cell-derived prostaglandin D_2 (PGD_2) may also contribute to eosinophil chemotaxis via the DP2/CRTH2 receptor. Interleukin-4 drives B-cell isotype switching and leads to Immunoglobulin E (IgE) synthesis. Specific IgE then binds to high-affinity IgE receptors on mast cells, leading to activation following allergen-mediated IgE cross-linking (Papi et al., 2018). Serum IgE is established as a biomarker for allergic, eosinophilic asthma.

The pathogenesis of non-allergic eosinophilic asthma is initiated following epithelial damage caused by viruses, microbes, smoke, and other pollutants, which release epithelium-derived alarmins IL-33, IL-25, and TSLP. Innate lymphoid type 2 cells then produce several different interleukins (IL-5, IL-13, IL-9, and IL-4) in response to these epithelial mediators, as well as activation by PGD_2, leading to eosinophil recruitment and activation in the lung (Mjösberg & Spits, 2016). This results in mucus hypersecretion, smooth muscle cell hyperreactivity, and airway remodelling with chronic eosinophilic inflammation as shown in **Figure 1**.

Eosinophils play a central role in type 2 inflammation and are used as defining biomarkers of this endotype when measured in blood, sputum, and tissues. Other biomarkers of type 2 asthma, which may be upregulated by the effects of IL-4 and IL-13 on epithelial cells include fractional exhaled nitric oxide (F_ENO) (Kuo et al., 2019) and periostin (Emson et al., 2018; Takayama et al., 2006). There is evidence that in type 2 asthma, IL-13 induces iNOS (inducible nitric oxide synthetase) expression, primarily through transcription in primary human airway epithelial cells, which results in an increase of F_ENO (**Figure1**) (Chibana et al., 2008). Periostin is an extracellular protein, which is expressed in multiple organs and plays a wide variety of roles in tissue development, for example, it influences extracellular matrix restructuring and tissue

remodelling. Elevated serum periostin levels are associated with type 2 asthma as evidenced by increased periostin expression in the airway epithelium of patients with eosinophilic asthma as well as another type 2 disease, chronic rhinosinusitis with nasal polyps (Ninomiya et al., 2018; Yilmaz et al., 2022), both of which are dependent on IL-4 and IL-13 signalling (Takayama et al., 2006).

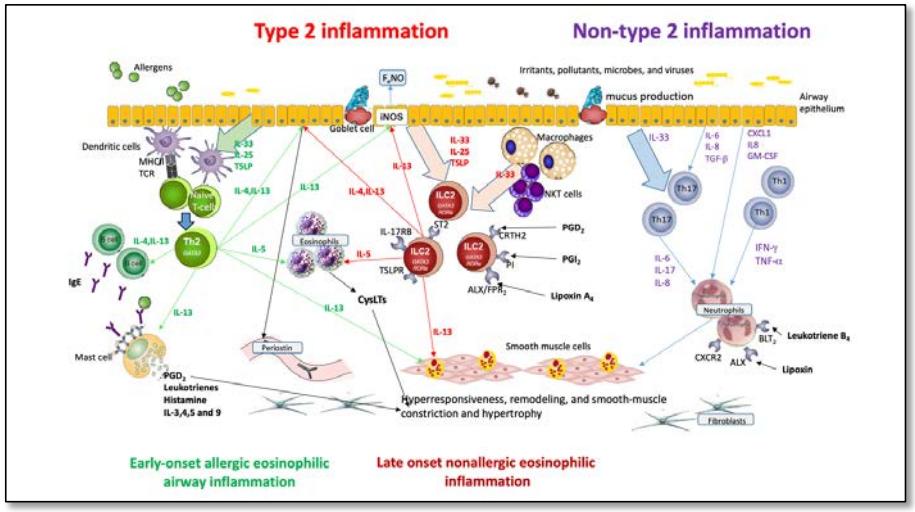


Figure 1 (modified from Bel E., ERS Open research 2015): Immunologic mechanisms and characteristic pathological features of asthma. IL=interleukin, ILC2=type 2 innate lymphoid cell, Th=T helper, TCR=T cell receptor, MHC-epitope speciphic T cells receptor on dendritic cell, CXCL=C-X-C motif chemokine ligand, TSLP=thymic stromal lymphopoetin, PGD_2=prostaglandin D_2, TGF= transforming growth factor, GM-CSF=granulocyte-macrophage colony-stimulating factor, INF=interferon, TNF= tumour necrosis factor, F_ENO=fractional exhaled nitric oxide, iNOS=inducible nitric oxide synthetase.

In recent decades, advances in the understanding of the pathophysiology of asthma have been made, and several different underlying molecular pathways (endotypes) have been proposed (Chung & Adcock, 2015). Sputum cytology provides evidence of eosinophilic, neutrophilic, and mixed complex inflammation, as well as an absence of inflammatory cells in certain patients (paucigranulocytic) (Papi et al., 2018; Simpson et al., 2006). Non-eosinophilic asthma occurs in adults and children but is poorly understood (Del Giacco et al., 2017; Green, Brightling, Woltmann, et al., 2002). A small proportion of patients in this group have a neutrophil-predominance according to induced sputum and this is linked to neutrophilic activation reflected by increased levels of IL-8 and IL-1β (Simpson et al., 2007). As shown in Figure 1, another possible pathway leading to neutrophilic inflammation is the induction of chemoattractants such as IL-8, leukotriene (LT) B_4, and CXCL-1 through the interaction of the airway epithelium with viruses, microbes, pollutants or allergens (Thomson, 2016) (Siddiqui et al., 2008). It is suggested that the immunological mechanisms that drive eosinophilic and non-eosinophilic inflammation in asthma may occur together, which can lead to mixed granulocytic inflammation, or by inflammatory mechanisms that merge and change inflammatory profile over time.

One of the most common features of asthma is AHR which occurs in patients of all ages and sometimes even without granulocytic inflammation in the airways. Airway hyperresponsiveness is defined as the predisposition of the airways to narrow excessively in response to stimuli that would produce little or no effect in healthy subjects (Cockcroft et al., 1977). This feature can be examined as the degree of airway obstruction and can be tested by measuring lung function by spirometry during bronchial challenges with bronchocontricting agents, usually methacholine (Joos & O'Connor, 2003). Airway hyperresponsiveness has long been considered a main feature of asthma and research into the mechanisms involved using various bronchoprovocation models have provided profound insights into the underlying pathophysiology of the disease.

Obstruction of the airway in asthma is due to smooth muscle hypercontractility that is most often associated with mast cell activation regardless of inflammation (Brightling et al., 2003). Chronic obstructivity can also lead to tissue remodelling, which is characterized by several structural changes, e.g. epithelial damage and ciliary dysfunction, goblet cell hyperplasia, increased thickness of the lamina reticularis and reticular basement membrane, increased vascularization and raised number of subepithelial myofibroblasts, fibroblasts and increased smooth muscle mass (Siddiqui et al., 2008). These processes lead to the thickening of the airway wall and reduction of the bronchial lumen, as well as increased mucus plugs, and can be seen upon computer tomography (CT)-chest examination (Hartley et al., 2016).

2.1.2 CLINICAL PHENOTYPES

The clinical phenotypes of asthma are defined by distinct clinical features. Common asthma symptoms must however be related to specific timings, trigger factors and response to treatment. Therefore, accurate history recording is important to assess the likelihood of respiratory symptoms being due to asthma rather than differential diagnoses or comorbidities. Sometimes the underlying immunological mechanisms can be linked to a particular asthma phenotype already at the time of the first clinical presentation, but usually some detective work is required to distinguish different types of asthma.

Table 1. Clinical phenotypes of asthma

Clinical phenotypes	Natural history	Clinical and physiological features	Pathobiology and proposed biomarkers
Early-onset allergic	Early onset; mild to severe	Allergic symptoms and other allergic diseases	Specific IgE; Type 2 cytokines; CD-sens
Late-onset eosinophilic	Adult onset; often sever	Sinusitis; nasal polyposis; less allergic; sometimes AERD	Eosinophilia and Type 2 cytokines
Obesity-related	Adult onset	Women are primarily affected; very symptomatic; AHR less clear	Lack of Type 2 biomarkers; oxidative stress
Neutrophilic		Low FEV1; more air trapping	Sputum neutrophilia T_H17 pathways; IL-8

In some patients, asthma is associated with severe bronchospasm after taking aspirin or other non-steroidal anti-inflammatory drugs. Aspirin exacerbated respiratory disease is more common in severe asthma (15% in people with severe asthma vs 7% in the general population)

and is usually associated with rhinitis and nasal polyposis (Rajan et al., 2015). This phenotype of severe asthma is considered a very distinct asthma entity, but the cause remains elusive (Langdon & Mullol, 2016). **Table 1** presents the most common clinical variants of asthma phenotypes, with links to underlying immunological mechanisms, trigger factors, and historical data.

2.2 CURRENT MANAGEMENT

The overall goal of asthma treatment is to achieve good control which means a reduction in symptoms and risk of exacerbation. Control of asthma includes a normal level of day-to-day activity, including exercise capacity (Carr & Bleecker, 2016; Papi et al., 2018). Therefore, asthma management must be personalized and multifaceted, composed of both pharmacological and non-pharmacological treatments, as well as the treatment of different comorbidities. The non-pharmacological approach includes education in self-management, a written asthma action plan, inhaler training, avoidance of tobacco exposure, weight loss, improving adherence, and removing relevant exposures, as well as physiotherapy and physical training.

The guidelines for pharmacological treatment involve a stepwise approach related to the severity of the disease (**Figure 2**), with evidence summarised by the Global Initiative for Asthma (GINA)-recommendations (Reddel et al., 2022). Anti-inflammatory and bronchodilator treatment is the mainstay of asthma therapy. In the stepwise approach, the use of controller medications is recommended, particularly inhaled corticosteroids (ICS), then titrating doses or adding additional therapies in a step-by-step fashion as required, to achieve an adequate level of symptom control (Papi et al., 2018).

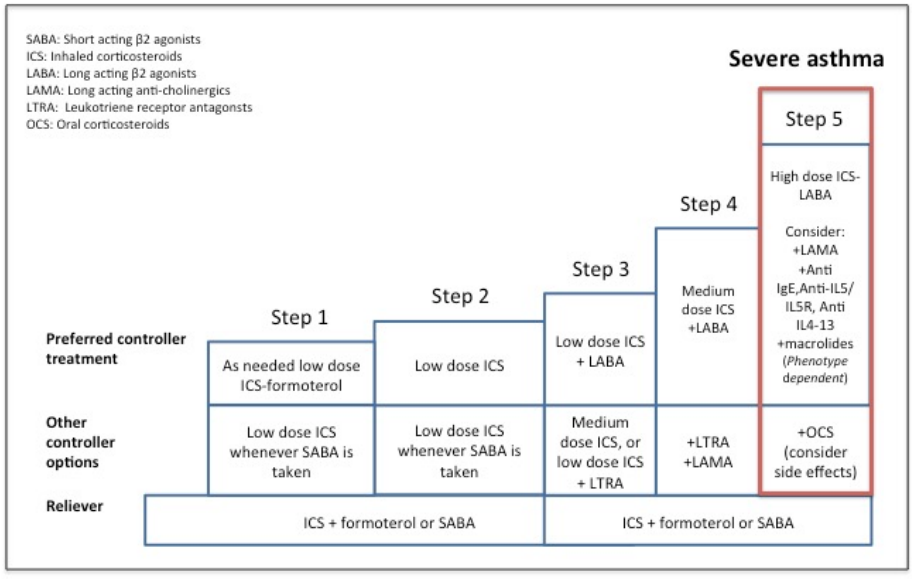


Figure 2. Treatment steps according to GINA.
(Modified from pocket-guide 2020, the Nordic severe asthma Network (NSAN)).

Add-on therapies including long-acting muscarinic antagonists and leukotriene receptor antagonists should be considered before systemic corticosteroids. Patients with severe asthma that is persistently uncontrolled (low lung function, poor symptom control or frequent exacerbations) on GINA step 5 treatments (high dose ICS + second controller) despite good adherence, correct inhaler therapy and attempts to optimise therapy with different inhalers and add-on therapies should be referred to a severe asthma centre for investigation of underlying phenotype. A decision can then be made regarding whether the patient is suitable for treatment with modern and expensive biologics (annual cost is between 110-280, 000 SEK per patient).

2.3 SEVERE ASTHMA

Severe asthma is defined according to the European Respiratory Society (ERS) and the American Thoracic Society (ATS) guidelines, as asthma that requires treatment with guideline-suggested medications (GINA, step 4) such as high dose ICS and a second controller during the previous year, and/or systemic corticosteroids for at least half of the previous year, to achieve control, or asthma which remains uncontrolled despite this therapy (Chung et al., 2014) (Holguin et al., 2020). Uncontrolled asthma is defined as the presence of at least one of the following characteristics: persistently poor symptom control, two or more exacerbations requiring bursts of systemic corticosteroids during the preceding year, at least one serious exacerbation requiring hospitalization in the previous year, or chronic airflow limitation as reflected by an FEV_1 (forced expiratory volume in one second) of < 80% predicted, with an FEV_1/FVC (forced vital capacity) ratio below the lower limit of normal (Chung et al., 2018)., Under-diagnosed or under-treated comorbidities are also know to affect the quality of life and asthma control (Boulet & Boulay, 2011).

In order to differentiate and exclude patients who have poor asthma control due to external factors, such as poor adherence or untreated comorbidities (difficult-to treat asthma), a systematic assessment should be performed (**Figure 3**). The prevalence of severe asthma has been estimated to be up to 4-12% of all asthma patients (Backer et al., 2020; Hekking et al., 2015; Larsson et al., 2018). The large variation in reported prevalence from different studies depends on the population studied as well as the type of calculation and reporting used, therefore the exact prevalence is difficult to conclude. According to one Swedish study, it has been shown that only one in five patients with severe asthma visited a specialist in secondary care, and the attendance rate to doctors in primary care was also low (Larsson et al., 2018). Therefore, the prevalence could be underestimated. However, it could also be overestimated if all patients with difficult-to-treat asthma are included before a systematic assessment, or if studies are based on prescribed medications (To et al., 2012).

In one recently published study from NORDSTAR (a pan-Nordic multi-party research collaboration platform) that is governed by the Nordic Severe Asthma Network (NSAN), 598 242 patients with current asthma were identified in Sweden (n=246 057), Norway (n=156 001) and Finland (n=196 184) 2018 (Hansen et al., 2023). The prevalence of severe asthma in these

countries was 3.5%, 5.4% and 5.2% respectively in adults, and 0.4%, 1.0% and 0.3% respectively in children. Most patients with severe asthma are still managed in primary care and only 37% of adult patients with severe asthma in Sweden and 40% in Finland, that have more than 2 exacerbations per year were treated in specialist care. The Clinical Lung and Allergy research unit (CLA) at Karolinska Institutet (KI), the Department of Medicine, Huddinge and the Department of Respiratory Medicine and Allergy at Karolinska University Hospital (KUH) is a member of the NSAN, and I am one of the co-authors of this publication.

Although the number of patients with severe asthma represents a relatively small proportion of the asthma population, their societal costs account for an unproportionally large share (> 50 %) (Canonica et al., 2020) of the total costs attributed to asthma.

Knowledge gap

There is a great need for increased knowledge regarding the prevalence, characteristics and treatment of patients with severe asthma across Europe.

Due to a lack of knowledge regarding the prevalence, characteristics and treatment of severe asthmatics across Europe, as well as the need for a more comprehensive and integrated partnership and shared expertise, improved coordination and agreement on prioritization of research, the ERS funded the Clinical Research Collaboration (CRC) named SHARP (Severe Heterogeneous Asthma Research collaboration, Patient-centered) in 2018 (Djukanovic et al., 2018). The main goal of this collaboration is to ensure the best possible care for all patients in Europe and to enable patient-related research. SHARP now covers 28 countries and Sweden is one of them. Immediately after the establishment of SHARP, the first study began with the aim to harmonize severe asthma management throughout Europe. The CLA research unit at KI, the Department of Medicine, Huddinge and the Department of Respiratory Medicine and Allergy at Karolinska University Hospital was a part of this first study, and the results of this survey are reported in my first publication.

A new paper describing the characteristics of severe asthma patients on biologics from the SHARP central register was published during last year (Principe et al., 2023). Results showed that there are differences among patients receiving biologics as part of routine clinical asthma care and those participating in phase III randomized controlled trials (RCTs) across Europe. In real-life, the population benefiting from these medications is more diverse and much broader.

2.3.1 SYSTEMATIC ASSESSMENT OF SEVERE ASTHMA

The systematic assessment of patients with severe asthma has proven to be effective in reducing the number of exacerbations, as well as the general utilization of healthcare resources (Chung, 2018). This process includes three steps, see **Figure 3**:

1. Confirming the asthma diagnosis, assessing asthma control, assessing clinical phenotype,
2. Reassessing potential treatment barriers like a poor inhalation technique and/or adherence,
3. Identification and removal of potential exposures whenever possible, assessing comorbidities.

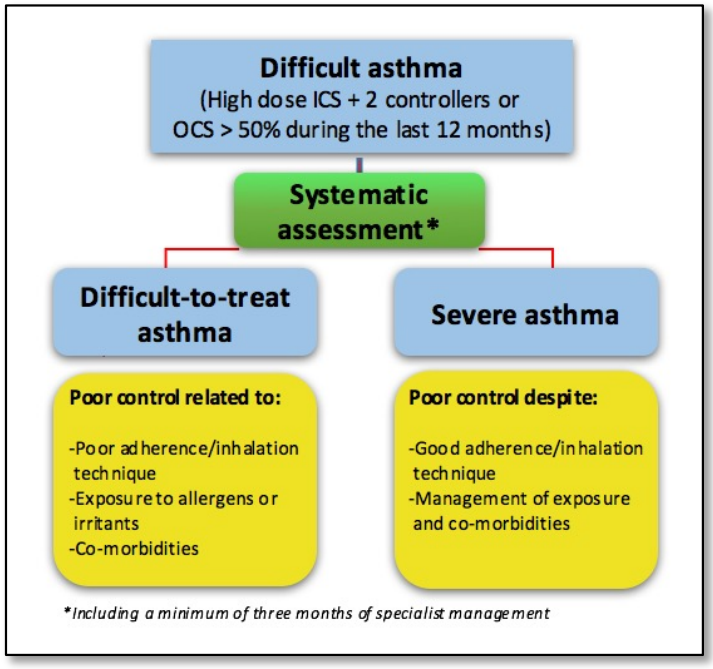


Figure 3. Diffentiation of difficult-to-treat versus severe asthma.
(Modified from pocket-guide 2020, the Nordic severe asthma Network (NSAN)).

Individuals with suspected severe asthma should undergo a thorough, systematic assessment to confirm their diagnosis. The investigation requires a detailed history of the occurrence of asthma symptoms in combination with objective confirmation of variable airflow limitation. This can be shown by worsening airflow obstruction in the setting of airway provocation or in association with a down-titration of asthma medication. A reversibility test with documented improved airflow after bronchodilator administration, or improvement after a course of oral steroids are other objective criteria.

2.3.2 STEROID EFFECTS IN SEVERE ASTHMA

As mentioned, inhaled corticosteroids (ICS) are currently the mainstay of asthma treatment. There is evidence that not only patients with moderate to severe disease, but also patients with mild asthma benefit from this treatment (O'Byrne et al., 2018), (Lazarinis et al., 2014). After the 2019 update of the GINA guidelines, low dose ICS treatment is now recommended as an "as-needed" controller therapy for mild asthma as early as step 1 of the asthma therapy ladder. According to GINA (Reddel et al., 2022), the dose of ICS can be increased if necessary. Patients with severe asthma may need high ICS-doses but may also still have uncontrolled asthma and need systemic corticosteroid treatment, if blood and/or sputum eosinophils are elevated despite high-dose ICS (Aleman et al., 2016). However, there are no randomized controlled trials of oral corticosteroid (OCS) use for the maintenance treatment of severe asthma, although the adverse effects of these drugs are well documented (Papi et al., 2018) (Lefebvre et al., 2015). Two small, randomized trials of intramuscular depot triamcinolone in adults with severe asthma (ten Brinke et al., 2004), (Mancinelli et al., 1997) have shown fewer exacerbations and thus fewer hospitalizations and emergency department visits, as well as increased lung function and decreased eosinophilic inflammation. Treatment with inhaled and oral corticosteroid doses tailored specifically to control sputum eosinophilia in asthma led to reductions in exacerbation rates (Green, Brightling, McKenna, et al., 2002) and this regimen is now included in the guidelines for adults with severe asthma (Reddel et al., 2022).

The largest challenge with OCS use is their side effects. Some are life-long and serious, such as secondary adrenal insufficiency, osteoporosis, corticosteroid-related diabetes, uncontrolled blood sugar level in diabetics, hypertension, glaucoma, and skin atrophy. The risk of side effects is however dose dependent. Because patients with severe asthma need high doses of ICS, frequent courses of OCS are often required as maintenance chronic OCS therapy, which run the highest risk of side effects. Biomarker-guided OCS therapy is an attractive option because of the need to consider the risks and benefits of corticosteroid therapy in severe illness (Papi et al., 2018).

It is known that the prevalence of asthma differs between men and women. Asthma is more prevalent and severe in young boys but after puberty, asthma is more severe and prevalent in women (Hekking et al., 2015), (Leynaert et al., 2012). The majority of these women have poor asthma control despite high doses of ICS and OCS. The influence of female sex hormones in sex-related asthma phenotypes have been discussed. However, the role of sex hormones in severe asthma and its underlying immunopathology are not clear and clinical studies are few.

Knowledge gap

What is the role of sex hormones in severe asthma?

In order to study sex differences in endogenous steroid hormones and their association with asthma severity and treatment with glucocorticoids, urine samples were collected and analysed in the U-BIOPRED (Unbiased Biomarkers for the Predictions of Respiratory Disease) study (Dominick E. Shaw et al., 2015). Interpretation of this analysis was included in one of my sub-studies, the BIOSTEROID project. The results of this study were recently published in ERJ Open Research (Yasinska et al., 2023).

2.4 CLINICAL PHENOTYPING OF OTHER RESPIRATORY DISEASES AND WHY THEY MATTER

Asthma symptoms such as shortness of breath, cough, and increased sputum are nonspecific and can be caused by other respiratory diseases, including COPD, interstitial lung disease, pulmonary fibrosis, bronchiectasis, and respiratory infections. In some systemic immune-related diseases such as hypereosinophilic syndrome, eosinophilic granulomatous with polyangiitis (EGPA), immunoglobulin G (IgG) 4-associated disease, the lung is one of the target organs, and thus part of a complex pathobiology (Janson et al., 2022). Furthermore, during the past decade, it has become increasingly evident that several other diseases, including COPD (Kostikas et al., 2018) and sarcoidosis (Heinle & Chang, 2014), are also heterogeneous in nature, similar to asthma and different phenotypes with underlying pathophysiological characteristics are also described. Adult, non-cystic fibrosis (non-CF) bronchiectasis is a further example of a complicating factor in asthma diagnostics, especially for the non-eosinophilic phenotype of asthma. The similarities in symptoms associated with different respiratory diseases and the need to investigate underlying differences in biomarkers was the reason why we started a clinical academic study, BIOCROSS (BIOmarkers in CROSS-sectional study), which forms the largest part of my research project. This study includes patients with severe asthma as the main group but comparisons with patients with other common diseases such as COPD, bronchiectasis, sarcoidosis and interstitial lung disease are secondary aims.

2.5 BIOLOGICS – THE NEXT STEP TOWARDS PERSONALIZED TREATMENT

Distinct clinical clusters or phenotypes, consisting of several inflammatory phenotypes each with different underlying molecular pathways [endotypes] are thought to explain different treatment responses. Therefore, the personalized medicine strategy aims to find therapeutic interventions that target underlying disease mechanisms more precisely (Holgate et al., 2019). This concept becomes increasingly important in severe asthma, where optimizing the balance between safety, efficacy, and cost for each therapeutic option are important considerations (Papi et al., 2018). The goal is to treat the right patients with the right intervention at the right time. New biological drugs for the treatment of severe asthma provide opportunities for individually tailored treatments and have a corticosteroid-sparing effect causing reductions in severe exacerbations without substantial adverse effects. There are already five approved therapies targeting type 2-mediated immunity (Papi et al., 2018), (Andrew Menzies-Gow et al.,

2020) (**Table 2**). Biological drugs such as anti-IL-5 treatments and anti-IL-13/anti-IL-4 are already used in secondary care for patients with typical clinical phenotypes supported by specific clinical biomarkers (increased blood eosinophils and high F_ENO levels) (**Table 2**).

Table 2. Biological drugs available in Sweden

Biological drugs targeting type 2 inflammation		
Biologic	Target	Effect of therapy (main response)
Omalizumab	Free IgE	Reduce exacerbation and OCS-use, reduce allergic symptoms
Dupilumab	IL-4Rα	Reduce exacerbations, improve FEV_1, reduce allergic symptoms, reduce mucus plugs
Mepolizumab	IL-5	Reduce exacerbations and OCS-use, improvement in FEV_1
Reslizumab	IL-5	Reduce exacerbations and improve FEV_1
Benralizumab	IL-5Rα	Reduce exacerbations and OCS-use, improve FEV_1
Biological drugs targeting type 2 inflammation with broader effects		
Tezepelumab	TSLP	Reduce exacerbations, reduce AHR, reduce mucus plugs

Other targets against type 2-mediated inflammation, such as TSLP (Corren, 2019; Gauvreau et al., 2014), have broader effects and is introduced in Sweden during this year. Even though patients in this group are clinically well-characterized, there are however responders and non-responders.

Knowledge gap

How can we improve assessment of response to biologics?

Previous studies targeting non-type 2 asthma were not considered to be clinically useful, for example anti-TNF (Tumour necrosis factor)-α showed little benefit compared to its side effects (Wenzel et al., 2009) and anti-CXC motif chemokine receptor 2 was without beneficial effects (O'Byrne et al., 2016). Results from other programs targeting IL-17 and IL-23 have also been negative (Busse et al., 2013), (Brightling et al., 2021). However, there is also a need to identify the most appropriate patients for these treatments and to better understand how to measure response to treatment (Brightling, 2017). Moreover, biologics are very expensive, requiring structured implementation and predictive biomarkers.

The analysis of data obtained from different registries regarding the effects of treatment with biological therapy have generated important results that have been published. Results from the nationwide Danish Severe Asthma Register have shown that there were no non-responders to anti-IL-5 treatment after 12 months treatment (Soendergaard et al., 2022). All patients were

distributed in 2 groups of response: Complete responders and non-complete responders. The assessment was made in accordance with two criteria: exacerbation rate and OCS use.

During 2020, an international European project was started entitled 3TR (Taxonomy, Treatment, Target and Remission) that was financed by the IMI (Innovative Medicines Initiative) and also supported by the European Union Horizon 2020 research and innovation programme and EFPIA (European Federation of Pharmaceutical and Industries and Associations). This project aims to provide fundamental new insights into the molecular mechanisms of response and non-response to treatment, relapses and remission in autoimmune, inflammatory, and allergic conditions. The COMSA (Core Outcome Measures sets for paediatric and adult Severe Asthma) Working Group is a part of the 3TR project aimed to develop Core Outcome Measures (COM) sets to facilitate better synthesis of data and appraisal of biologics in paediatric and adult asthma clinical studies. The result of collaboration within this working group includes a systemic review with definitions of non-response and response to biological therapy and development of the COMSA set for assessment of response (Ekaterina Khaleva et al., 2023), (E. Khaleva, A. Rattu, C. Brightling, A. Bush, A. Bourdin, et al., 2023). Our research group is a part of the 3TR project, and I have actively participated in the COMSA working group and am co-author of several publications. Paper III is the first attempt to test the COMSA set in a real-life clinical study.

2.6 CLINICALLY APPLICABLE BIOMARKERS, OVERVIEW

2.6.1 DEFINITION OF BIOMARKER

A biomarker may be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (National Institutes of Health (NIH) definition) (Atkinson et al., 2001). However, a biomarker needs to be qualified in terms of its use, i.e. for diagnostic purposes, for choice of treatment and evaluation of treatment response, for monitoring of disease progression and prognosis, or for early detection of exacerbations. In order to qualify for clinical use, new biomarker candidates should be better or easier to collect and analyse than already existing options, more cost effective and the analysis technology should be easy to implement in a clinical laboratory (Amur et al., 2015), (Diamant et al., 2019), (Hollander et al., 2017).

2.6.2 CURRENT CLINICALLY USED BIOMARKERS

The most useful and clinically available biomarkers in asthma are blood eosinophil counts (B-eos) and total/specific IgE in peripheral blood, and F_ENO (Andrew Menzies-Gow et al., 2020). All of these relate to type 2 inflammation. Peripheral blood can easily be obtained for the measurement of eosinophils, and blood eosinophils have been shown to correlate well with eosinophil counts in sputum (Diamant et al., 2019). However, the relationship between eosinophils in blood and eosinophils in lung tissue is less clear (Ullmann et al., 2013).

Total and specific IgE are advantageously used for diagnostics in patients with allergic eosinophilic asthma. F_ENO is used in clinical practice, and in the majority of asthma studies for the diagnosis of airway inflammation, but it is not precise. The interpretation of F_ENO is often hindered by several confounding factors including age, smoking status, atopy, and anti-inflammatory therapy (especially corticosteroids) (Dweik et al., 2010). Combining all three markers increases specificity and sensitivity. The UK RASP (Severe Asthma Research Programme) programme has used blood eosinophils, F_ENO and periostin to increase precision (Heaney et al., 2021). However, studies such as RASP are based on populations and there is a great need for new validated biomarkers that can be used on individual patients at the point of care.

2.6.3 CANDIDATE BIOMARKERS IN RESEARCH

This is currently a great deal of effort being put into research to identify new biomarkers that may aid the diagnosis of asthma and its sub-phenotypes, as well as for the stratification and follow-up of specific targeted treatments. The candidate markers being explored range from specific genetic signatures to metabolites that either cause certain biological effects or provide evidence that specific reactions have occurred (**Figure 4**).

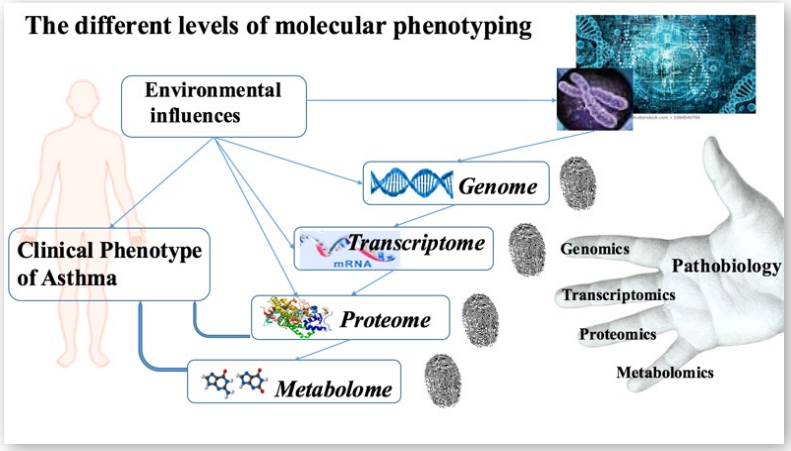


Figure 4. The different levels of molecular phenotyping

Concerning genetic determinants of asthma phenotypes, there are many complex gene-environment interactions that affect risk of asthma, allergy, and other clinical expressions of respiratory diseases (Papi et al., 2018). However, these findings remain at the research level, and we do not yet have gene signatures that aid diagnosis or stratification for treatment. The main role of genetics in asthma research so far has been to reveal new targets associated with different phenotypes. There are many basic science investigations of how transcription from genes to the synthesis of proteins is regulated in normal physiology and altered in disease. In the clinical research setting, studies aim to identify transcriptomic patterns linked to specific phenotypes. Such studies are often directed to the tissues of interest, such as airway biopsies or

isolated cells from sputum or blood. Publications reveal many interesting associations, but the field is in its infancy.

Studies of the proteome include a large number of different molecules with functions ranging from structural elements to enzymes, receptors and control of cell signalling. So far, it is mostly the latter group of proteins that have been examined as biomarkers or regulators of biomarker biosynthesis. Lipid mediators are one particular example belonging to the "cell signalling" group. Their production occurs via specific enzymes that control the release of arachidonic acid (AA) and related polyunsaturated fatty acids from the cell membrane to initiate biosynthesis of lipid-based molecules, which in turn induce potent biological actions via specific receptors throughout the airways and on inflammatory cells. The metabolome also includes small molecular metabolites of intermediary metabolism and signalling molecules such as histamine and serotonin released from mast cells and platelets, respectively. The following account will give some examples of current biomarker candidates from different pathways included in my studies.

2.6.3.1 PLASMA PROTEOMICS

Using the Human Protein Atlas (HPA) resource of the Science for Life Laboratory in Sweden, an antibody based, proteomic bead-array has been developed, with a panel comprising 377 antibodies directed against 177 proteins potentially involved in airway or systemic inflammation. This panel was applied to plasma samples from U-BIOPRED and BIOAIR, two well-characterized EU cohorts including subjects with severe and mild-to-moderate asthma, chronic obstructive pulmonary disease, and healthy controls (Mikus et al.). This study confirmed associations between established markers with asthma, but also identified potential new biomarkers. From the protein profiles measured, asthmatic subjects could be grouped into six clusters that appeared to represent clinically distinct phenotypes of asthma. An updated proteomic panel will be used for analysis in the BIOCROSS study.

2.6.3.2 LIPID MEDIATORS

All immune cells participating in the pathobiology of asthma are dependent on interactions between multiple cell types. These interactions are mediated by cell-to-cell contact and soluble mediators such as proteins and lipids. Arachidonic acid is the most well-studied lipid mediator that originates from phospholipids in the cell membrane. Its downstream metabolites are known to play key roles in mediating inflammatory signals, such as pain, vasodilation/constriction, recruitment of inflammatory cells and plasma leakage (Dennis & Norris, 2015), (Johan et al., 2021). Metabolism of AA follows two major pathways, the cyclooxygenase (COX) and lipoxygenase (LOX) pathways which lead to the production of eicosanoids including prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) (Dahlén et al., 1986) (**Figure 5**).

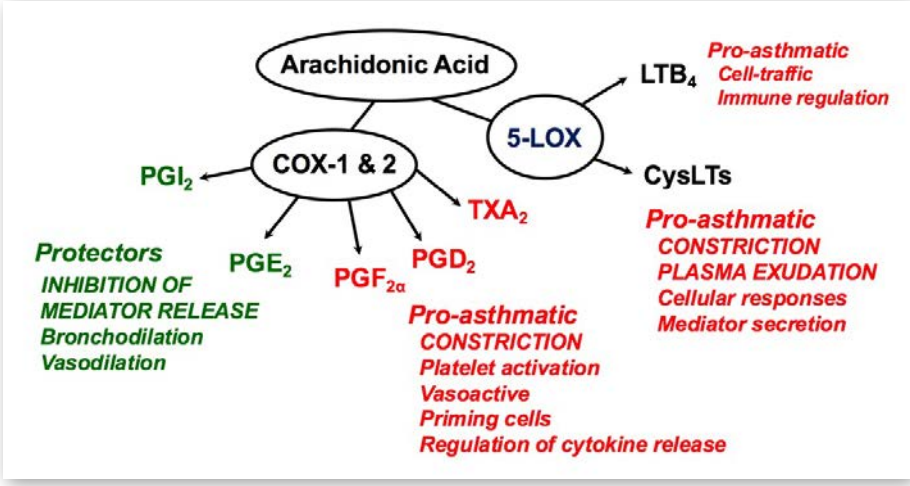


Figure 5. Two major pathways of metabolism of arachidonic acid: the cyclooxygenase (COX) and lipoxygenase (LOX) pathways. PGs - prostaglandins, TXs - thromboxanes, LTs - leukotrienes.

The lipoxygenase (LOX) pathway

Following cellular activation, five lipoxygenase activating protein (FLAP) binds AA in the cellular nuclear membrane (Miller et al., 1990). AA is transferred to the co-localised 5-LOX enzyme, which produces 5-hydroxyeixosatetraneoic acid (5-HETE) and the unstable product leukotriene A_4 (LTA_4) (Smith, 1989). In cells containing LTA_4 hydrolase (e.g. neutrophils), LTA_4 can be converted to LTB_4 and in cells containing LTC_4 synthase (e.g. mast cells and eosinophils) (Haeggström et al., 2007), LTA_4 can be converted to the first of the cysteinyl leukotriene (CysLT) family, LTC_4 (Welsch et al., 1994). Following extracellular excretion, LTC_4 is rapidly metabolised and converted to LTD_4 and then to LTE_4 by specific peptidases. Cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) are potent bronchoconstrictors (Dahlén et al., 1980) and pro-inflammatory mediators (Peters-Golden & Henderson, 2007).

PGD_2 on the other hand is the major product of the COX pathway in mast cells with bronchoconstrictive and pro-inflammatory actions (Lazarinis et al., 2015), (Kolmert et al., 2018) (Figure 1&5). The eicosanoids have a short half-life in the tissue in which they are biosynthesised, and they are rapidly removed from the circulation for filtration by the kidney and excretion into the urine. The measurement of eicosanoid metabolites in the urine reflects activation of different biosynthetic pathways and is a reliable method to assess in vivo production of primary eicosanoids (Kumlin et al., 1992), (O'Sullivan et al., 1996), (Gomez et al., 2019). In contrast, levels of these metabolites in the blood are low, fluctuating, and interpretations can be complicated by artefactual formation during sampling. In the European study, U-BIOPRED, the largest evaluation to date of multiple urinary eicosanoid metabolites in healthy and asthmatic adults was recently published (Johan et al., 2021). Levels of LTE_4 were significantly higher in all asthma groups relative to healthy participants, with the strongest difference being for LTE_4 in the severe asthma groups. PGD_2 metabolites were also elevated

in relation to asthma severity as compared to healthy individuals. There was a good correlation between urinary eicosanoids (LTE_4 and metabolites of PGD_2) and clinical biomarkers for eosinophilic type 2 inflammation, such as F_ENO, blood and sputum eosinophils, serum periostin, and IL-13. This study concluded that eicosanoids could be new, non-invasive biomarkers for molecular phenotyping of type 2 asthma (Johan et al., 2021).

In **Paper III**, which is the first report from the BIOCROSS study, eosinophilic markers were analysed during treatment with the biologic mepolizumab and compared to baseline. All 77 patients in **Paper III** had evidence of eosinophilic inflammation prior to treatment. Results showed that both blood eosinophil counts and F_ENO levels were significantly decreased after 12 months of treatment. Eosinophil derived neurotoxin (EDN), which is a marker of eosinophil activity, was also significantly decreased in both serum and urine. Furthermore, there was a numericaly reduction in urinary LTE_4 on group level, although this effect did not reach statistical significance. The urinary mast cell marker tetranor-PGDM remained unchanged during follow-up visits.

3 RESEARCH AIMS

The overall purpose of my research project was aimed at studying clinical features in patients with severe asthma and how these change during treatment with biological drugs to increase understanding of underlying mechanism for different sub-phenotypes of severe asthma. The main focus was to validate different clinical outcomes and assess their importance in analysis of response to therapy as well as for severe asthma management.

2.1 Specific aims	*Paper*
• To investigate whether there are differences or not in characteristics of severe asthmatics between current registries from the 11 different European countries that were members of SHARP collaboration 2018, also evaluate severe asthma and compare their treatment before starting biologics.	**I**
• To investigate whether there are strengths or weaknesses in the aforementioned registries across Europe.	**I**
• To study the urinary concentrations of endogenous steroids, including cortisol and androgens in patients with different severity of asthma compared to healthy controls	**II**
• To investigate how asthma treatment including both inhaled corticosteroids and oral corticosteroids affect levels of androgens and whether there are gender related differences	**II**
• To investigate if composite Core Outcome Measure (COM) including patient reported outcomes is a good algorithm to identify non-responders/responder to biologic mepolizumab.	**III**
• To assess if modified COMSA (Core Outcome Measure in Severe Asthma) can be useful in real world.	**III**

4 MATERIALS AND METHODS

4.1 ETHICAL CONSIDERATIONS

All studies in this thesis were conducted in line with the ethical principles of the Declaration of Helsinki(ref) ("Declaration of Helsinki. Ethical principles for medical research involving human subjects," 2009) and were approved by the Swedish Ethical Review board, Stockholm, Sweden (with the approval numbers 2012/1235-32 (**Paper II**) and 2017/832-31/1 (**Paper I** and **Paper III**). All subject enrolled were informed about the studies and thereafter provided their written consent to participate. To ensure the security of the study participants' data, in compliance with the General Data Protection Regulation (GDPR) legislation, all retrieved information was pseudonymized and located on a secure server, accessible to the Clinical Lung-Allergy (CLA) research Unit (**Paper III**). For **Paper II**, for analysis of clinical outcomes and biomarker results, the original data from the U-BIOPRED TranSMART data handling system was used without access to source data from different countries. For **Paper I**, that includes data from 11 countries across Europe, the Swedish data was presented in aggregate form such as counts (percentages), mean (with standard deviation) and median (with interquartile range (IQR)).

4.2 STUDY DESIGN AND POPULATIONS

Paper I. This study was the first study started by SHARP (Severe Heterogeneous Asthma Research collaboration, Patient-centred), which was established within the ERS (European Respiratory Society). The study was a cross-sectional, retrospective analysis of aggregated registry data that included data from a total of 3236 adult patients classified as having severe asthma by the 11 different European registries used for the analysis (**Figure 6).**

Figure 6. Participating countries in the current study in the European Respiratory Society Severe Heterogeneous Asthma Research collaboration, Patient-centred (SHARP) (van Bragt et al., 2020).

Figure 7 shows the inclusion criteria for patients in different registries differed between countries. No general inclusion criteria were provided, and each country had their own basis for inclusion (*e.g.,* ERS/ATS guidelines or national guidelines). However, most patients included in the registers used fulfilled the criteria for severe asthma.

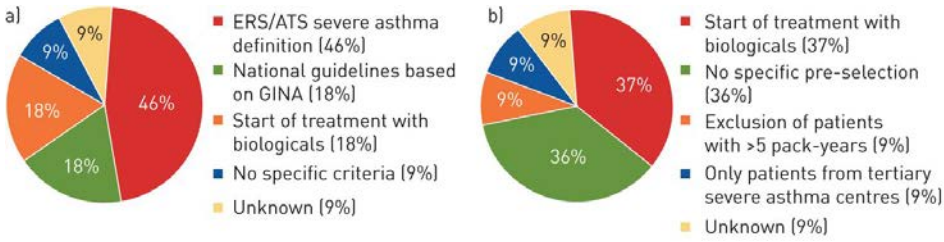


Figure 7. a) Inclusion criteria and b) criteria for pre-selection of patients in the different registries. ERS: European Respiratory Society; ATS: American Thoracic Society; GINA: Global Initiative for Asthma. (van Bragt et al., 2020)

The patients we had reported from our research group at the CLA research unit at KI, the Department of Medicine, Huddinge, fulfilled the ERS/ATS guidelines.

The main purpose of this study was to investigate differences between different registers across Europe and compare the characteristics of patients with severe asthma who began high-cost therapies (e.g., biologics or thermoplasty). **Paper I** analysed information regarding the baseline characteristics of patients upon register entry including gender, smoking history, BMI (Body Mass Index), lung function, level of blood eosinophils, F_ENO, total IgE level and asthma treatment which was collected from registers in 11 countries across Europe.

Paper II.

Material used in this study originates from U-BIOPRED (Unbiased BIOmarkers for the Prediction of Respiratory Disease outcome), a European multicenter observational study supported by IMI ([http://www.imi.europa.eu)]www.imi.europa.eu). In **Paper II**, 576 participants from the U-BIOPRED study were included with complete data for urinary steroid metabolites. 408 of them were patients with severe asthma (SA), 70 patients with mild-to-moderate asthma (MMA) and 98 healthy controls (HC) (**Figure 8A**).

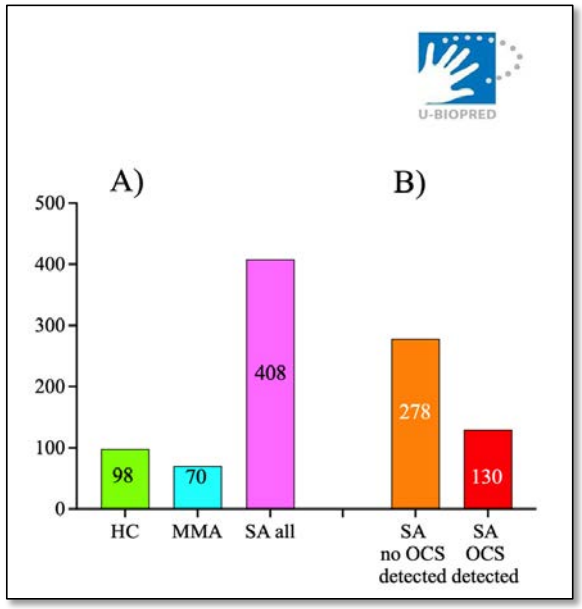


Figure 8: A) Study population: SA - severe asthmatics, MMA - mild-to-moderate asthmatics, HC – healthy controls. B) Stratification of severe asthma patients depending on detection of prednisolone metabolites in urine: SA OCS detected – severe asthmatics with prednisolone metabolites detected in urine; SA no OCS detection – severe asthmatics without.

The goals of **Paper II** were:

- to measure urinary concentrations of endogenous steroids, including androgens, in severe asthmatics patients and compare them with patients with mild-to-moderate and HCs, and also to compare differences between males and females.
- to investigate how exposure to exogenous corticosteroids affect urinary concentrations of endogenous steroids and specific androgens.

The SA group included patients who fulfilled the criteria for severe asthma according to ERS/ATS guidelines (Chung et al., 2018) and who required daily dose fluticasone propionate ⩾1000 µg or an equivalent dose of other ICS. A regular use of OCS was also allowed in the SA group. In the MMA group patients required <500 µg ICS daily. All participants who fulfilled the inclusion criteria during a screening visit were invited to attend a baseline visit

(**Figure 9**). During the baseline visit, clinical endpoints examined included lung function, F_ENO, and patients-reported data (*e.g.,* asthma control questionnaire (ACQ), asthma quality of life questionnaire (AQLQ), as well as biosamples (D. E. Shaw et al., 2015).

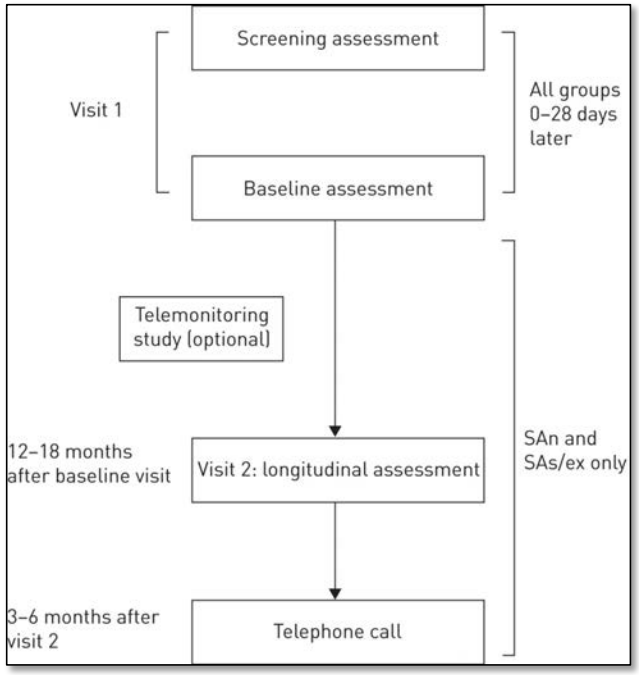


Figure 9: U-BIOPRED study flow chart. (Dominick E. Shaw et al., 2015)

Patients with severe asthma were also invited to a follow-up visits (**Figure 9**) after 12-18 months where study procedures were repeated (D. E. Shaw et al., 2015).

In order to investigate how treatment with oral corticosteroids affects steroids hormones including androgens, patients from the SA group were stratified in to two groups depending on the presence of urinary prednisolone metabolites; OCS detected or not (**Figure 8B**).

Paper III.

The BIOCROSS (BIOmarkers in CROSS-sectional study) study is a Swedish, multicenter, cross-sectional, prospective, observational, real-life study that includes patients with asthma of varying severities, as well as other respiratory diseases such as chronic obstructive lung disease (COPD), bronchiectasis, sarcoidosis, interstitial lung disease and healthy controls, with the aim to investigate specificity and patterns of biomarkers expression among the different groups (and in response to treatment). **Figure 10** shows study design.

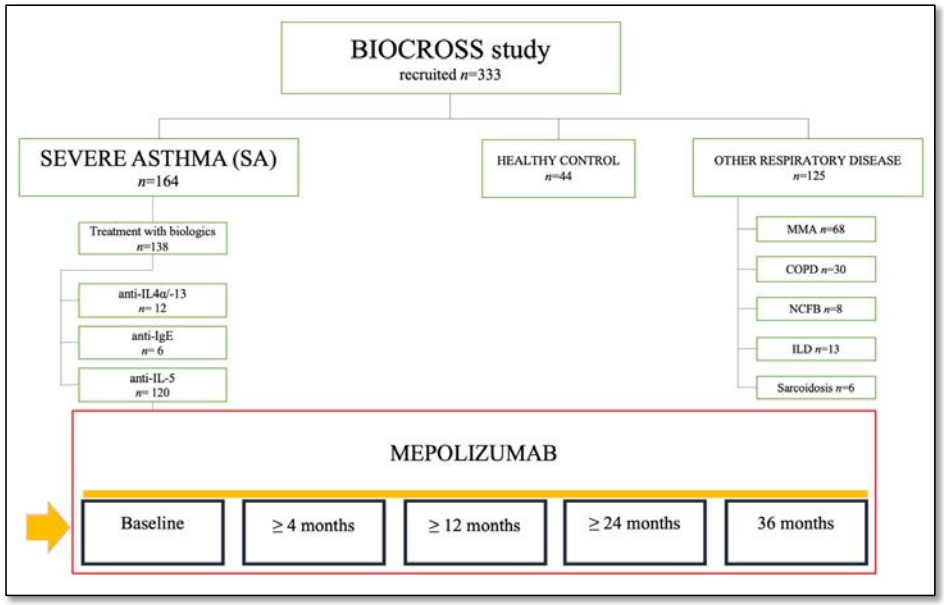


Figure10. Flow chart for the BIOCROSS study, focusing on the mepolizumab arm.

The main part of the BIOCROSS study involves a longitudinal arm where severe asthmatics treated with biologics at the Severe Asthma Center at Karolinska University Hospital and the Lung-allergy Department of the Academic Hospital in Uppsala, are followed over time. During the treatment period, biological samples were collected for biomarkers analysis alongside monitoring of clinical response. In **Paper III**, 77 patients were included who fulfilled the criteria for severe asthma according to ERS/ATS guidelines. These patients all had evidence of underlying eosinophilic inflammation, uncontrolled asthma and met the requirements for starting treatment with mepolizumab. After including into the BIOCROSS study they completed at least 1 year of follow-up visit until the end of May 2023 (**Figure 10** and **Figure 11**).

The **Figure 11** shows the examinations and sampling that was carried out before starting treatment with mepolizumab, and at follow-up visits after 4, 12, 24, 36 months, as well as the number of patients who attended the various visits during this analysis.

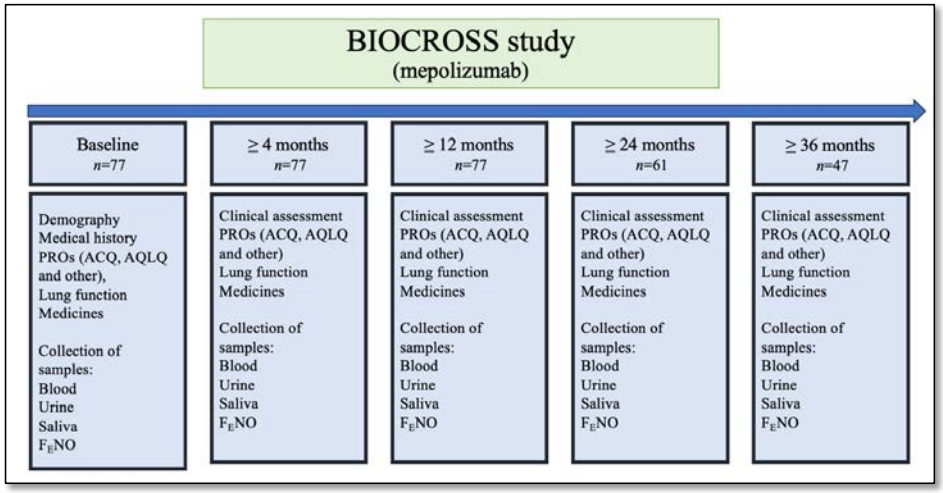


Figure 11. The clinical examinations and biosamples at baseline and during follow-up visits in the BIOCROSS study. This current study includes patients with severe asthma treated with mepolizumab and who completed at least 12 months follow-up visits until the end of May 2023.

4.3 M-COMSA SCORE FOR DEFINITION OF COMPOSITE RESPONSE TO BIOLOGICAL THERAPY

The analysis carried out in **Paper III** is the first attempt to implement a new evidence-based and patient-centered composite outcomes with the main aim of identifying patients who respond and do not respond to the anti-IL-5 drug mepolizumab in a prospective real-life study. This model was developed within the EU consortium 3TR (Taxonomy, Treatments, Targets and Remission) collaboration, and named Core Outcome Measures for Severe Asthma (COMSA) (E. Khaleva, A. Rattu, C. Brightling, A. Bush, A. Bossios, et al., 2023). The development process was extensive and included a systematic review of development, validity, and reliability of selected outcomes measures. To provide a better understanding of patient and carer opinions regarding outcome measures, a narrative review and a pan-European survey were performed with patient participation. Subsequently, consensus criteria were discussed in stakeholder groups on several occasions and were finally chosen after anonymous voting (E. Khaleva, A. Rattu, C. Brightling, A. Bush, A. Bourdin, et al., 2023). Finally, five definitions of response were selected according to **Figure 12**. These definitions were tested in our BIOCROSS study in relation to the effect of mepolizumab.

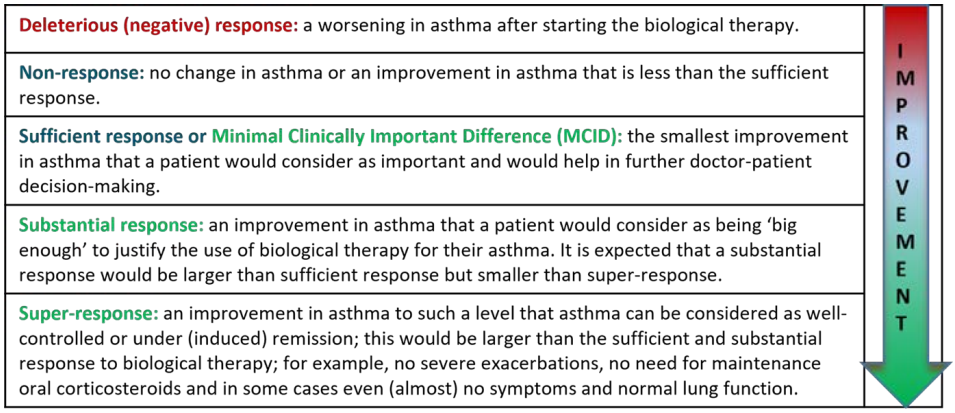


Figure 12: Definitions of response (3TR, Khaleva et al, unpublished).

In **Paper III**, for assessment of response to treatment with mepolizumab, we used a modified Core Outcomes Measures for Severe Asthma (M-COMSA) that was applied in the BIOCROSS study, and which included three clinical outcomes (FEV_1, exacerbations per year, and maintenance oral corticosteroids (OCS) use) as well as two patient reported outcomes (ACQ-5 and AQLQ). The differences between M-COMSA and COMSA were that we used ACQ-5 instead of ACQ-6 and AQLQ instead of severe asthma questionnaire (SAQ). The reason for this is described in more detail in **Paper III**. Data collected at the 1-year follow-up was used to test the M-COMSA strategy (**Table 3**).

Table 3: Overview of the modified COMSA (M-COMSA) scoring criteria used in the BIOCROSS sub-study of mepolizumab.

Score	OCS-dose	Exacerbations	FEV_1 %pred	AQLQ	ACQ-5
-1	Increase	Increase	Decrease >10%	Decrease ≥ 0.5 point	Increase ≥ 0.5 points
0	No change	No change	No change	No change	No change
1	Reduction < 50%	Reduction < 50%	10% <FEV_1 %pred increase ≤ 15%	Increase ≥ 0.5 point and total score < 5	Decrease ≥ 0.5 point, and mean score ≥1.5
2	Reduction 50% - <100%	Reduction 50% - <100%	15% <FEV_1 %pred increase ≤ 20%	Increase ≥ 0.5 point and total score < 6	Decrease ≥ 0.5 point, and mean score > 0.75 - <1.5
3	Reduction 100%	Reduction 100%	Increase > 20%	Increase ≥ 0.5 point and total score ≥ 6	Decrease ≥ 0.5 point, and mean score ≤ 0.75

The change from baseline for each outcome was measured at the one-year follow-up. We used an assessment scale where each domain was scored between -1 and +3, and the sum of the score for the five domains provided the overall response quantification which thus could range from -5 to +15 as each domain was given equal importance. To assess the stability of M-COMSA groups, we used total M-COMSA scores at 24- and 36-months follow-up visits.

4.4 LUNG FUNCTION MEASUREMENTS

Spirometry was performed at all study visit according to ERS/ATS guidelines (Graham et al., 2019; Miller et al., 2005). Following a maximal inspiration, subjects performed a fast and powerful exhalation manoeuvre whilst connected to a mouthpiece and wearing a nose clip. The forced expiratory volume in one second (FEV_1), the forced vital capacity (FVC), the forced expiratory flow between 25% and 75% of FVC (FEF25-75) and peak expiratory flow rate (PEFR) were recorded.

4.5 MEASUREMENTS OF F_ENO

Nitric oxide (NO) is produced by airway epithelial cells, mostly by inducible NO synthase (iNOS), which is upregulated in asthmatic inflammation and suppressed by corticosteroid treatment (Dweik et al., 2011). Measurement of the fraction of exhaled NO (F_ENO) at a fixed flow rate is non-invasive test that is widely considered to be a surrogate marker of eosinophilic inflammation (Dweik et al., 2010; Malinovschi et al., 2015). In **Paper I** and **Paper III** F_ENO measurements (NIOX analyzer, Aerocrine AB, Solna, Sweden) were performed at a flow rate of 50 mL/s according to ATS guidelines (Dweik et al., 2011) at all study visits.

4.6 PATIENT-REPORTED OUTCOMES

Patient-reported outcomes were recorded using the short Juniper asthma control questionnaire ACQ (Juniper, O'Byrne, et al., 1999), the asthma quality of life questionnaire AQLQ (Juniper, Buist, et al., 1999), and the hospital anxiety and depression scale (HADS) (Herrmann, 1997).

In **Paper I**, some registries recorded only ACQ results whereas others recorded only ACT (Asthma control test) scores but in 3 registries (out of 11), both were recorded. In **Paper II**, ACQ, AQLQ and HADS scores were recorded in the TranSMART data system. In **Paper III**, ACQ and AQLQ score were collected at all study visits and were included in a combined score, M-COMSA, to assess mepolizumab treatment effect (see above).

4.7 MEASUREMENTS IN BLOOD

In **Paper I**, blood eosinophil count and total IgE were recorded in some registries. In **Paper II**, blood eosinophil count and total IgE were recorded in the TranSMART data system as well as other blood-mics results. In **Paper III**, routine blood samples were collected during the screening visit to exclude comorbidities that might eventually influence the study results. Blood samples were also collected at all study visits for analysis of eosinophil counts and total IgE.

4.8 URINE COLLECTION AND ANALYSIS

In **Paper II**, one spot urine sample was split into five 8 mL tubes (Sarstedt, Nümbrecht, Garmaty), placed into -20°C freezers at the study sites and kept at -80°C following shipment to the central analysis site in Stockholm. Under such conditions, steroid conjugates have been documented to be intact for $\geq$ 10 years, and they are also stable during several thaw-freeze cycles (De Wilde et al., 2022). The quantification of endogenous and exogenous steroids was

performed at the World Anti-Doping Agency (WADA) by Ultra High-Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) as previously described (Schulze et al., 2012). Metabolites of urinary prednisone are highest during the first 24-36 hours after oral intake but may be found up to a week after the last dose with the high sensitivity method used (limit of detection [LOD] 1 ng/mL for all analytes) (Coll et al., 2021).

Urine was collected for **Paper III** to investigate how lipid mediator levels change during treatment with mepolizumab as a marker of eosinophil activity. Urine collection was performed at the beginning and at the end of each study visit. Collected samples were distributed into smaller plastic tubes, initially stored at -20°C, followed by -80°C, until time of analysis. The quantification of eicosanoids was performed using liquid chromatography coupled to tandem mass spectrometry using an Acquity UPLC system connected to an Xevo TQ-XS (Waters, Milford) instrument system operated in negative electrospray mode. Details of the method are presented elsewhere (Kolmert et al., 2014). Concentrations were normalized to specific gravity (Atago UG-) and expressed in ng/mL. This platform measured the main urinary metabolites of prostaglandins (PG), thromboxanes (TX) and the cysteinyl leukotrienes (CysLTs).

In **Paper III**, eosinophil derived neurotoxin (EDN) was measured in plasma and urine using an ImmunoCAP research assay (Thermo Fisher Scientific, Uppsala, Sweden). The EDN assay has been described elsewhere (Rydell et al., 2019).

4.9 STATISTICAL ANALYSES

Descriptive statistics

Overall, continuous variables were present as median with interquartile range (IQR) or means and standard deviations (SD), where appropriate (Papers I-III), while numbers (n) and percentages (%) were used to describe dichotomous variables (**Papers I-III**). In addition, median with interquartile range (IQR) was used to describe continuous variables where appropriate, e.g., when presenting data with a skewed distribution (**Papers I-III**).

In **Paper I**, the clinical characteristics in the different registries were compared using descriptive statistics. To describe differences in treatment regimens and biomarkers, comparisons were made before starting high-cost therapies. Treatment with biologics, bronchial thermoplasty or high-altitude treatment were defined as high-cost therapies.

In **Paper II** and **Paper III**, statistical evaluation was performed using GraphPad Prism (v9, GraphPad). Descriptive statistics were performed to compare all patient characteristics, where a p-value <0.05 was considered statistically significant. Multi-group comparisons of non-normally distributed variables were assessed using the Kruskal-Wallis-test. Pair-wise analyses between groups were performed using the Mann-Whitney U-test. For comparisons of outcomes changing over time within the same group, Wilcoxon matched-pair signed-rank tests were performed. Chi-squared tests were used for comparisons of proportions.

In **Paper II**, all clinical outcomes and biomarker results were retrieved from the U-BIOPRED TranSMART data handling system (De Meulder et al., 2018). The presence of prednisone metabolites in urine was used to stratify the participants into two groups regarding OCS detection: yeas or no. Relations between the urinary concentrations of DHEA (Dehydroepiandrosterone) and clinical outcomes were evaluated using an extreme group analysis, where asthmatic subjects were stratified into high (above 75th percentile) or low (below 25th percentile) DHEA-S groups.

In **Paper III**, the M-COMSA score for all included patients was divided into four responder groups depending on the distribution of score sum (**Figure 18**). Severe asthma patients with a low score (below the 25th percentile) were assessed as non-responders; patients with a high score (above 75th percentile) were assessed as super responders; patients with a score ≥25th percentile and <50th percentile were judged to belong to the group of sufficient responders and patients with a score ≥50th percentile and ≤75th percentile to the group of substantial responders.

The stability of the M-COMSA responder groups was analyzed in R Statistical Software using an alluvial plot (ggalluvial package,(Brunson, 2020)), allowing for changes in the flow of patients between response groups over time to be visualized (**Figure 18**).

5 RESULTS AND DISCUSSION

Main findings of the three included studies (**Paper I-III**).

5.1 CHARACTERISTICS AND TREATMENT REGIMENS ACROSS ERS SHARP SEVERE ASTHMA REGISTRIES (PAPER I).

In **Paper I** we analyzed data from different European registries that included a total of 3236 patients with severe asthma. Most registries enrolled patients undergoing treatment in tertiary care centers, and most (>90%) of these patients were treated according to GINA step 4 or 5 guidelines (Reddel et al., 2022) in Denmark it was 77.6% and in six registries (Hungary, Poland, Sweden, Germany, Italy and Slovenia) 100% of patients were at GINA step 4 or 5.

5.1.1 DIFFERENCES IN BASELINE CLINICAL CHARACTERISTICS

There were differences in baseline clinical characteristics (**Paper I: Table 1**) between different registries regarding mean age of patients, and percentages of current smokers and ex-smokers. "Based on mean forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) (% pred), patients in the Dutch registry had the best lung function" (van Bragt et al., 2020), whereas patients in Hungary had "the worst lung function (FEV_1 76.9% versus 56.0%, and FVC 98.3% versus 76.6%) (van Bragt et al., 2020). It was however difficult to explain these differences in lung function results. The highest FEV_1 (% pred) expressed pre-bronchodilator was noted in the Netherlands and differed by as much as 20.9% from the lowest value in the Hungarian register. Notably, patients in both registries had similar biomarkers like F_ENO levels and blood eosinophil counts and similar treatment regimens.

Possible explanations might relate to differences in disease duration and time since start of maintenance ICS treatment, which would result in progressive flow limitation, or differences in OCS use between Hungary (60%) compared to the Netherlands (26%) before the start of biological therapy. Exposure to asthma triggers such as outdoor and/or indoor pollutions (including cigarette smoke) that lead to a decline lung function, could also be another potential explanation, but these factors were not assessed as the relevant data was not collected. Another possible explanation for these differences may be generally that the inclusion criteria in 9% of cases were unknown and in 9% of cases there were no inclusion criteria, moreover patients in some countries were included to register from secondary care before they were referred to tertiary care, and it is unclear if there an investigation was made to exclude other respiratory diseases, especially COPD. In addition, this analysis included pre-bronchodilator lung function and information on reversibility is not available.

In most registries, differences in clinical inflammatory biomarkers were observed; median blood eosinophil levels ranged from 230 $x10^9$ (Netherlands) to 800 $x10^9$ cells/L^{-1} (Sweden), median fraction of exhaled nitric oxide (F_ENO) ranged from 25 (Belgium) to 66 ppb (Slovenia), and median serum total IgE varied from 144 (Netherlands) to 275 IU/mL (Sweden). For more information about biomarkers, see **Table 3** in **Paper I (van Bragt et al., 2020)**.

The proportions of patients with uncontrolled asthma based on questionnaires and asthma-related hospital stays over the past 12 months differed between registries and could depend on possible differences in access to specialist care. According to patient-reported questionnaire scores (ACQ or ACT), 54.6% of patients had uncontrolled asthma in Italy and 100% in Hungary and Sweden.

5.1.2 DIFFERENCES IN HIGH-COST THERAPIES

Anti-IgE was the most frequently prescribed biological in seven registries including Belgium, Spain, Hungary, Germany, Italy and Slovenia (van Bragt et al., 2020). Some countries had already started anti-IL-5 biologics and in three registries these drugs were the most frequently used (Netherlands, UK and Denmark). Generally, the highest percentage of patients on biological treatment was in Poland (71.0%), whereas in Sweden, register patients were enrolled who were planning to start biologics. Some countries also used other types of non-medical treatment such as bronchial thermoplasty and high-altitude treatment. The information about patients treated with bronchial thermoplasty was recorded in registries from the Netherlands, Belgium and Slovenia, and patients who received high-altitude treatment were enrolled in the Netherlands (14%).

5.1.3 DIFFERENCES IN MAINTENANCE TREATMENT

Figure 13 provides information about the maintenance therapy as severe asthmatics used before starting treatment with anti-IL-5 biologics (mepolizumab, reslizumab or benralizumab; n=617). Treatment regimens differed between registries. Although all patients were treated with ICS in almost all registries (except for Denmark, where 94.7% of patients used ICS), the doses of ICS differed greatly, ranging from 1335 ± 529 (Spain) to 700 ± 118 μg/day (Slovenia). Potential explanations for these deviations could be the different interpretations of what high dose ICS actually is, ERS/ATC guidelines state a dose of >1000 μg/day fluticasone equivalent (Chung et al., 2018) and GINA guidelines a dose of >500 μg/day fluticasone equivalent. Only four registries (Spain, Hungary, Sweden, and Slovenia) included patients where LABA was used prior to starting anti-IL-5 treatment, whereas in other countries this ranged from 25.0% (Poland) to 94.4% (Netherlands). Treatment with LAMA ranged from 0% (Hungary) to 79.1% (Slovenia). This difference is striking and the cause of it is unclear. According to the above-mentioned, possible explanation would be an overlap with COPD in patients included to registries. In all registries, OCS was used as a maintenance treatment before starting anti-IL-5, however, OCS use varied from 21.0% (Belgium) to 63.0% (Sweden) of the population. Daily doses of mOCS also ranged widely between registries (**Figure 13**). It also was striking how mOCS use before treatment with biologics differed between different countries. The proportion of patients on mOCS was highest in the UK, differing by 52.2% from clinical practice in Belgium. The reason for these differences is unclear and more focused investigations within the SHARP CRC are required to address these.

More information regarding medications is shown in **Table 2, Paper I**. The most frequently used reliever medications were short-acting β-agonists (SABAs) in all countries, except Spain, where short-acting muscarinic antagonists (SAMAs) were the most frequent relievers.

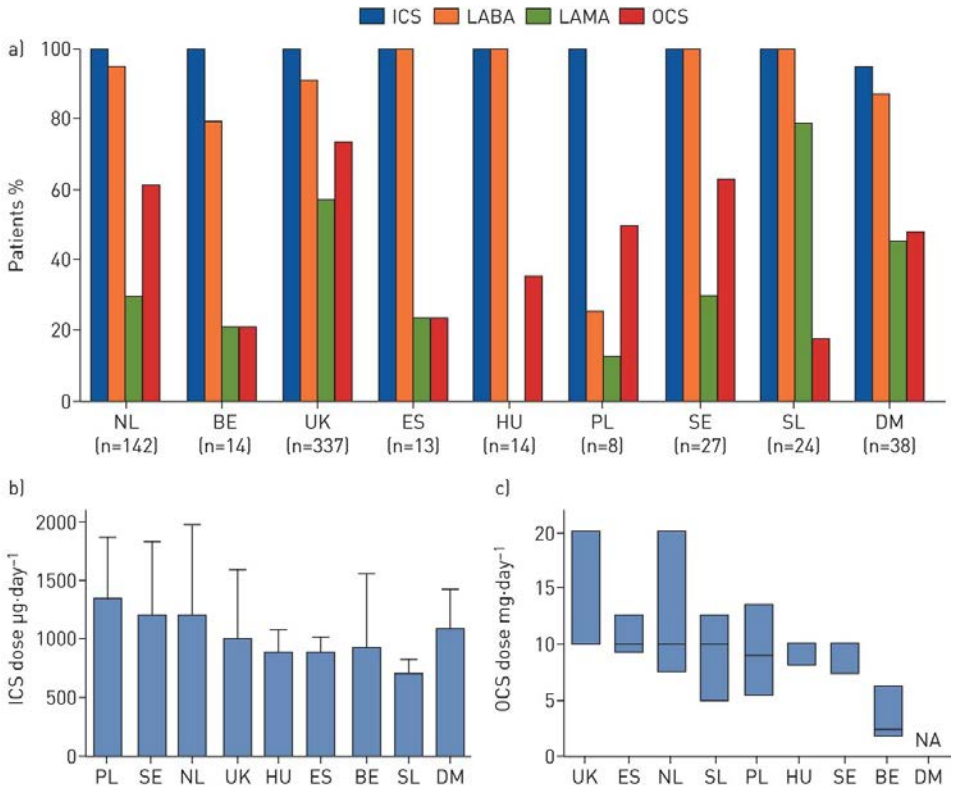


Figure13. a) Overview of maintenance treatment in patients starting anti-interleukin (IL)-5 biological therapy. NL: Netherlands; BE: Belgium, ES: Spain; HU: Hungary; PL: Poland; SE: Sweden; SL: Slovenia; DM: Denmark; ICS: inhaled corticosteroid; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist; OCS: oral corticosteroid; NA: not available. B) Mean±SD ICS dose (fluticasone equivalent) in patients that start with anti-IL-5 biological therapies. C) Median (interquartile range) maintenance OCS dose (prednisolone equivalent) in patients that start with anti-IL-5 therapies. Median values: UK 10 mg/; Spain 12.5 mg/day; Netherlands 10 mg/day; Slovenia 10 mg/day; Poland 9 mg/day; Hungary 10 mg/day; Sweden 10 mg/day and Belgium 2.5 mg/day. (van Bragt et al., 2020)

Differences in maintenance treatment between registries could depend on differences in the definitions of severe asthma in different countries. Commonly used definitions include those used in ERS/ATS and GINA guidelines (**Figure 7**). Nevertheless, some patients starting treatment with biological therapies, and therefore considered to have severe asthma, did not meet the criteria for severe asthma as defined by ERS/ATS and GINA. One possible explanation for these differences could be that some patients were considered to have severe asthma by clinical experts from certain countries. Such differences would require more analysis, including the processes whereby biologics were offered to patients. The most important criteria for starting treatment with biologics were the frequency of exacerbations

(three or four) in the previous 12 months or mOCS, which were required in several countries, for example in the UK by the National Institute of Clinical Excellence (NICE) (Bermejo et al., 2018; Cooper et al., 2018). These recommendations are implemented rigorously by the commissioning groups that regulate the use of biologics. Theoretically, differences could also be reflected by differences in the severity of the broader asthma population in each country. However, the data collected in **Paper I** did not allow us to address this question. Furthermore, patients who started treatment with biologics did not always fulfil the criteria used to recruit patients into RCTs. Taken together, there is a general need for observational studies investigating the use of biologics in real-life patients, with different comorbidities, to provide better understanding about the efficacy of biologics and their possible side effects in daily practice.

5.1.4 SUMMARY

Several important observations were made in **Paper I**, the first collaborative study in the SHARP CRC.

Firstly, **Paper I**, reveals that the population of severe asthmatics in Europe is heterogeneous and differs regarding clinical characteristics, lifestyle factors and treatment. Differences in treatments between countries meant that the results of single-centre trials, or even multicentre studies in the same country, were difficult to compare.

Secondly, Paper I highlights that fact that the definition of severe asthma in current guidelines is not in accordance with the characteristics of real-word, severe asthmatics.

Thirdly, **Paper I** emphasizes the importance of harmonizing severe asthma databases across Europe, and the need for long-term follow-up of this group of patients.

In conclusion, the subjects covered **Paper I** could be an important stepping-stone towards future discussions aimed at standardizing severe asthma care in Europe. An international agreement “on a minimal set of well-defined key variables is needed to increase the utility of the SHARP platform” (van Bragt et al., 2020) and provide opportunities for future research. Such an agreement could provide solutions to the challenges described in **Paper I** and be a logical next step for the SHARP collaboration.

5.2 LOW LEVELS OF ENDOGENOUS ANABOLIC ANDROGENIC STEROIDS IN FEMALES WITH SEVERE ASTHMA TAKING CORTICOSTEROIDS (Paper II).

The concentrations of endogenous steroids were quantified in urine by mass spectrometry according to WADA panel in 478 well-characterized patients with asthma of varying severity and 98 healthy subjects. Data for endogenous steroids were separated by sex into two groups, as males generally have higher urinary concentrations of androgens (**Paper II: Table 2**). There were 253 females in one group and 155 males in another.

5.2.1 URINARY EXCRETION OF ENDOGENOUS STEROID METABOLITES IN SEVERE ASTHMA

Individuals with SA, regardless of sex had significantly lower concentrations of endogenous cortisol and androgen metabolites compared to MMA and HC (**Table 4A** and **Table 4B**).

Table 4A. Concentrations of urinary steroids (ng/mL) in U-BIOPRED study groups at baseline

Females							
Endogenous steroids (ng/mL)	HC (n=39)	MMA (n=32)	SA (n=253)	p-value	SA *High dose ICS OCS negative* (n=177)	SA *High dose ICS OCS positive* (n=76)	p-value All 4 groups*
Cortisone	206 (139–263)	222 (146–270)	95 (0.5–190)	<0.0001	122.2 (55.4–203.6)	0.5 (0.5–65.4)	<0.0001
<LOD n (%)	4 (10%)	4 (13%)	81 (32%)		30 (17%)	51 (67%)	
Cortisol	68 (34–156)	87 (52–173)	30 (0.5–79)	<0.0001	44.9 (0.5–83.7)	0.5 (0.5–9.1)	<0.0001
<LOD n (%)	6 (15%)	4 (13%)	104 (41%)		47 (27%)	57 (75%)	
Dehydroepiandrosterone (DHEA)-S	275 (46–915)	172 (74–593)	39 (11–104)	<0.0001	50 (16–125)	11 (2–44)	<0.0001
<LOD n (%)	0	0	9 (4%)		2 (1%)	7 (9%)	
DHEA-G	44 (19–62)	36 (20–55)	12 (4–28)	<0.0001	17 (6–34)	4 (2–12)	<0.0001
<LOD n (%)	0	0	19 (8%)		9 (5%)	10 (13%)	
Androsterone-G	2688 (1468–4506)	1991 (1154–3908)	745 (203–1905)	<0.0001	1002 (391–2203)	214 (80–797)	<0.0001
<LOD n (%)	0	0	0		0	0	
Androsterone-S	403 (202–722)	299 (129–700)	109 (38–272)	<0.0001	134 (59–337)	41 (13–113)	<0.0001
<LOD n (%)	0	0	0		0	0	
Testosterone-G	11 (8–14)	8 (5–13)	6 (4–10)	<0.0001	6 (4–10)	5 (3–8)	<0.0001
<LOD n (%)	0	0	1 (0.4%)		0	1 (1%)	
Testosterone-S	0.5 (0.5–1.7)	0.7 (0.5–1.8)	0.5 (0.5–0.5)	<0.0001	0.5 (0.5–0.5)	0.5 (0.5–0.5)	<0.0001
<LOD n (%)	20 (51%)	15 (47%)	165(65%)		99 (56%)	66 (87%)	
DHT-G	2.9 (2.0–6.5)	1.8 (0.5–4.1)	1.1 (0.5–2.6)	<0.0001	1.1 (0.5–2.6)	1.2 (0.5–2.4)	<0.0001
<LOD n (%)	3 (8%)	12 (31%)	89 (35%)		66 (37%)	23 (30%)	

Epitestosterone-G	15 (6–25)	14 (7–28)	7 (3–18)	0.006	9 (3–19)	4 (1–13)	0.0009
<LOD n (%)	4 (10%)	3 (26%)	36 (14%)		22 (12%)	14 (18%)	
Epitestosterone-S	1.5 (0.5–3.1)	2.9 (0.5–8.6)	0.5 (0.5–1.6)	<0.0001	0.8 (0.5–1.9)	0.5 (0.5–0.9)	<0.0001
<LOD n (%)	12 (31%)	11 (34%)	133 (53%)		81 (46%)	52 (68%)	
Etiocholanolone-G	2441 (1783–4625)	2450 (1424–3780)	833 (271–1844)	<0.0001	1113 (457–2072)	337 (105–901)	<0.0001
<LOD n (%)	0	0	0		0	0	
Etiocholanolone-S	209 (82–308)	170 (95–410)	67 (27–154)	<0.0001	87 (37-208)	41 (10–78)	0.0003
<LOD n (%)	0	0	1 (0.4%)		0	0	
5alpha-Androstane-3α,17β-diol 17β-D-glucuronide (AAB-17G)	10 (4–25)	8.3 (4–16)	4 (2–12)	0.06	6.2 (3–13)	3 (1–6)	0.02
<LOD n (%)	1 (3%)	2 (6%)	16 (6%)		7 (4%)	9 (12%)	
5β-Androstane-3α,17β-diol-3α-glucuronide (BAB-3G)	21 (13–55)	23 (17–43)	11 (4–24)	<0.0001	13 (6–27)	5 (2–13)	<0.0001
<LOD n (%)	0	0	21 (8%)		11 (6%)	10 (13%)	
5β-Androstane-3α,17β-diol 17β-D-glucuronide (BAB-17G)	68 (26–125)	36 (17–93)	16 (5–41)	0.17	21 (8–47)	7 (3–26)	0.27
<LOD n (%)	0	0	2 (1%)		0	2 (3%)	
5β-Androstane-3α,17β-diol-3α-glucuronide (ABB-3G)	13 (6–25)	11 (6–22)	5 (2–12)	0.0007	6.8 (2.2–13.9)	2.2 (0.5–5.8)	0.0009
<LOD n (%)	2 (5%)	2 (6%)	41 (16%)		21 (12%)	20 (26%)	

Patients with severe asthma, all of whom received high doses ICS, were stratified as OCS negative, or OCS positive, according to detection of urinary OCS metabolites. Glucuronated metabolites are given suffix G, and sulphated S. Occasional values below LOD: limit of detection. Data are presented as n, median (interquartile range) or n (%). Significance was evaluated using the Kruskal-Wallis-test. (Yasinska et al., 2023)

Table 4B. Concentrations of urinary steroids (ng/mL) in U-BIOPRED study groups at baseline

Males							
Endogenous steroids (ng/mL)	HC (n=59)	MMA (n=38)	SA (n=155)	p-value	SA *High dose ICS OCS negative* (n=101)	SA *High dose ICS OCS positive* (n=54)	p-value All 4 groups
Cortisone	193 (132–260)	172 (107–231)	78 (0.5–170)	<0.0001	120.1 (46.1–211.9)	0.5 (0.5–77.7)	<0.0001
<LOD n (%)	1 (2%)	3 (8%)	56 (36%)		19 (19%)	37 (69%)	
Cortisol	94 (65–139)	82 (44–114)	25 (0.5–72)	<0.0001	49.4 (0.5–98.7)	0.5 (0.5–0.5)	<0.0001
<LOD n (%)	2 (3%)	4 (11%)	71 (46%)		27 (27%)	44 (82%)	
Dehydroepiandrosterone (DHEA)-S	1080 (284–3668)	673 (89–2822)	41.1 (17–204)	<0.0001	81 (22–287)	31 (14–55)	<0.0001
<LOD n (%)	0	0	0		0	0	
DHEA-G	52 (32–72)	48 (29–69)	13 (7–29)	<0.0001	17 (8–38)	10 (5–21)	<0.0001
<LOD n (%)	0	0	1 (0.6%)		0	1 (2%)	

Androsterone-G	4778 (3184–7438)	4808 (3058–6851)	2131 (1413–3252)	<0.0001	2328 (1659–4163)	1650 (1137–2721)	<0.0001
<LOD n (%)	0	0	0		0	0	
Androsterone-S	951 (507–1556)	781 (233–1444)	280 (126–528)	<0.0001	299 (141–598)	248 (116–404)	<0.0001
<LOD n (%)	0	0	0		0	0	
Testosterone-G	62 (38–94)	50 (32–87)	51 (34–84)	0.32	53 (36–86)	47 (29–85)	0.47
<LOD n (%)	0	0	0		0	0	
Testosterone-S	3.1 (1.7–7.7)	3.5 (1.0–6.9)	1.3 (0.7–2.7)	<0.0001	1.4 (0.6–3.0)	1.0 (0.7–1.6)	<0.0001
<LOD n (%)	3 (5%)	4 (11%)	35 (23%)		23 (23%)	12 (22%)	
DHT-G	8.3 (4.6–13.8)	7.9 (4.8–12.8)	6.2 (3.0–10.7)	0.07	6.4 (3.2–11.7)	6.0 (2.9–9.0)	0.095
<LOD n (%)	3 (7%)	4 (11%)	7 (5%)		5 (5%)	2 (4%)	
Epitestosterone-G	72 (51–114)	78 (43–123)	76 (49–112)	0.93	84 (50–119)	71 (48–98)	0.67
<LOD n (%)	0	0	0		0	0	
Epitestosterone-S	10 (6–16)	11 (5–16)	7 (4–9)	<0.0001	7 (4–10)	6 (4–9)	<0.0001
<LOD n (%)	0	0	2 (1%)		1 (1%)	1(2%)	
Etiocholanolone-G	3516 (2491–5135)	3083 (2179–4467)	1853 (1200–2814)	<0.0001	1913 (1206–2889)	1672 (1126–2542)	<0.0001
<LOD n (%)	0	0	0		0	0	
Etiocholanolone-S	231 (78–396)	77 (42–308)	69 (32–200)	<0.0001	73 (31-209)	67 (32–183)	0.0001
<LOD n (%)	0	0	0		0	0	
5alpha-Androstane-3α,17β-diol 17β-D-glucuronide (AAB-17G)	88 (41–117)	79 (44–123)	60 (37–106)	0.07	62 (41–109)	47 (25–92)	0.03
<LOD n (%)	0	0	0		0	0	
5β-Androstane-3α,17β-diol-3α-glucuronide (BAB-3G)	65 (36–116)	54 (37–77)	43 (28–72)	<0.0001	39 (26–71)	50 (33–78)	<0.0001
<LOD n (%)	0	0	0		0	0	
5β-Androstane-3α,17β-diol 17β-D-glucuronide (BAB-17G)	208 (107–336)	204 (127–315)	167 (91–286)	0.22	163 (109–308)	169 (70–273)	0.32
<LOD n (%)	0	0	0		0	0	
5β-Androstane-3α,17β-diol-3α-glucuronide (ABB-3G)	42 (25–70)	44 (30–63)	25 (16–39)	0.001	25 (18–39)	25 (15–40)	0.001
<LOD n (%)	0	0	0		0	0	

Patients with severe asthma, all of whom received high doses ICS, were stratified as OCS negative, or OCS positive, according to detection of urinary OCS metabolites. Glucuronated metabolites are given suffix G, and sulphated S. Occasional values below LOD: limit of detection. Data are presented as n, median (interquartile range) or n (%). Significance was evaluated using the Kruskal-Wallis-test. (Yasinska et al., 2023)

Our explanation for the differences in androgenic steroid concentration is a dose-dependent suppression of corticosteroid exposure, i.e., patients treated with the higher dose of corticosteroids have a lower level of androgens. Severe asthmatics take by definition a high daily dose of ICS ⩾1000 μg fluticasone propionate or equivalent dose of other ICS and some

of them also take OCS. In this group the concentration of androgens metabolites was lower compared to the other groups.

Accordingly, the severe asthmatics who used both high dose ICS and OCS had lower levels of androgens compared to severe asthmatics that only had high dose ICS. In fact, 75% of females and 80% of males in the OCS-positive group had undetectable levels of cortisol as signs of adrenal insufficiency with (**Figure 15A** below; **Paper II: Table 2B**). Furthermore, severe asthmatics who only used high dose ICS had lower concentrations av DHEA compared to patients with MMA. The patients with MMA treated with a daily dose of ICS <500 μg had higher concentration of androgens compared to severe asthmatics, but they still had a lower concentration compared to HC (**Table 4A** and **Table 4B**), supporting a systematical effect also of lower doses of ICS. In the next section of this thesis, there is a more detailed explanation for dose-dependent influence of exposure with exogenous steroid on androgenic steroids.

However, urinary testosterone in males did not differ between the three study groups, which could reflect its gonadal origin in men (**Figure 14** below) (Alemany, 2022; O'Byrne & Pedersen, 1998).

As shown in **Paper II** (**Table S2**), the levels of most steroid metabolites in urine in both sexes did not differ between MMA and HC which is in line with other reports (Lipworth, 1996; O'Byrne & Pedersen, 1998). Moreover, the differences in steroid levels remained between all study groups regardless of age and BMI according to expanded statistical analyses.

5.2.2 DATA STABILITY AT LONGITUDINAL FOLLOW-UP

Of the 408 patients with severe asthma, 289 (71%) were able to participate in a follow-up visit and repeat all measurements. The clinical outcomes and patterns of EAAS (Endogenous androgenic anabolic steroid) metabolites were replicated and stable (**Paper II: Table 3**).

5.2.3 RELATIONSHIP BETWEEN ENDOGENOUS ANABOLIC STEROIDS AND CORTICOSTEROID TREATMENT

Detectable urinary prednisolone metabolites were present in 75% of females and 82% of males with severe asthma, and these patients who were taking OCS had undetectable concentrations of cortisol (**Table 4A** and **Table 4B**). Adrenal insufficiency is a known side effect of treatment with corticosteroid that is a result of suppression of cortisol biosynthesis (Gurnell et al., 2021), but the extent to which this occurred was greater than anticipated. Results also showed low concentrations of androgens measured in the WADA panel not only in group of patients treated with OCS, but in patients treated with high dose ICS, which were not previously reported.

Figure 14 shows how the natural steroid hormones are synthesized from cholesterol. This biosynthesis take place in the adrenal cortex and in the testis in males, and ovary in females (Alemany, 2022; O'Byrne & Pedersen, 1998). Cortisol synthesizes from pregnenolone and thereafter is converted into cortisone in peripheral tissues. Both cortisol and cortisone are found in the blood circulation and secreted to the urine. In the adrenal cortex, pregnenolone is also metabolised to DHEA (Alemany, 2022). In the ovary or testis, the DHEA is metabolized to

androstenedione or androstenediol via a pathway for biosynthesis of the male sex hormone testosterone, and via further metabolism to the female sex hormones, estrogens.

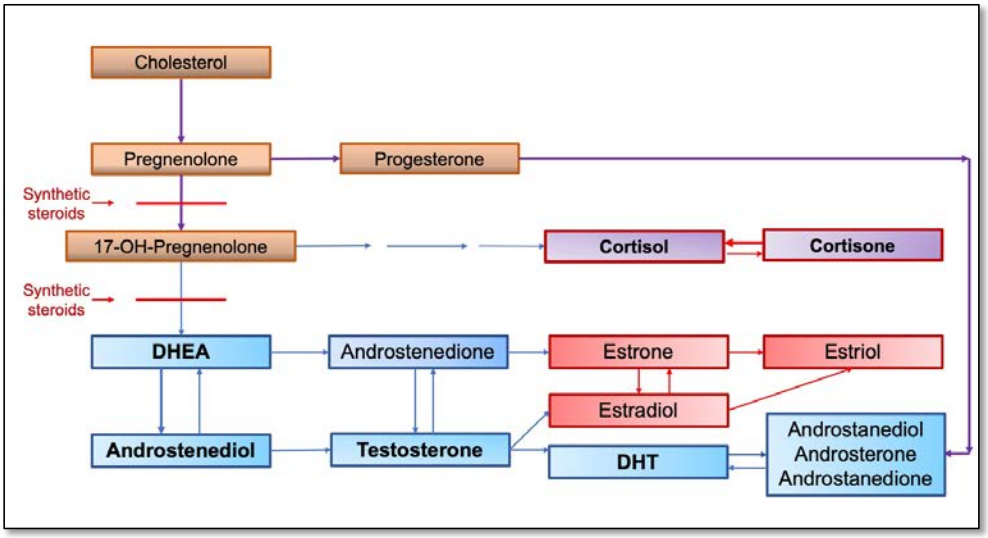


Figure 14. Overview of the biosynthesis of the natural steroid hormones from cholesterol and inhibition of this biosynthesis by synthetic steroids.

The **Figure 14** also shows how synthetic steroids (oral corticosteroids) inhibit the biosynthesis of both cortisol and DHEA in one step, leading to a reduced level of both cortisol and consequently cortisone, and DHEA. In addition, the reduced levels of DHEA lead to inhibited biosynthesis of other androgens including testosterone.

However, this inhibition is more pronounced in females than in males since in fertile women 70-80% of testosterone is produced by adrenal glands, while in men testosterone is mainly produced in testicles (Alemany, 2022).

Our results show dose-dependent effects of corticosteroid treatment on endogenous steroid levels, which is in line with previous data (Lipworth, 1996). Patients with SA that were treated with high doses ICS and were OCS-positive showed the most profound suppression of cortisol and androgens regardless of sex, whereas severe asthmatics treated with only high dose ICS showed less pronounced suppressions in androgens. Nevertheless, patients treated with high dose ICS and without detectable urinary prednisone metabolites had substantially lower levels of androgens and cortisol compared to HC and patients in the MMA group. (**Paper II: Figure 2, Table 2A-2B**). Moreover, in patients who reported OCS use at least once a week, detectable urinary prednisone metabolites were found in 70 (43%) out of 162 patients at baseline, and 51 (49%) out of 104 at the follow-up visit after 12-18 months (**Figure 15**).

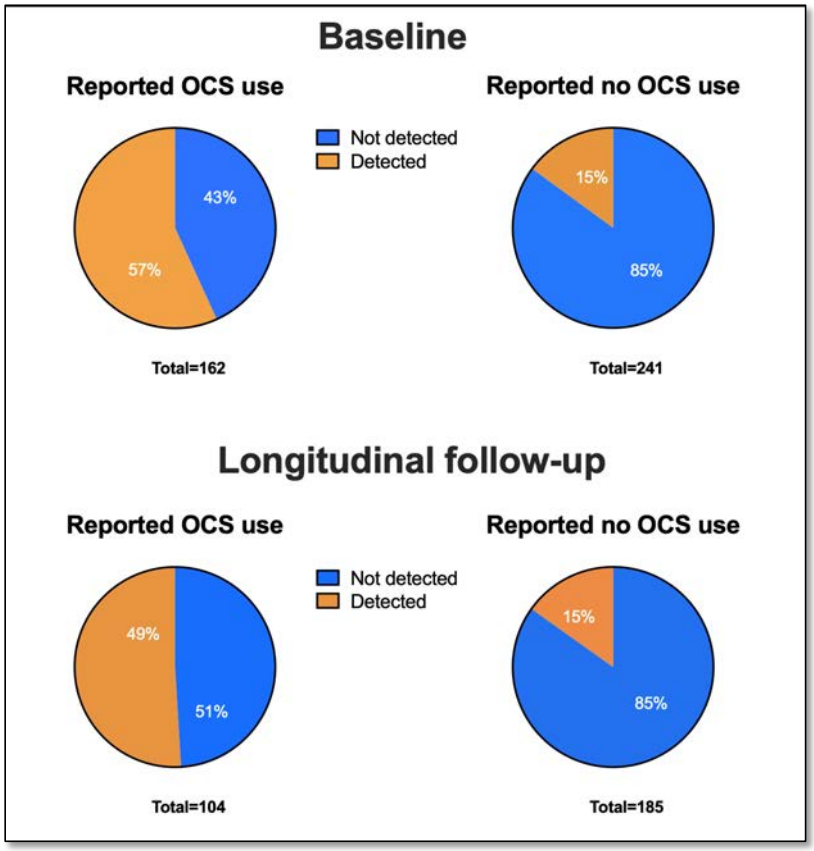


Figure 15. OCS detected versus reported at baseline and at follow-up visit after 12-18 months.

This observation may suggest adherence issues (Alahmadi et al., 2021). However, since the prednisone metabolites are undetectable after a few days according to most of published data (Coll et al., 2021), the possibility remains that the prednisone metabolites would not have been detected at the clinical visit when urine samples were collected, even if OCS were taken in the past week. Detectable prednisone metabolites were presumably most likely to originate from the most severe, steroid-dependent SA patients, who used OCS daily. Consequently, the patients in this group also had higher blood neutrophil counts, lower eosinophil counts, and high serum MMP-3 (Matrix Metalloproteinase 3) (Hathout et al., 2016) (**Paper II: Table S7**), which are all markers for recent exposure to corticosteroids. On the other hand, the systemic effect of corticosteroids would explain low levels of steroid-sensitive type 2 markers in severe asthmatics with detectable OCS. **Table 4** in **Paper II** shows that F_ENO, periostin and blood eosinophils were not raised. It is interesting that 15% of patients who reported no OCS use also had detectable prednisone metabolites in their urine, as well as some patients in the MMA group (**Paper II: Figure 3, Table S6**). This observation could be explained by the fact that it is common for general practitioners (GPs) to prescribe OCS to patients with asthma to take it as a self-management during exacerbation.

5.2.4 SEX DIFFERENCES

The levels of androgens were low in both females and males with severe asthma (**Table 4A** and **Table 4B**). However, the levels for all androgens in females with SA were more suppressed compared to males regardless of they were OCS-positive and OCS-negative. (**Paper II: Table 2B, Table S2**). These differences are likely to depend on differences in the inhibition of the biosynthesis of androgenic steroids in women and men due to different sites of biosynthesis (**Figure 14**).

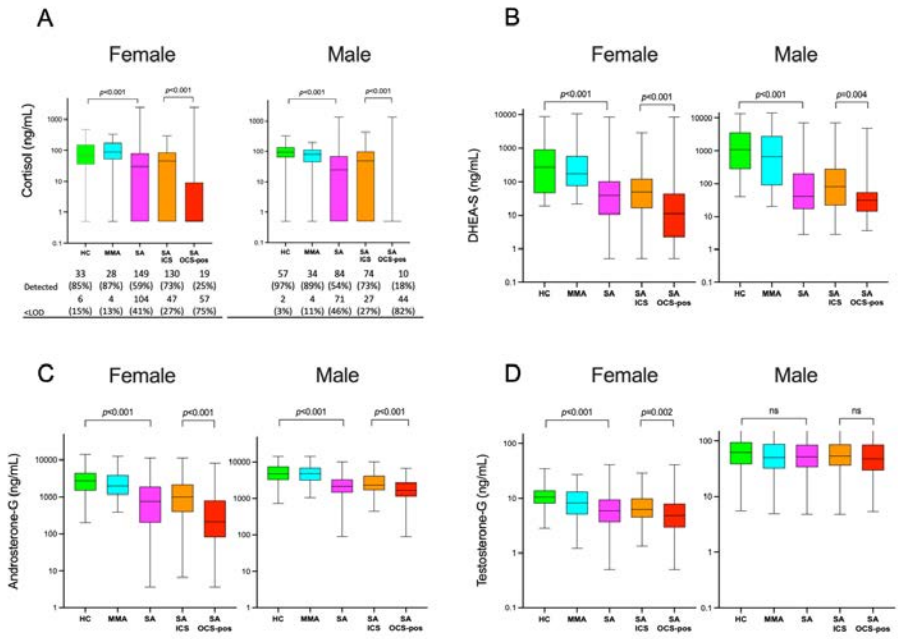


Figure 16. Levels of endogenous steroids in the urine of female and male study participants. HC=healthy controls, MMA=mild to moderate asthma, SA=all severe asthma, SA ICS=severe asthma with no detectable metabolites of oral corticosteroids in the urine, SA OCS-pos=severe asthmatics with detectable metabolites of oral corticosteroids in the urine. A. Cortisol; B. DHEA-S; C. Androsterone-G; D. Testosterone-G. Data are presented as median (interquartile range). Significance was evaluated using a Mann-Whitney U-test for comparisons between 2 groups, and a Kruskal-Wallis-test for comparisons of 3 groups. (Yasinska et al., 2023)

All patients in the group with the lowest DHEA-S levels were female severe asthmatics with poorer asthma outcomes that in the high DHEA group (**Figure 17**, **Paper II: Table 4**).

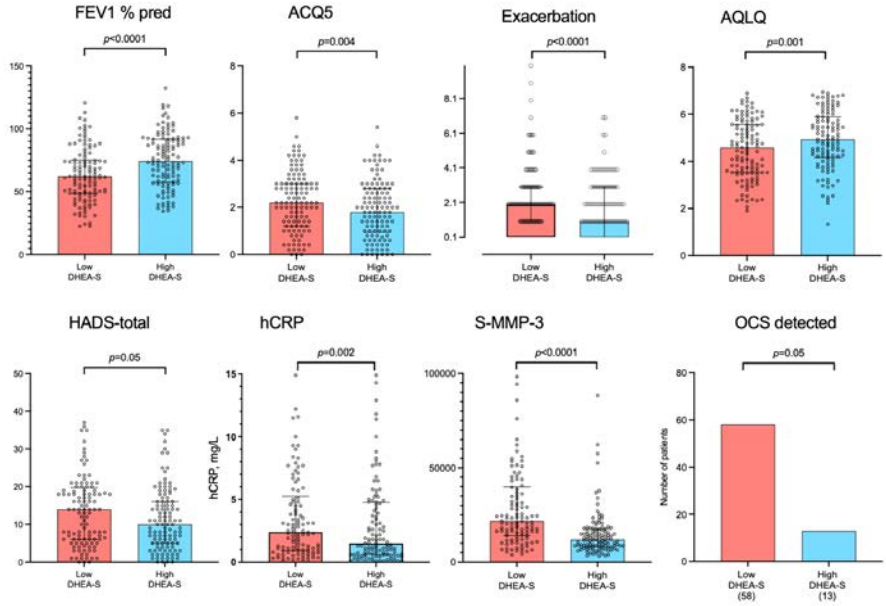


Figure 17. Clinical variables shown in patients stratified according to DHEA-S levels (lowest and highest quartiles). AQLQ, FEV_1 % predicted, exacerbations and ACQ-5, hCRP, HADS total score, S-MMP3, and number of individuals with detectable urinary oral corticosteroid metabolites. Data are presented as median and IQR. Significance was evaluated using a Mann-Whitney U-test (or Chi-squared test to compare numbers with OCS detected).(Yasinska et al., 2023)

5.2.5 RELATIONSHIP BETWEEN ENDOGENOUS ANDROGENS AND CLINICAL OUTCOMES

We stratified all asthmatics according to the key androgen DHEA-S (**Figure 17**, **Paper II: Table 4**) and examined clinical and biomarker outcomes in relation to its concentrations. The results in **Paper II** had shown that patients with more severe disease according to clinical outcomes such as more increased ACQ-5, more decreased AQLQ, higher HADS score, poorer lung function, highest number exacerbation per year, as well as higher proportion of OCS-positive asthmatics (**Figure 17, Paper II: Table 4**) had also the lowest levels of DHEA. There was an association between lung function and DHEA concentrations in urine in both sexes. This finding was in line with observations from other studies. For example, report from the US SARP (NHLBI funded Severe Asthma Research Programme) consortium had shown that DHEA concentrations in blood were associated with patient-reported symptoms and lung function regardless sex, and asthmatics with high expression of androgen receptors in bronchial epithelial had better lung function (Zein et al., 2021). Moreover, some experimental asthma models reported several beneficial effects of androgens (Cephus et al., 2017; Fuseini et al., 2018; Koziol-White et al., 2012). Finally, an RCT study showed improvement in asthma control in patients with moderate and severe asthma after six weeks treatment with nebulised DHEA, but without change in lung function (Wenzel et al., 2010). Given that the previous

studies had shown signs that androgens have favorable anti-asthmatic properties and the pronounced suppression of androgenic level according to the results in our study, it is considered important to assess not only the adrenal function, but also levels of androgen steroids in severe asthmatics treated with high doses of oral corticosteroids. At this time, we don't have enough evidence that androgen steroids can be used as a treatment of asthma, although there are indications of an anti-asthmatic property of androgens. The previous studies are too few and not large enough, so more studies are required. However, patients with insufficient production of androgen steroids may be referred to endocrinologist for assessment whether indication exists for substitution therapy.

5.2.6 SUMMARY

Severe asthma was associated with pronounced suppression of biosynthesis of androgens and cortisol, which had been shown in the U-BIOPRED study through intensively measurements of endogenous and exogenous steroid metabolites in urine.

The data show that this adrenal suppression is mainly caused of treatment with exogenous corticosteroids in dose depended manner, which is in line with a previous small study (Coll et al., 2018).

Our findings lend support to the hypothesis that sex differences in asthma severity and prevalence (Leynaert et al., 2012; The, 2003) may partly be related to the greater relative deficiency in production of androgens in women in comparison to men.

The evaluating corticosteroid exposure from only patient's self-reported data and from prescription records without measurement of urinary OCS metabolites may be insufficient.

Our study supports that the decrease of high dose ICS, and in particular, the tapering of OCS, can reduce the side effects of corticosteroids and should be the clinical goal (Gurnell et al., 2021). Adrenal function and androgen levels should be assessed in all patients with severe asthma treated with maintenance oral corticosteroids.

5.3 A QUANTITATIVE SCORE ALGORITHM TO REFINE RESPONSE EVALUATION IN MEPOLIZUMAB TREATED SEVERE ASTHMATICS (Paper III).

Paper III included data from 77 severe asthma patients enrolled in the BIOCROSS study. All patients were eligible for specific therapy targeting type 2 inflammation and therefore started treatment with the biologic mepolizumab and completed at least 12-months of follow-up.

5.3.1 EFFECTS OF 12 MONTHS OF MEPOLIZUMAB IN ALL 77 PATIENTS (TABLE 5)

An analysis of clinical outcomes in the BIOCROSS sub-study at the 1-year follow-up visit showed a positive, group-level effect of treatment with mepolizumab. Median blood eosinophilic count (B-eos) decreased to 50 counts per μL as expected, due to the anti-IL-5 effect of this drug. The proportion of patients on mOCS was halved (26%) compared to baseline (52%) (**Table 5**), the median dose of mOCS per day decreased by 74% and exacerbation rate decreased by 90%. For the entire group, a significant improvement was seen in FEV1 % predicted by 6%, ACQ-5 by -1.4 and AQLQ by 1.3 (**Table 5**).

Table 5. Clinical characteristics of severe asthma patients at baseline and after 1 year of mepolizumab treatment in the BIOCROSS study

Variable	Baseline (n=77)	1 year (n=77)	*p*-value
Number of patients with exacerbation	72 (94%)	12 (16%)	<0.0001
Exacerbations number/year	4 (3-6)	1.5 (1-3)	0.0005
$FEV_{1,}$ (L)	2.31 (1.86-2.89)	2.59 (2.12-3.16)	0.002
FEV_1, (%) pred	84 (61-96)	90 (76-104)	0.0001
FEV_1/FVC	64 (54-71)	66 (58-72)	0.01
FEV_1 >80% (n)	44 (57%)	53 (69%)	0.13
ACQ-5 score	2.0 (1.4-3.0)	0.6 (0.2-1.3)	<0.0001
0.75<ACQ-5 ≤ 1.5 (n)	8 (10%)	20 (26%)	0.005
ACQ-5 ≤ 0.75 (n)	12 (16%)	40 (52%)	<0.0001
AQLQ score	5.0 (4.2-5.6)	6.3 (5.3-6.8)	<0.0001
mOCS (n)	40 (52%)	20 (26%)	0.001
OCS dose, median (mg/day)	10.0 (5.0-10.0)	3.1 (2.5-5.0)	<0.0001
ICS, budesonide-equivalent dose (μg)	2350 (1800-2840)	2300 (1800-2750)	0.17
Blood eosinophils (counts $x10^9$ L^{-1})	330 (200-610)	50 (29-90)	<0.0001
Blood eosinophils (n=counts ⩾$0.3x10^9$ L^{-1})	46 (60%)	2 (3%)	<0.0001
IgE ($IU \cdot mL^{-1}$)	190 (79-275)	160 (64-270)	0.27
IgE ⩾150 $IU \cdot mL^{-1}$ (n)	38 (60%)	40 (52%)	0.25

F_ENO (ppb)	36 (26-66)	31 (19-53)	0.007
hs-CRP (mg/mL)	1.3 (0.5-3.2)	1.4 (0.6-3.2)	0.8
EDN-plasma (ng/mL)	35.5 (22.2-53.5)	12.0 (9.9-16.6)	<0.0001
LTE_4 (pg/mL)	101 (68-207)	72 (53-138)	0.0002
Tryptase (µg/L)	4.5 (3.3-5.7)	4.5 (3.2-5.8)	0.06
TetranorPGDM (pg/mL)	2400 (1600-3900)	2100 (1400-4100)	0.48

Data are presented as n (%) or median (interquartile range). Significance evaluated by Mann-Whitney U-test and Chi-squared test.

However, a deeper analysis of individual patients' responses revealed that some patients displayed remarkably large improvements, whereas others achieved only sufficient differences in clinical symptoms. In order to better understand differences in response among patients and evaluate the efficacy of mepolizumab, we tested a new quantitative algorithm, the so called M-COMSA score.

5.3.2 DISTRIBUTION OF M-COMSA SCORES FOLLOWING 12 MONTHS OF TREATMENT

After calculating total M-COMSA score for each patient, the minimum M-COMSA score sum was 1 and the maximum was 15 (**Figure 18B**). Since all 77 patients included in this sub-study had a reduction, either in OCS use or/and in exacerbation rate, and their M-COMSA score sum was higher than 0, no patients were judged to belong to a negative response group (**Figure 18B**). Subsequently, all patients were divided into four groups depending on the distribution of their M-COMSA score sum. The median M-COMSA score sum was 8, with a 25th percentile of 5.0 and a 75th percentile of 10.

Of the 77 patients that started mepolizumab treatment, 14 (18%) were classified as super responders (**Figure 12**). They had an average M-COMSA sum score of 12.9 (**Figure 18A**) which was the highest sum score (above 75th percentile). All patients in this group showed the greatest improvement in all five outcomes at the 12 month follow-up visit; a significant reduction from 2.2 to 0.1 in ACQ-5 score (p=0.0002), a significant reduction in OCS-use (median dose, mg/day) by 94% compared to baseline (p=0.0002), a significantly improved quality of life (AQLQ) (p=0.0001) and lung function regarding FEV_1 % pred (p=0.003) from 64% to 88% (**Figures 19A-C, Figures 20A-B**). They had no exacerbations during the first year of mepolizumab treatment (**Table 6**).

Patients with an M-COMSA score ≥ 50th percentile and ≤ 75th percentile were assessed as substantial responders. Thirty patients (39%) belonged to this group with an average value of 9.0 in M-COMSA score sum (**Figure 18A**). These patients also showed improvement in all five clinical outcomes at the 12-month follow-up visit with significantly decreased ACQ-5 (p<0.0001) and significantly increased AQLQ (p<0.0001) and FEV1 % predicted (p=0.0002)

from 85% to 92%. Exacerbations were reduced by 98% and OCS use by 68% in this group, with significant differences compared to baseline (**Figures 19A-C**, **Figures 20A-B**, **Tables 6-7**). However, the improvement was lower than in the super responder group (**Paper III: Table S3B).**

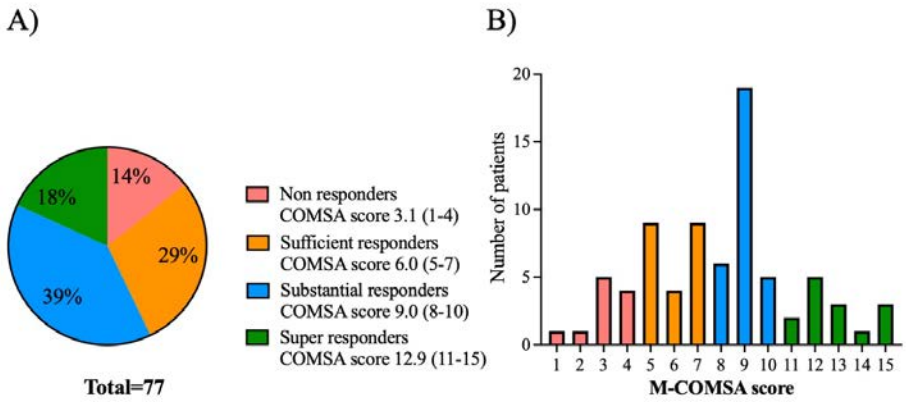
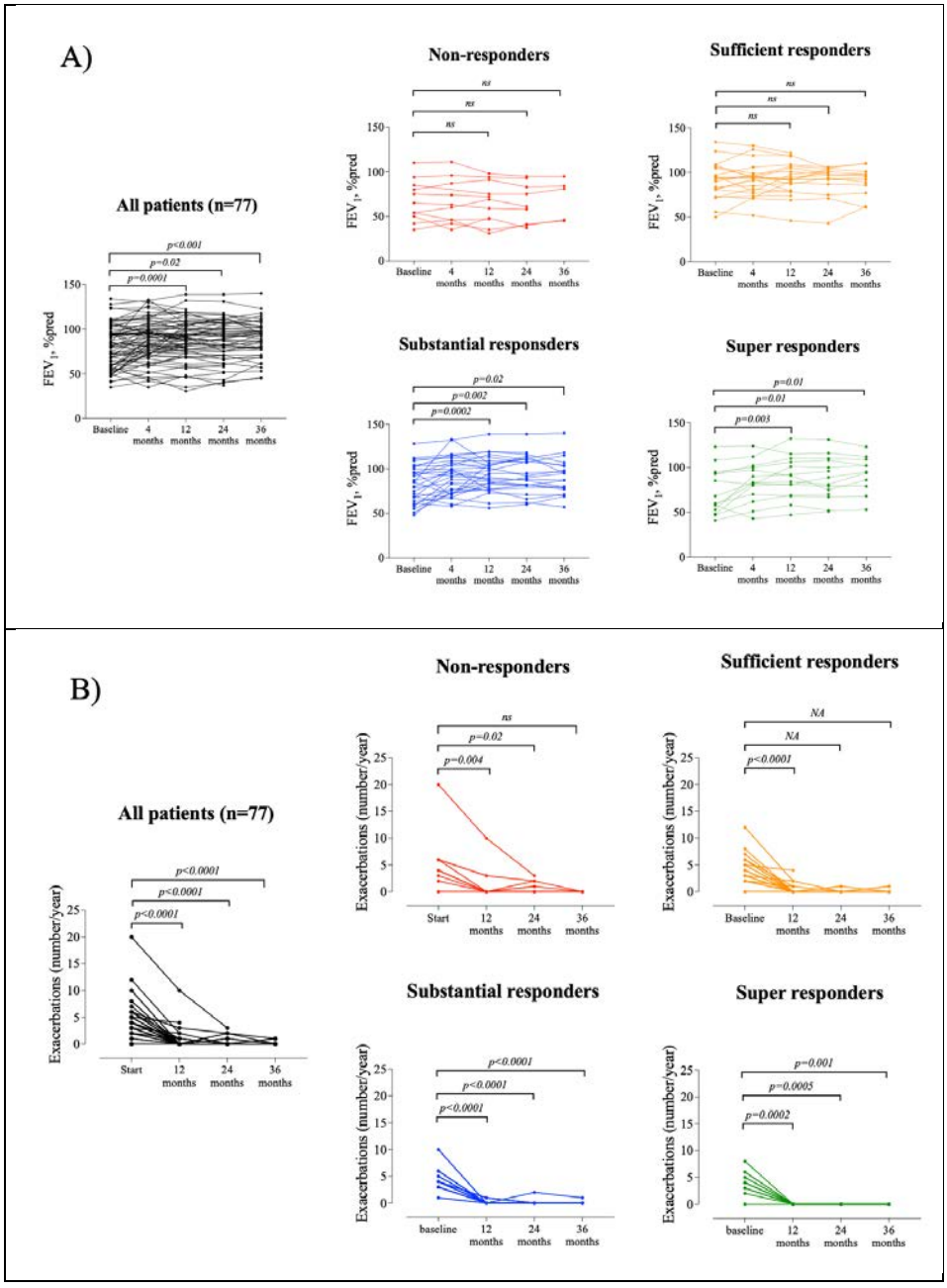


Figure 18: M-COMSA score distribution at 12 months showing (A) proportion of patients in the responder groups and (B) frequency of M-COMSA score in the BIOCROSS study.

Twenty-two patients (29%) had an M-COMSA score sum ≥ 25th percentile and < 50th percentile, with an average score between 5 and 7 (**Figures 18A-B**). They were judged to belong to the group of sufficient responders. They had a significantly decreased ACQ-5 ($p=0.0001$), significantly increased AQLQ ($p<0.0001$) and exacerbations were reduced by 77% ($p<0.0001$), as well as use of mOCS by 74% ($p=0.01$). However, they did not experience any change in lung function (**Figures 19A-C**, **Figures 20A-B**, **Tables 6-7**; **Paper III: Table S4C**).

Finally, non-responders had the lowest total M-COMSA score sum (below the 25th percentile). This group consisted of 11 patients (14% of all 77) with a sum score between 1 and 4 (**Figures 18A-B**). Exacerbations were significantly reduced in this group by 83% ($p=0.03$) and mOCS use by 67% ($p=0.004$), but there was no improvement in ACQ-5. Lung function was found to decline by 86 mL on average, AQLQ was decreased by 0.5, ICS dose was increased by 11%, LAMA by 33% and the use of reliever medications by 5% (**Figures 20A-B**, **Tables 6-7**; **Paper III: Table S4D).**


A)
All patients (n=77)
FEV1, %pred
p<0.001
p=0.02
p=0.0001
Baseline 4 months 12 months 24 months 36 months
Non-responders
ns
Sufficient responders
Substantial responsders
p=0.02
p=0.002
p=0.0002
Super responders
p=0.01
p=0.01
p=0.003
B)
All patients (n=77)
Exacerbations (number/year)
p<0.0001
Start 12 months 24 months 36 months
Non-responders
p=0.02
p=0.004
Sufficient responders
NA
Substantial responders
baseline 12 months 24 months 36 months
Super responders
p=0.001
p=0.0005
p=0.0002

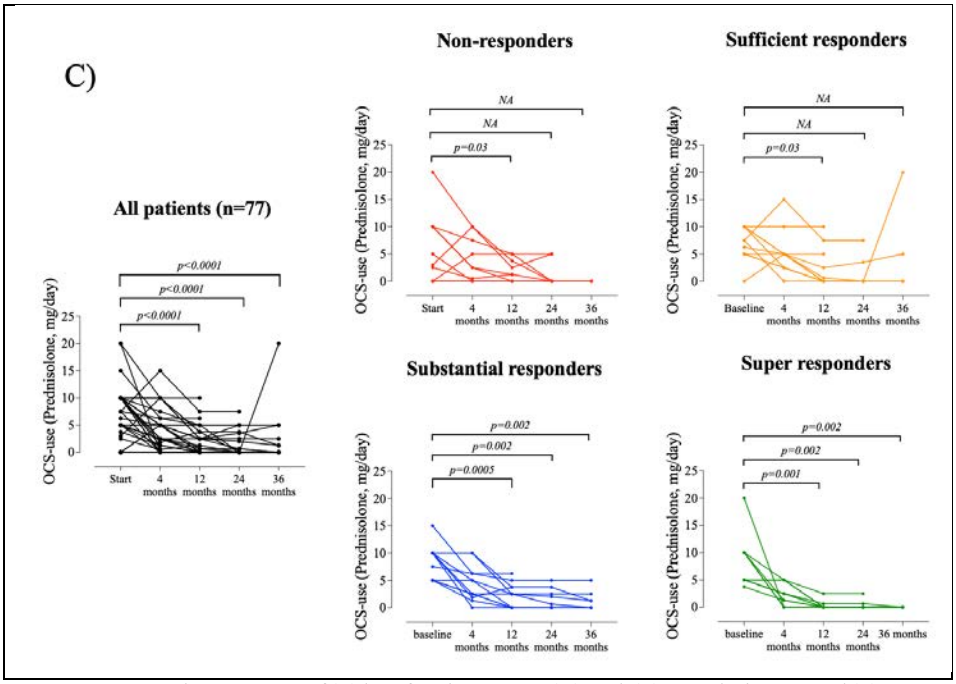


Figure 19: Repeated measurement of (A) lung function as FEV_1 (%) pred, (B) exacerbation rate, and (C) mOCS use during 36 months of mepolizumab treatment in the four M-COMSA groups from the BIOCROSS study.

5.3.3 DIFFERENCES IN CLINICAL AND PATIENT REPORTED OUTCOMES BETWEEN RESPONSE GROUPS AT THE 12 MONTHS FOLLOW-UP

As shown in **Figures 19A-C** and **Table 6**, there were differences in clinical results between all responder groups after 12 months of treatment with mepolizumab in each of three clinical outcomes. Lung function (FEV_1 % pred) was increased in both super responders and substantial responders by 25% and 14% respectively, whereas in non-responders lung function declined by 3% and minimal increased with 4% in the sufficient responders. Super responders showed the greatest significant improvements in all clinical outcomes (**Paper III: Table S3B**).

Moreover, the super responders and substantial responders had significantly increased FEV_1/FVC ratios, as a sign of reduced obstruction which could indicate a reduction in airway remodeling (**Table 6**). However, the FEV_1/FVC ratio did not change in the non-responders and sufficient responders.

Table 6. Change from baseline in main clinical outcomes per modified COMSA group.

Group	Exacerbations (n)				Change in FEV_1 % predicted (% change)				Change (Δ) in FEV_1 in mL			
Visit	*4*	*12*	*24*	*36*	*4*	*12*	*24*	*36*	*4*	*12*	*24*	*36*
Non-responders	*NA*	**-83%** (*n*=9)	**-64%** (*n=8*)	**-100%** (*n=4*)	**0%** (n=11)	**-3%** (n=11)	**-4%** (n=9)	**5%** (n=5)	**-4** (n=11)	**-86** (n=11)	**-110** (n=9)	**-38** (n=5)
Sufficient responders	*NA*	**-77%** (*n*=22)	**-95%** (*n*=17)	**-80%** (*n*=14)	**2%** (n=22)	**4%** (n=22)	**1%** (n=17)	**3%** (n=14)	**30** (n=22)	**-15** (n=22)	**-15** (n=17)	**9** (n=14)
Substantial responders	*NA*	**-98%** (*n*=30)	**-99%** (*n*=23)	**-99%** (*n*=19)	**14%** (n=30)	**14%** (n=30)	**11%** (n=23)	**12%** (n=20)	**259** (n=30)	**259** (n=30)	**191** (n=23)	**213** (n=20)
Super responders	*NA*	**-100%** (*n*=14)	**-100%** (*n*=13)	**-100%** (*n=11*)	**19%** (n=11)	**25%** (n=11)	**25%** (n=11)	**23%** (n=9)	**360** (n=11)	**462** (n=11)	**426** (n=11)	**390** (n=9)
Group	**Change in FEV1/FVC ration (% change)**				**Change (Δ) ACQ-5 score**				**Change (Δ) AQLQ score**			
Visit	*4*	*12*	*24*	*36*	*4*	*12*	*24*	*36*	*4*	*12*	*24*	*36*
Non-responders	**-1%** (n=10)	**-2%** (n=11)	**-2%** (n=9)	**4%** (n=5)	**0.1** (n=10)	**0.1** (n=11)	**0.4** (n=9)	**0.6** (n=5)	**0.1** (n=10)	**-0.5** (n=11)	**0.4** (n=9)	**0.6** (n=5)
Sufficient responders	**-1%** (n=17)	**0%** (n=17)	**0%** (n=13)	**0%** (n=11)	**-0.6** (n=22)	**-0.6** (n=22)	**-0.7** (n=17)	**-0.5** (n=14)	**1.0** (n=22)	**0.8** (n=22)	**-0.7** (n=17)	**-0.5** (n=14)
Substantial responders	**6%** (n=23)	**5%** (n=23)	**3%** (n=16)	**5%** (n=13)	**-1.5** (n=30)	**-1.5** (n=30)	**-1.4** (n=21)	**-1.4** (n=19)	**1.3** (n=29)	**1.5** (n=29)	**1.4** (n=21)	**1.4** (n=19)
Super responders	**11%** (n=14)	**13%** (n=14)	**11%** (n=13)	**12%** (n=11)	**-1.7** (n=13)	**-2.3** (n=14)	**-2.3** (n=14)	**-2.6** (n=11)	**1.6** (n=13)	**2.1** (n=14)	**2.3** (n=14)	**2.6** (n=11)

The modified COMSA group assignment based on 12-month visit (Values based on average % change for FEV_1 % predicted and for FEV_1/FVC ratio. For FEV_1 in mL, values based on average of change. For ACQ-5 and AQLQ score, values are based on average point change).

Furthermore, the super responders showed the greatest improvements in both patient reported outcomes as compared to other response groups, with $p<0.0001$ for both ACQ-5 and AQLQ (**Figures 20A-B** and **Table 6**).

We also tested the hypothesis that the M-COMSA algorithm would allow responses to be assessed as early as the 4-month follow-up visit. In fact, the super responders and substantial responders did show significant improvement in all five clinical outcomes already after 4 months of treatment with mepolizumab, with increases in lung function (FEV_1) of 360 mL and 259 mL respectively, whereas lung function did not improve in the sufficient responders and non-responders (**Table 4 and Table 4A-D**). Moreover, the majority of patients obtained similar results at the 12-month follow-up. The results therefore indicate that might be possible to predict response to therapy as early as the 4-month follow-up.

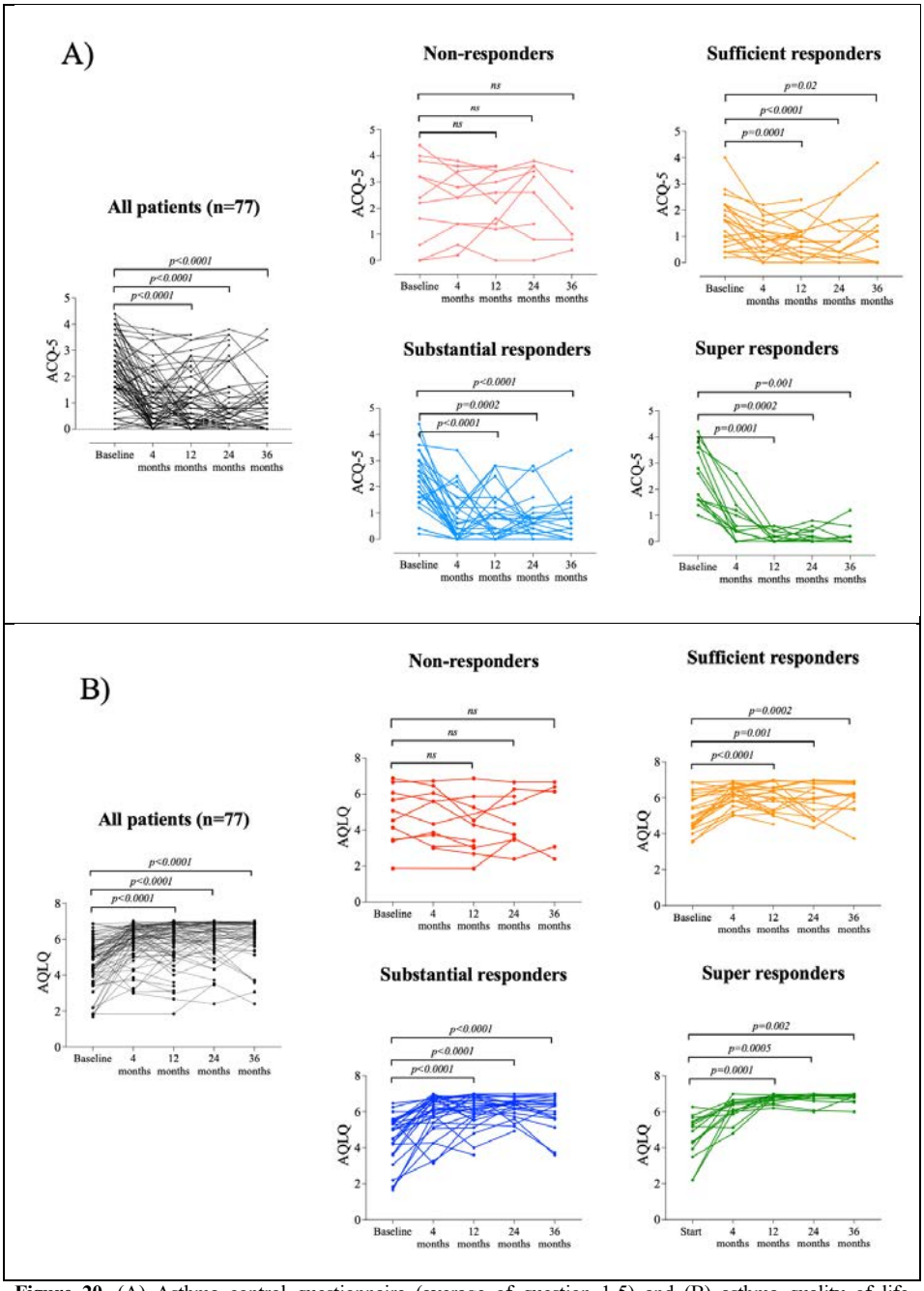


Figure 20. (A) Asthma control questionnaire (average of question 1-5) and (B) asthma quality of life questionnaire, at baseline and at 4-36 months of mepolizumab treatment in the BIOCROSS study.

Thus, the use of the M-COMSA algorithm as composed of five domains led to a better assessment of response to mepolizumab treatment and enabled classification of all 77 patients into different groups depending on response: super responders, non-responders, and two intermediate groups, sufficient responders and substantial responders.

5.3.4 DIFFERENCES IN MAINTENANCE TREATMENT BETWEEN RESPONSE GROUPS AT THE 12 MONTH FOLLOW-UP

Super responders were also able to reduce both their dose of mOCS and ICS at the 12-month control, compared to the three other groups (Table S4). In addition, they could also reduce other maintenance asthma medications such as LABA, LAMA and relievers to a greater extent than other responder groups (**Figure 7**).

Table 7. Group average change from baseline in medications in the BIOCROSS study.

Group	**mOCS**				**ICS**				**Reliever**			
Visit	***4***	***12***	***24***	***36***	***4***	***12***	***24***	***36***	***4***	***12***	***24***	***36***
Non-responder	**-33%** (*n*=7)	**-67%** (*n*=7)	**-75%** (*n*=6)	**-100%** (*n*=2)	**0%** (*n*=10)	**11%** (*n*=11)	**4%** (*n*=9)	**-16%** (*n*=5)	**-7%** (*n*=10)	**5%** (*n*=11)	**-28%** (*n*=9)	**-23%** (*n*=5)
Sufficient responder	**-30%** (*n*=10)	**-74%** (*n*=9)	**-76%** (*n*=7)	**-25%** (*n*=4)	**-5%** (*n*=22)	**-1%** (*n*=22)	**-11%** (*n*=17)	**-6%** (*n*=14)	**-15%** (*n*=22)	**-25%** (*n*=22)	**-34%** (*n*=17)	**-23%** (*n*=14)
Substantial responder	**-43%** (*n*=13)	**-68%** (*n*=13)	**-84%** (*n*=10)	**-88%** (*n*=9)	**-2%** (*n*=30)	**5%** (*n*=30)	**3%** (*n*=23)	**-2%** (*n*=19)	**-29%** (*n*=30)	**-30%** (*n*=30)	**-32%** (*n*=21)	**-36%** (*n*=19)
Super responders	**-44%** (*n*=11)	**-94%** (*n*=11)	**-94%** (*n*=10)	**-100%** (*n*=9)	**-6%** (*n*=14)	**-13%** (*n*=14)	**-14%** (*n*=13)	**-18%** (*n*=11)	**-22%** (*n*=13)	**-44%** (*n*=14)	**-32%** (*n*=14)	**-61%** (*n*=11)
Group	**LABA**				**LAMA**				**Montelukast**			
Visit	***4***	***12***	***24***	***36***	***4***	***12***	***24***	***36***	***4***	***12***	***24***	***36***
Non-responders	**-5%** (*n*=10)	**0%** (*n*=11)	**-14%** (*n*=9)	**-20%** (*n*=5)	**0%** (*n*=2)	**33%** (*n*=4)	**67%** (*n*=3)	**67%** (*n*=3)	**0%** (*n*=6)	**0%** (*n*=8)	**-17%** (*n*=6)	**20%** (*n*=4)
Sufficient responders	**-10%** (*n*=22)	**2%** (*n*=22)	**2%** (*n*=17)	**5%** (*n*=14)	**25%** (*n*=4)	**-25%** (*n*=4)	**40%** (*n*=5)	**25%** (*n*=4)	**-6%** (*n*=17)	**-21%** (*n*=17)	**-27%** (*n*=13)	**-36%** (*n*=11)
Substantial responders	**-1%** (*n*=30)	**-1%** (*n*=30)	**-12%** (*n*=22)	**-14%** (*n*=19)	**-25%** (*n*=11)	**-50%** (*n*=11)	**-33%** (*n*=7)	**-50%** (*n*=8)	**-7%** (*n*=23)	**-15%** (*n*=23)	**-13%** (*n*=16)	**-15%** (*n*=13)
Super responders	**-8%** (*n*=14)	**-12%** (*n*=14)	**-14%** (*n*=13)	**-14%** (*n*=11)	**-33%** (*n*=6)	**-33%** (*n*=6)	**-33%** (*n*=6)	**-40%** (*n*=5)	**0%** (*n*=10)	**10%** (*n*=10)	**5%** (*n*=10)	**0%** (*n*=8)

Values based on average % change.

In fact, non-responders significantly increased their dose of ICS from 2300 µg to 2880 µg (**Paper III: Table S4D**), and three patients (27%) added LAMA to their treatment regimens due to insufficient control of asthma symptoms. So, despite managing to reduce OCS and/or exacerbations, they still increased their dose of ICS. It is known (Maijers et al., 2020) that high doses of ICS in asthma do have an oral steroid-sparing effect, which means that these higher ICS doses partly replaced the reduced oral corticosteroid intake. This is also in line with the observations we made in **Paper II**, when high doses ICS caused suppression of endogenous cortisol and androgens much like OCS use.

5.3.5 DIFFERENCES BETWEEN RESPONSE GROUPS AT BASELINE

The baseline clinical characteristics were similar for the four identified response groups regarding age, body mass index and age at onset of disease (**Paper III: Table 4**). However, there was a significant difference in the percentage of women between groups and fewer women were super responders (21%) compared to non-responders (55%), sufficient responders (41%) and substantial responders (57%). Moreover, the super responder and the substantial responder groups only consisted of never-smokers, whereas there were ex-smokers among the non-responders and sufficient responders (**Paper III: Table 4**).

The four response groups did not differ with respect to the number of exacerbations in the previous 12 months before start of mepolizumab treatment, the daily dose of maintenance oral corticosteroids and AQLQ scores. However, lung function differed significantly between all four groups, both for FEV_1 measured in L as well as % predicted. Interestingly, lung function does not differ significantly between non-responders and super responders at baseline. Response groups also differed regarding the presence of comorbidities (**Paper III: Table S2**). There were significant differences for the incidence of nasal polyps ($p=0.02$), allergic rhinitis ($p=0.002$) and aspirin sensitivity ($p=0.01$). The incidence of nasal polyps and allergic rhinitis were highest in the super responder group, and the incidence of aspirin sensitivity was lowest in the non-responder group. The levels of inflammatory markers were similar in all response groups with respect to baseline blood eosinophils and IgE, although F_ENO levels were significantly highest in the super responders. The proportion of patients with high levels of F_ENO (>40 ppb) was also significantly higher in super responder group compared to other response groups.

Further analyses comparing non-responders and super responders showed that the odds for patients with a high F_ENO (>40 ppb) to be super responders were 11-fold higher (Chi-squared test) than for patients with a low F_ENO level. Similar results were found for patients with aspirin sensitivity (5.6-fold higher odds), nasal polyps (2.0-fold higher odds) and allergic rhinitis (15-fold higher odds) where patients with these comorbidities were more likely to be super responders. Thus, the higher the expression of type 2 inflammation, the greater the likelihood of being a super responder. This observation confirms previously published results, including RCTs (Pavord et al., 2012; Szefler et al., 2012)

5.3.6 STABILITY OF M-COMSA GROUP ASSIGNMENT OVER TIME

In total, 47 (61%) patients of the 77 included in this sub-study attended both 2- and 3-year follow-ups, providing complete clinical evaluations and biological sample collection at these time points. We tested the M-COMSA algorithm to investigate the stability of response groups after two and three years of mepolizumab treatment (**Figure 21**). Results showed that all super responders maintained their group. None of these patients experienced any exacerbations and stopped using mOCS during the 3-year follow-up period. Their asthma was well-controlled according to ACQ-5, they also had good quality of life according to AQLQ and improved lung function (**Paper III: Table S4A**). Most of the substantial responders also maintained their

group. However, certain substantial responders (47%) increased their total M-COMSA scores during year 2 and were able to transition to the super responder group (**Figure 21, Paper III: Table S4B**).

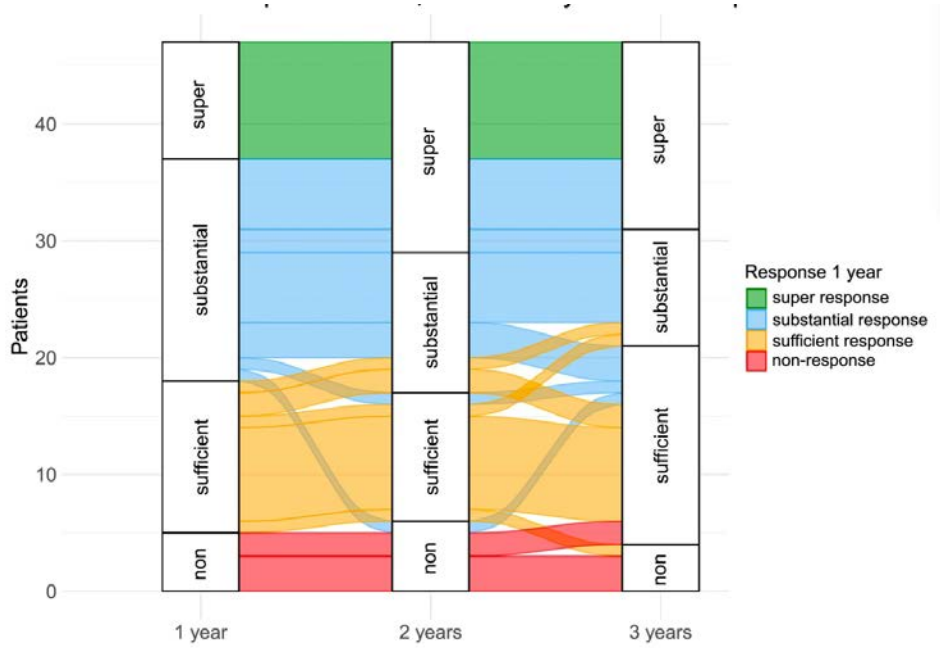


Figure 21. Changes in BIOCROSS responder groups at two- and three-year follow-up. Please note that some patients un the substantial responder group reached super responder score after 2 and 3 years.

Moreover, most of the sufficient responders (64%) remained unchanged regarding their M-COMSA scores at the 2- and 3-year follow-up, whereas most non-responders experienced continued exacerbations, loss of asthma control according to ACQ-5 and were switched to a different biologic during the 3 years of mepolizumab treatment.

Overall, 72% of patients remained in their M-COMSA group at 24-36 months. Furthermore, the super responders and most of the substantial responders had improved lung function and achieved well-controlled asthma, did not have asthma exacerbations and stopped use of OCS for 3 years. This type of treatment response should be the ultimate treatment target for expensive biological therapies and is the reason we need ongoing discussions around the world about treatment-induced remission (A. Menzies-Gow et al., 2020; Thomas et al., 2022). The M-COMSA algorithm would be one suggestion for further evaluation in clinical practice and observational studies, with the goal to refine assessment of response.

5.3.7 SUMMARY

The modified COMSA scale that includes composite clinical outcomes was able to quantify response to an expensive biological treatment and identify four groups with different degrees of response to mepolizumab: super response, substantial response, sufficient response, and non-response. The super responder group had the greatest improvement in lung function, AQLQ, ACQ-5 and the highest reductions in exacerbation and OCS use, whereas the non-responders lost asthma control, discontinued mepolizumab treatment and switched to other biologics.

The modified COMSA might be useful for early response assessment since the super responders had already after 4 months of mepolizumab treatment significant improvements with greater increases in all domains compared to sufficient responder and non-responder groups.

This new, quantitative algorithm including patient reported outcomes is shown to be a more individual-adapted assessment of treatment response and highlights non-responders in need of revised treatment.

Implementation of the M-COMSA strategy in clinical practice would allow greater precision in early clinical decision-making, regarding the use of biological therapy, and could be the algorithm of choice for assessment of treatment-induced remission.

6 CONCLUSIONS

6.1 Paper I.

Through analysis of data from different registries of severe asthma across Europe, we could:

1. Discover and describe strengths/weaknesses in those registries.
2. Describe differences in the characteristics of patients in European severe asthma registries and compare their treatment regimens before and after starting treatment with biologics.
3. Show the importance of harmonization of severe asthma databases across Europe and the need for long-term follow-up of this group of patients.
4. Reveal that the definition of severe asthma in current guidelines does not comply with the characteristics of real-word severe asthmatics.

6.2 Paper II.

1. By measuring and analyzing the concantrations of endogenous steroids, including androgens in urine in patients with different severities of asthma and healthy controls we could show that:
   a) Severe asthma is associated with pronounced suppression of biosythesis of both androgens and cortisol.
2. By measuring and analyzing the concentrations of exogenous steroids (prednisone metabolites) in urine, present in severe asthmatics, and by studying their relationship with endogenous steroids, we could show that:
   a) The adrenal suppression is mainly caused of treatment with exogenous corticosteroids.
   b) The corticosteroids have a dose-dependent effects on the adrenal insufficience.
   c) The evaluating corticosteroid exposure from only patient's self-reported data and from prescription records without measurement of urinary OCS metabolites may be insufficient.
3. Stratification of all asthmatics according to levels of the androgen DHEA revealed “a correlation between urinary DHEA and FEV_1 (% predicted)”(Yasinska et al., 2023).
4. In addition, having low levels of DHEA-S and other androgens was associated with more severe asthma, poorer asthma control and worse quality of life.
5. The findings in our study lend support to the hypothesis that a greater relative deficiency in production of androgens in women in comparison to men, may in part explain sex differences regarding asthma severity and prevalence.

6.3 Paper III.

By modification of the COMSA strategy into a quantitative algorithm with five equally weighted clinical outcomes, and implementation of this in a prospective, observational, real-life study BIOCROSS, we were able to refine assessment of the treatment effect of mepolizumab and show that:

I. A composite core outcome measure including patient reported outcomes is a good algorithm to identify not only non-responders, but even patients with varying degrees of response to mepolizumab.
II. This new quantitative algorithm was shown to be a more individually adapted assessment of treatment response, that provides early identification of non-responders in need of revised treatment.
III. The Modified Core Outcome Measure in Severe Asthma (M-COMSA) strategy can be useful in a real world setting.

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7 GENERAL DISCUSSION

Asthma is a heterogeneous disease with various underlying phenotypes. Several mechanistic discoveries have been made during recent decades facilitating the development of new biological drugs that bind to very specific targets at a molecular level, such as interleukin (IL)-5, the IL-4 receptor (IL-4R), IL-5R, IL-13, and TSLP, all of which have been introduced for the treatment of severe asthma. For these new expensive medicines to achieve their expected effects and be able to help the most severe asthmatics that suffer daily from breathing problems, it is essential that the right medicine is given to the right patient. This requires in-depth knowledge of biological drugs, and also how these work in a real-world setting. In addition, it is important to understand how best to assess and evaluate the effects of these drugs in clinical practice, and systematic follow-up of patients treated with biologics is required. Therefore, my research project focuses on investigating various phenotypes of severe asthma through clinical studies and linking these phenotypes to clinical outcomes and molecular mechanisms. Consequently, this thesis includes three clinical studies that highlight different issues regarding the management and care of severe asthma, all of which focus on clinical outcomes.

The first paper in this thesis was result of an early study within SHARP (Severe Heterogeneous Asthma Research collaboration, Patient-centered), which was a Clinical Research Collaboration (CRC) on severe asthma initiated by the European Respiratory Society (ERS) in 2018. One of the specific goals of SHARP was to provide a thorough description of the severe asthma cohorts, along with treatment effects in severe asthma groups from across Europe. As stated in Results and Conclusions, this goal was achieved, but this initial attempt to integrate register data obtained throughout Europe had both limitations and strengths.

This study included over 3000 patients with severe asthma and was “one of the largest comparisons of this population, which provided insight into the characteristics and treatments of this heterogeneous group across Europe” (van Bragt et al., 2020). The study had a good geographical distribution “with representation from Southern, Western, Eastern, and Northern Europe” (van Bragt et al., 2020) which includes the influence of differences in environmental and genetic factors and in healthcare systems, which contributed also to the observed heterogeneity in the asthma population.

One of the largest weaknesses is the retrospective nature of the study, which lead to significant variation in inclusion criteria, and only half of the registers used the joint ERS/ATS definition of severe asthma. In addition, not all patients were treated in a specialized asthma center, which reflected diversity regarding what clinics “in different European countries consider to be severe asthma” (van Bragt et al., 2020). A further important restriction is that some registers are focused on the inclusion of patients who have begun biological therapies, such as those in the Netherlands, Sweden, and Slovenia, which is expected to result in cohorts consisting of the most severe patients. Since the data from Sweden only was from one hospital, Karolinska University Hospital in Stockholm, it was also judged that this data did not necessarily reflect the situation throughout the country.

One of the main strengths of this study is that the issues that were highlighted in **Paper I** demonstrate that all asthma research must take into account the complexity and heterogeneity of this disease in different populations. The greatest strength of this descriptive study however was that, according to my opinion, it generated new hypotheses and highlighted issues that formed the basis for future SHARP collaborations and led to the development of new studies. In addition, the study also highlights important unmet needs regarding healthcare for the most severely ill patients in Europe. These included:

- The need for discussions about more standardized practices for severe asthma care in Europe.
- The need for an international agreement on a minimal set of well-defined key variables to increase the utility of the SHARP platform and provide opportunities for future research (van Bragt et al., 2020).

In my opinion, the most surprising finding regarding the treatment aspect of **Paper I,** was the use of maintenance oral corticosteroids and the variations in dose of OCS. Since one of the SHARP visions was to end dependency on oral corticosteroids to achieve asthma control, it was natural that the second study (**Paper II**) in this thesis was about the negative side effects of mOCS treatment, which also revealed sex differences in the context of asthma from a new angle.

This study has both strengths and weaknesses. One limitation was that we had no information about females' sex hormones as the WADA doping panel was designed to measure EAAS only and a relationship between androgens and females sex hormones cannot be calculated. This would have led to a better understanding of clinical outcomes and could help predict response to novel treatments. Similarly, no data was collected regarding menstrual cycle, the use of contraceptives or hormone replacements. However, in another study using the WADA platform, it was shown that concentrations of androgen metabolites did not change so much during a normal menstrual cycle (Mullen et al., 2017). Another of the weaknesses in this study is that the exact intake ICS dose for each patient did not record, thus a statistical method of correlation analysis between ICS dose and DHEA could not be provided.

The probability that the use of contraceptives could be a major confounder was not so great in our study, as there were only 27% of patients below the age of 45 years in SA group, where the greatest effects on androgens were observed.

The greatest effects on androgens in our study were observed in SA where only 27% of patients were below the age of 45 (**Paper II: Table S2**), making it unlikely that the use of contraceptives could be a major confounder. However, the use of hormone replacement therapy (HRT) could be a problem. In a large study based on the UK database in the primary care was estimated that 16% of women aged 46-70 used HRT (Shah et al., 2021). However, given the prevalence of severe asthma in the UK, we do not believe that this small subgroup would to be cause to the differences in the concentrations between women and men with SA. Another limitation was

that there were too few current smokers included in this study and we could not to assess the potential influence of smoking on steroid hormone levels.

The strengths of this prospective study included the amounts of data collected in large cohorts of well-characterized asthmatics of different severities compared to healthy controls. In fact, we know of no larger study of steroid hormones in asthma general and SA in particular. Moreover, a validated quantitative method was employed, also used by an international sports authority to disqualify athletes due to doping. The measurements also demonstrated the relative stability of urinary androgen metabolites analysed in two samples collected at different time points with 12-18 months between them.

In conclusion, this study demonstrates that treatment with mOCS is the main cause of not only adrenal suppression, but also decreased levels of endogenous androgens. Moreover, this suppression is more pronounced in women with significant differences in clinical outcomes compared to men. Finally, this study was able to demonstrate the association between severe asthma and suppressed androgen levels which led to the conclusion that the deficiency in androgen levels during steroid treatment in women could contribute in part to sex differences in asthma severity and prevalence.

The third study in this thesis is the first analysis from the ongoing BIOCROSS study which forms the main part of my research project. The BIOCROSS study was originally designed as a multicentre cross-sectional and observational study. When mepolizumab was introduced in Sweden in 2017 as the first new biologic for the treatment of severe asthma, a prospective design was added to follow patients undergoing treatment with biologics, with repeated visits to register clinical response and collect biological samples for biomarker analyses. During the period that patients were recruited and the BIOCROSS cohorts were built, the 3TR project was initiated by EU. Our research unit was involved in this project, and I participated in the COMSA Working Group, that sought to develop Core Outcome Measures (COM) sets to facilitate improved data synthesis, and appraisal of biologics in paediatric and adult asthma clinical studies. As a result of this collaboration, five definitions of response were selected and developed as the COMSA set.

While the COMSA set was being developed, we started compiling data for the first 77 patients from the BIOCROSS study who completed at least the 1-year follow-up visit, to plan for various analyses. Since the purpose of this study was to evaluate new biological drugs, including an assessment of their effects and of clinical response, it became obvious that COMSA should be tested in our real-life study. As a next step, the COMSA set was modified and developed into a new quantitative algorithm to assess response in the 77 patients included in the first analysis of clinical data in BIOCROSS. This method has now been shown to be useful for refining response evaluation in severe asthmatics treated with mepolizumab.

One limitation with our study is that we did not attempt to try different response scales for the five domains, e.g., seven or ten points per domain. Using a scale with greater resolution could perhaps have achieved greater precision in each patient. Furthermore, a domain-weighing

function would allow a rebalancing regarding the importance of each of the included five domains, perhaps better reflecting current clinical practice. On the other hand, the results indicate that the current approach works, and could easily be implemented in clinical practice and used during a patient visit.

8 POINTS OF PERSPECTIVE

Paper I

When paper was published in 2019, the urgent need to harmonize severe asthma datasets throughout Europe was highlighted, and a particular challenge was harmonization of the definitions of different variables. Since then, important work has been carried out to achieve an international consensus, which is now a reality. Moreover, following an agreement on the management of patient data between countries that participate in SHARP, an integration platform has been established, named SHARP Central. It is a central registry of patients with severe asthma in European countries, to assist countries that did not yet have their own severe asthma registry. The platform is linked to other countries that do have a national registry. According to agreement, both SHARP Central and other national registries include the same sets of variables, thereby providing opportunities for new studies on severe asthma across Europe. So far, the SHARP collaboration has generated seven different research projects, which have resulted in a few publications including **Paper I**, where our clinical unit is a co-author.

The future clinical perspective

Several different countries have now joined SHARP and to date, this collaboration involves 28 countries across Europe. In September 2023, national leaders for the 28 SHARP-federated European countries met at the ERS Congress in Milan to discuss current needs and possible solutions, to improve the care of people living with severe asthma.

The future research perspective

During the past year several new studies have been initiated and our research unit is part of three of them. Our severe asthma centre, in collaboration with the clinical lung and allergy research unit at Karolinska Institutet, was the only site in Sweden that joined SHARP at the beginning of this collaboration in 2018. After an agreement regarding the management of patient data within SHARP was established, and we received ethical approval, we used this opportunity to build the severe asthma cohort in a more centralized way, within the integration platform, SHARP Central registry. All university hospitals in Sweden now have the opportunity to participate in this registry. The biggest challenge facing each centre that wishes to join to the register is, in my opinion, a lack of resources. However, some sites have already joined. In the current environment where continuous discoveries are being made about the molecular mechanisms underlying severe asthma, and where new expensive biological treatment are becoming available, large amounts of clinical data are required to facilitate these processes that can only be collected via collaborations across borders and as part of international consortia.

Paper II

The future clinical perspective

Since this study demonstrated that treatment with OCS suppresses not only cortisol, but even androgen levels, which is of clinical relevance, we suggest that an assessment of adrenal function and androgens should be performed in all patients with severe asthma treated with maintenance oral corticosteroids.

The future research perspective

Regarding future perspectives, there are several opportunities to expand this research in a similar area. Firstly, the results of this study should be validated in other external cohorts to add strength to the results. According to previous discussions within our research group, an opportunity exists to perform similar analysis on material collected from the intervention phase of a previous study, BIOAIR. In this study, urine samples were collected before and after a two week OCS intervention. Another possibility could be the analysis of urine samples collected during the ongoing study, BIOCROSS. In this study urine samples were collected before the start of treatment with biologics, and then at 1-year, 2-year and 3-year follow-up visits. Some patients participating in this study were taking both high doses of ICS and mOCS, that were reduced during the follow-up period. Future studies will evaluate if there will be less suppression of endogenous steroid levels during treatment with biologics. Another interesting research question that could be evaluated in the BIOCROSS is if biologics have some influence on steroid hormones or not.

Paper III

The future clinical perspective

Implementation of the M-COMSA strategy in clinical practice will allow greater precision in early clinical decision-making regarding biological therapy. Moreover, the algorithm can refine the traditional way of assessing treatment response. Evaluation can also be carried out earlier in treatment, as a proposal, at the 4 months follow-up visit, in my opinion. This earlier assessment is necessary to highlight non-responders in need of revised treatment, which is important from a socio-economic perspective, as well as to provide improved patient care. M-COMSA could also be the algorithm of choice for assessment of treatment-induced remission.

The future research perspective

One obvious future research perspective will be to elaborate on and pursue the findings of Paper III. Since Paper III is still in manuscript form, it is an ongoing work with areas that could be improved, despite nearing completion. Before publication of this manuscript, internal interpretation will determine which predictive markers should be used to assess treatment

response, and it is possible that these could possibly achieve even higher predictive value than the results demonstrated in this thesis.

A further step towards future research perspectives will be to test this new algorithm in other groups of patients within BIOCROSS, such as those treated with biologics other than mepolizumab to provide a validation of the new method. As a more general and distant future research perspective, it can also be mentioned that the BIOCROSS study has generated a vast collection of both clinical outcomes and biological samples for biomarker research, and following analysis more interesting findings may well emerge in the future.

My overall conclusions of the work presenting in this thesis are:

The three clinical studies included in the thesis contributed to an increased understanding of the clinical phenotypes of severe asthma.

Longitudinal, prospective studies carried out in a real-world setting are important for evaluation of response to treatment with new drugs since the differing responses of well-characterized and phenotyped patients can reveal clinical sub-phenotypes and their relationship to underlying molecular mechanisms.

The longitudinal clinical studies also allow for the utility of different clinical outcomes and their importance for asthma management assessed.

Patient-centered research contributes to a better understanding of patient needs, and thereby facilitates refined assessment of clinical response to treatment.

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