Pathophysiological Mechanisms in Asthma Phenotypes: An International Multicentre Study

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Abstract

Background: Differences in asthma prevalence have been reported in high-, medium- and low-income countries. While different asthma phenotypes may be involved, little is known about the underlying mechanisms or if they vary between countries. **Aims:** To assess in high-income (New Zealand, UK) and medium- to low-income (Brazil, Uganda, Ecuador) countries, whether: 1) levels of sputum inflammatory, remodelling, or neural mediators differ between asthma phenotypes within countries; and 2) levels of sputum inflammatory, remodelling, or neural mediators differ between specific asthma phenotypes between countries. **Methods:** Questionnaires and clinical assessments (including skin prick test, spirometry, and sputum induction) were conducted in 527 asthmatics and 191 non-asthmatics aged 8 – 27 years (Brazil: 91 and 11, Ecuador: 55 and 11, Uganda: 68 and 16, New Zealand: 204 and 103, United Kingdom: 50 and 22, respectively). Asthma subgroups (eosinophilic and non-eosinophilic asthma) and inflammatory phenotypes (eosinophilic, neutrophilic, paucigranulocytic, and mixed granulocytic asthma) were identified based on ≥2.5% eosinophils and/or ≥61% neutrophils in sputum. Twenty sputum mediators were analysed using Luminex MAGPIX or ELISA. **Results:** Sputum levels of eosinophil-associated mediators (ECP, PGD-2, periostin) were higher in eosinophil-associated asthma groups, and neutrophil-associated mediators (IL-1β, IL-6, IL-8, NE) were higher in neutrophil-associated asthma groups, irrespective of country. Nociceptin was increased in EA compared to other phenotypes in 4/5 countries (e.g. UK EA vs NA (562.9 vs 67.2 ng/mL, $p<0.05$). Sputum mediator levels varied significantly between specific phenotypes when comparing countries, but in PGA (the most prevalent phenotype overall (49.5%)), there was no evidence of increased inflammatory, neural, or remodelling mediators. **Conclusions:** Similar associations between specific mediators and asthma phenotypes were found across countries, suggesting common underlying mechanisms. Mediator levels in the same phenotypes varied across countries, which may be due to methodological variations or differences in environmental exposures. While PGA is very common across all countries studied, the underlying pathophysiology remains unknown.

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Abbreviations

ACQ	Asthma Control Questionnaire
AHR	Airway Hyperreactivity
APC	Antigen-Presenting Cell
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar Lavage
BDNF	Brain Derived Neurotrophic Factor
BDR	Bronchodilator Response
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CT	Computerised Tomography
DC	Dendritic Cells
DCC	Differential Cell Count
DTT	Dithiothreitol
EA	Eosinophilic Asthma
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
ECRHS	European Community Respiratory Health Survey
EDN	Eosinophil Derived Neurotoxin
ELISA	Enzyme-Linked Immunosorbent Assay
ERS	European Respiratory Society

FcεRI	High-affinity IgE Receptor
FeNO	Fractional Exhaled Nitric Oxide
FEV_1	Forced Exhaled Volume in 1 second
FVC	Forced Vital Capacity
GINA	Global Initiative for Asthma
GNI	Gross National Income
HIC	High-Income Country
ICS	Inhaled Corticosteroids
IFN-γ	Interferon Gamma
IgE	Immunoglobulin E
IL	Interleukin
ILC2	Innate Lymphatic Cells
ISAAC	International Study of Asthma and Allergies in Childhood
IS	Induced Sputum
IQR	Interquartile Range
LPS	Lipopolysaccharide
LSHTM	London School of Hygiene and Tropical Medicine
MGA	Mixed Granulocytic Asthma
MLIC	Middle- to Low-Income Country
MMP	Matrix Metalloproteinase
NANC	Non-Adrenergic-Non-Cholinergic Nervous System

NA	Neutrophilic Asthma
NE	Neutrophil Elastase
NEA	Non-Eosinophilic Asthma
NF-KB	Nuclear Factor - KB
NGF	Neural Growth Factor
NO	Nitric Oxide
N/OFQ	Nociceptin/orphanin FQ
NKA	Neurokinin A
NSAID	Non-Steroid Anti-Inflammatory Drugs
NZ	New Zealand
NZD	New Zealand Dollar
OR	Odds Ratio
PAF	Platelet Activating Factor
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PEF	Peak Expiratory Flow
PGA	Pauci-Granulocytic Asthma
PM	Particulate Matter
PRR	Pathogen Recognition Receptor
PGD-2	Prostaglandin-D2
RBM	Reticular Basement Membrane

SPT	Skin Prick Test
TCC	Total Cell Count
Th2/1	T-helper type 2/1
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor Alpha
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide
WHO	World Health Organisation
WASP	World Asthma Phenotype Study

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1. Introduction

Burden of asthma worldwide

Asthma is one of the most common non-communicable diseases affecting the lungs and represents a substantial global public health burden. Globally, around 300 million people have asthma and the prevalence is still increasing in some areas (1) with associated social and financial burdens (2). Although asthma mortality has reduced significantly in most high-income countries (HIC) over recent decades, there were still an estimated 445,000 asthma-related deaths worldwide in 2019 (3). In New Zealand (NZ), approximately 20% of the population has asthma (4) with an associated cost estimated to be approximately NZ$1.18 billion per year (5).

The considerable global burden of asthma is also reflected by the large amount of research conducted, with a search for “asthma” on PubMed (6) identifying more than 200,000 articles, with over 10,000 published in 2021 alone. Although this has resulted in an increased understanding of asthma, it has yet to result in a cure or effective prevention strategy, and although treatment has improved, it is not fully effective for all asthmatics. This is likely due to asthma being a more heterogeneous condition than previously thought.

What is asthma?

The World Health Organisation (WHO) defines asthma as ‘’*a heterogeneous disease, characterized by chronic airway inflammation’’,* and ‘’*defined by a history of respiratory symptoms, including wheeze, shortness of breath and chest tightness*’’ (3). Objective tests can be used to assess clinical aspects of asthma, such as lung function. However, most objective or subjective approaches currently used in the clinic to assess asthma do not provide evidence regarding aetiology or pathophysiology. Although asthma is "…*usually characterized by chronic airway inflammation*" (7), the assessment of inflammation and pathophysiology underlying asthma was, until relatively recently, rarely done, and

for many years was limited to examination of atopy status (8). This was of particular interest as it was believed that asthma was predominantly an allergic disease involving eosinophils (9), often referred to as eosinophilic asthma (EA).

Increasing evidence of heterogeneity in asthma

For decades, the overriding dogma was that asthma was primarily EA, an allergic disorder associated with infiltration of inflammatory cells including eosinophils, mast cells, and various T-helper 2 (Th2) cells (10). However, since the early Twentieth Century (11), it has been clear that not all asthma is associated with allergic inflammation. In some patients with symptoms characteristic of asthma, there is little evidence of accompanying airway inflammation, eosinophilic or otherwise (12). This led to the identification of asthma sub-types better known as allergic asthma and non-allergic asthma. However, after years of research focussing on predominantly allergic, inflammatory asthma, the causes and underlying pathophysiology in these non-allergic individuals are still poorly understood (13).

Researching asthma heterogeneity

One of the major problems with examining asthma pathology and airway inflammation is that direct examination of the airways is often not feasible. Approaches such as airway biopsies, bronchial brushings, and bronchoalveolar lavage (BAL) (which all require bronchoscopy) can be used to assess asthma pathology in greater detail; however, these are invasive technical procedures. A less invasive method of studying asthma inflammation involves the use of induced sputum (IS). Since Morrow Brown first identified eosinophils in IS of people with wheeze in the late 19th century (14), clinicians have been interested in the potential of IS in the assessment of underlying mechanisms involved in respiratory diseases (8). The more recent development of hypertonic saline-induced IS allows the sampling of the central airways and can provide valuable information about the presence of specific immune cells in this compartment (15). Recent studies using IS have increasingly shown that there is

little evidence of eosinophilic airway inflammation in approximately 50% of asthma cases (9). This asthma sub-group (variously described as either inflammatory phenotype or endotype) is generally referred to as non-eosinophilic asthma (NEA). EA and NEA are often associated with differences in skin prick test (SPT) positivity, serum IgE levels, and Th2 and Th1 cells (alongside associated inflammatory mediators) (16). Nonetheless, relatively little is known about the NEA phenotype, although evidence suggests that, at least in some cases, NEA may be characterised by neutrophilic inflammation, and could potentially be associated with environmental exposure to smoking, air pollutants, or the presence of pathogens in the airway microbiota (17).

Sputum analysis improves our understanding of different asthma pathologies

Although early sputum studies often used this EA/NEA classification, subsequent research has categorised four different asthma phenotypes on the basis of cellular profiles. These phenotypes are eosinophilic asthma (EA), mixed granulocytic asthma (MGA), neutrophilic asthma (NA), and paucigranulocytic asthma (PGA) (18). These phenotypes are now widely recognised in asthma research.

Soluble biomarkers

In addition to solely assessing cells in the airways to assess asthma pathology, it is also possible to look at levels of proteins. Soluble biomarkers, including cytokines, growth factors, and inflammatory proteins can be secreted by various immune cells and subsequently detected in IS samples in asthma. These biomarkers may provide further insight into asthma pathophysiology than inflammatory cells on their own (8). This is the focus of the study described in this thesis. To date, several studies have assessed sputum mediators in the context of airway inflammation in asthma. For example, studies have found increased levels of Th2-related mediators such as Interleukin (IL)-13 in EA (19), or increased levels of the Th1-related mediator IL-8 in NA (20). However, despite the combined evidence

provided by cellular and soluble marker analysis, and as noted above, the underlying pathophysiological pathways of NEA – particularly PGA - remain largely unclear. However, recent evidence suggests that airway remodelling or neural mechanisms may be involved (21-23).

Airway remodelling (structural changes in the upper and lower lungs (24)) has long been observed in asthmatics, and is associated with increased levels of mediators such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) in IS samples (25, 26). More recently it has been suggested that increased airway sensory nerve reactivity might also play a role in some asthma patients (27). In further support for the role of neural mechanisms, increased levels of neural mediators such as substance P (28) and neurokinin A (NKA) (29) have been found in some asthmatics during exacerbations. However, it remains unclear whether neural and remodelling processes occur alongside or in the absence of inflammation, or whether these pathways are particularly important in phenotypes that are not associated with inflammation (i.e. PGA). Combining asthma phenotyping on the basis of inflammatory cells and measurement of various soluble mediators in IS related to phenotypes is likely to improve our understanding of asthma pathophysiology.

Global asthma patterns

High asthma prevalence has predominantly been observed in HICs; in parallel, and largely because HICs have adequate resources to do so, most of the research that examined asthma pathology (including the majority of IS studies) has been conducted in HICs. However, data provided by large, multinational studies such as the International Study of Asthma and Allergies in Childhood (ISAAC) (30) have shown that while asthma prevalence in HICs has increased considerably over the past few decades, it has now plateaued or is even decreasing. Additionally, there is increasing (although mixed) evidence showing that high asthma prevalence is not limited to HICs, and asthma prevalence appears to be high and/or increasing in many middle- and low-income countries (MLICs). The reasons for these

trends are unclear. By using non-invasive techniques such as assessing inflammatory cells or soluble mediators in IS, it may be possible to provide more insight into the underlying causes of the changes in asthma prevalence observed, as well the underlying pathophysiological pathways causing asthma, and whether these vary across different geographical regions.

The current study

The research described in this thesis is part of a larger multicentre collaborative study named the World Asthma Phenotypes Study (WASP) (31). WASP is a collaboration between five research centres: NZ (Wellington), UK (Bristol), Brazil (Salvador), Uganda (Entebbe) and Ecuador (Esmeraldas), which aims to better understand asthma phenotypes in different geographical locations representing HICs and MLICs. In each centre, participants with and without asthma were recruited and information was obtained, such as symptoms, atopy, exhaled nitric oxide, and risk factors. IS was used to assess asthma phenotypes.

The research described in this thesis will build on previous WASP findings showing that most asthma is non-eosinophilic across the various centres, irrespective of geographical location or socioeconomic status. However, EA was more prevalent in HICs compared to MLICs (32). The current research will add to these findings by analysing soluble inflammatory, remodelling, and neurological mediators in IS supernatants, with the aim of further understanding and clarifying the pathophysiological pathways of asthma and its phenotypes in HICs and MLICs. To the author's knowledge, this is among one of the very few studies to assess asthma pathophysiology in this much detail in MLICs. This is important as the causes and mechanisms underlying asthma in these countries may potentially be different from those in HICs. Subsequently, current asthma treatment and management regimes, which are largely based on those in HICs, may therefore be less effective in MLICs.

The specific aims of this study were to assess:

1. whether levels of sputum inflammatory, remodelling, or neural mediators differ between asthma phenotypes within countries;
2. whether levels of sputum inflammatory, remodelling, or neural mediators differ between specific asthma phenotypes between countries.

2. Literature Review

2.1 Asthma

2.1.1 Definition

The word "*asthma*" originates from the Greek verb "*aazein*," meaning "*to exhale with open mouth, to pant*" (33). The clinical characteristics of asthma were undescribed up until the first century, when Aretaeus Cappadox, a Greek clinical physician, described asthma broadly. *"If from running, gymnastic exercises, or from any other work, the breathing becomes difficult, it is called asthma"*. Additionally, he described asthma as:" *heaviness of the chest; sluggishness to one's accustomed work and to every other exertion; difficulty of breathing when running or on a steep road; they [the patients] are hoarse and troubled with cough".* Since then, many clinicians, researchers, and authors have provided further insight into asthma (34) so it comes as no surprise that the description of asthma has evolved over the years.

The current definition of asthma described by the Global Initiative for Asthma (GINA), in collaboration with the WHO is: "*…a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms, such as wheeze, shortness of breath, chest tightness and cough, that vary over time and in intensity, together with variable expiratory airflow limitation "* (7).

2.1.2 Global trends

According to the WHO (35), approximately 300 million people globally have asthma, and with the current incidence trends, another 100 million are expected to be affected by 2025 (36). Globally, chronic respiratory diseases, including asthma, accounted for 4.1 million deaths in 2019. However deaths due to chronic respiratory diseases declined by 37% between 2000 and 2019, most notably in

Westernised HICs (35). While asthma mortality may be decreasing, asthma symptom prevalence over the last few years has increased substantially in childhood according to the ISAAC study (30), and asthma morbidity and mortality still remain high, particularly in adults compared to children (36, 37). These patterns are observed globally, but geographical variation is observed, with asthma symptom prevalence highest, yet decreasing, in English-speaking and more developed countries and asthma symptom prevalence increasing in some African, Latin American, and Asian countries (30). Even in the developed world e.g. Europe, asthma prevalence varies between different countries, with asthma prevalence lower in northern, central, and southern Europe than in western Europe. It also appears higher in English-speaking countries such as the UK, Australia, NZ, and the United States of America (USA) (38). In NZ in particular, one in seven have respiratory diseases, including asthma. Economically this costs NZ roughly 1.18 billion dollars annually, with asthma prevalence, hospitalisation, and mortality being significantly higher in Māori and Pasifika than non- Māori and Pasifika (39).

2.1.3 Asthma diagnosis and characteristics

The WHO definition of asthma states that asthma is ‘’*heterogeneous*’’; i.e. it can be present in various forms and can have different underlying causes or triggers among different individuals. This means that asthma is not always easily diagnosed. Because there is no definitive test to diagnose asthma, a combination of assessments is often required. Guidelines for clinical asthma assessment vary worldwide, but clinical diagnosis is generally focussed on a combination of the patient’s clinical history, physical assessment, and the use of objective measures to test airway obstruction and bronchial hyperresponsiveness (40). For example, according to a recent GINA report (7), asthma diagnosis should be based on the presence of characteristic asthma symptoms, such as wheezing, dyspnoea (shortness of breath), chest tightness, cough, the history of these symptoms (on the basis of questionnaires), and the assessment of variable airflow obstruction. This combined approach is

important clinically, as other conditions, such as chronic obstructive pulmonary disease (COPD), can also cause similar respiratory symptoms (7).

Measuring airway obstruction

Airway obstruction can occur through various pathophysiological pathways (discussed in more detail in chapter 2.2.3). As described above, measuring aspects of airway obstruction, generally using spirometry, is important for diagnosing respiratory diseases such as asthma (7) or COPD (41). To ensure global standardisation of spirometry assessments for this purpose, guidelines are available from the American Thoracic Society, European Respiratory Society (ERS) (42), and GINA (7). Spirometry is generally used to measure airflow over time and airway volume, which are indicative of general airway health and degree of airway obstruction (42). However, baseline lung function assessment may be less valuable in children than in adults, as children with asthma often have normal lung function between exacerbations (41). During spirometry, pre-bronchodilator forced exhaled volume in 1 second (FEV_1) and the forced vital capacity (FVC) are two variables commonly used. Reduced FEV_1 values and FEV_1/FVC ratio can be seen in many other airway illnesses, such as COPD (43), but asthma is commonly associated with variable and reversible airway obstruction when compared with healthy subjects (7, 44). Assessing bronchodilator response (BDR) is particularly useful in identifying asthma. A 200mL or 12% improvement in FEV_1 after inhalation of short-acting β_2-agonist (a type of bronchodilator) indicates reversible airway obstruction and is considered an indicator of asthma. However, the presence of irreversible airway obstruction does not rule out asthma but suggests the more likely cause is COPD (44). Peak expiratory flow (PEF) measurement is another way of measuring airway obstruction (7). Regularly monitoring the variability or reversibility of airway obstruction is useful, as one spirometry reading might not be sufficient to determine asthma status.

Airway hyperreactivity

Airway hyperreactivity (AHR) is another characteristic used in some asthma definitions and describes the increased propensity of the lungs to react to a specific dose of stimuli, which can result in respiratory symptoms and airway limitation (45). AHR intensity is commonly measured in terms of dose-response effect, with people with greater AHR reacting to lower stimuli concentrations (46). If this results in a reduction of FEV_1 by more than 20%, this is considered a positive test in some guidelines (46).

There are two types of stimuli commonly used for AHR measurement in the clinic and research settings: direct and indirect stimuli, both of which have pros and cons. Direct stimuli, such as methacholine and histamine, act directly on the airway smooth muscles (ASM), causing bronchoconstriction (47). Indirect airway stimuli do not directly act on the ASM, but is believed to work by causing osmotic changes in the lungs, triggering an inflammatory response leading to the release of a range of soluble mediators by airway inflammatory cells (such as eosinophils and mast cells), which in turn causes bronchoconstriction (48, 49). The most common indirect stimuli used are hypertonic saline (50) and mannitol (51). Like direct stimuli, indirect AHR tests work on a dose-response principle, with those with AHR reacting to lower doses of stimuli, as observed in severe asthma (49).

Skin prick testing

Asthma is often associated with atopy (52) and allergies (53), and the presence of atopy in a patient with respiratory symptoms is considered suggestive of asthma (54). Atopy describes an allergic reaction to specific environmental allergens and can be measured by SPT. These involve controlled and standardised skin exposure to various common allergens (e.g. birch trees, grass mix, cat, dog, dust mites, and cockroaches) after which the allergic response is measured based on wheal size (the red

''allergic'' circle around the allergen on the skin). If the wheal size diameter is ≥3mm, it is considered a positive test (55), and the patient is deemed atopic. On the basis of SPT, asthmatics can be referred to as atopic- or non-atopic asthmatics. However, a positive SPT does not necessarily mean that these allergens cause asthma, and asthma symptoms can occur independently of atopy (56).

Fractional Exhaled Nitric Oxide

Fractional exhaled nitric oxide (FeNO) is an objective way of measuring airway inflammation. Increased FeNO levels are often observed in asthma (57) and are indicative of eosinophilic airway inflammation (57). Studies have found that FeNO levels strongly correlate with increased blood eosinophils and sputum eosinophils in children with atopic asthma (58), and high FeNO levels are also associated with atopy (positive SPT) (59), AHR, and disease severity (60).

Asthma questionnaires

While many of the objective tests described above are useful for asthma diagnosis, they are not always easily available or convenient to conduct. However, ''*the history of asthma symptoms*'' is also critically important in asthma diagnosis (3), and can be cheaply and conveniently assessed using questionnaires.

In extensive population-based studies, such as the ISAAC or WASP studies (31), questionnaires are commonly used, as it is not always possible to perform objective tests such as spirometry and AHR testing in large populations and in different countries. Questionnaires can provide valuable information regarding asthma symptom history, current symptoms, severity, and asthma control. Two well-standardised and validated questionnaires commonly used in asthma research are: the ISAAC survey for children (30, 61) and the European Community Respiratory Health Survey (ECRHS) for adults

(62). Another commonly used questionnaire (for both children and adults), is the asthma control questionnaire (ACQ) (63), which is often used in clinical settings. However, as with objective measures of asthma, variability plays a role in questionnaire response. Subjectivity, due to variations in human interpretation and perception, can also be a problem (64).

2.1.4 Treatment

As asthma is highly variable, ongoing symptom assessment, treatment adjustments, and treatment reviewing are constant cycles that are recommended in asthma guidelines (7, 65, 66). Conventional asthma treatment (in both children and adults) involves the use of asthma preventers, which generally target the inflammatory component of asthma (67), and relievers, which act directly on the ASM, causing bronchodilation (7). Inhaled corticosteroids (ICS) are the most commonly used conventional asthma preventers and, alongside relievers, are often considered the first line of defence in asthma treatment. They reduce Th2 inflammation by acting upon the pro-inflammatory transcription factor nuclear factor-KB (NF-KB) and activator protein-1 associated pathways in the airways, resulting in a reduction of inflammatory Th2 mediator release (67). In some studies, corticosteroids have been shown to greatly reduce numbers of Th2-associated cells (such as mast cells and eosinophils) in the airways by 60-80% (68). ICS has also been shown to improve pre-bronchodilator FEV_1 in children and adults with mild asthma (69). However, approximately 30-50% of patients show either no or reduced improvement in either asthma control or FEV_1 when using ICS (70). There is also some evidence that long-term ICS use may be associated with adverse side effects (71).

Relievers (also called bronchodilators and including β_2-agonists) are prescribed to improve airflow and reduce bronchoconstriction. β_2-agonists cause ASM relaxation by interacting with the β_2-receptor on ASM (72). There are two types of β_2-agonists commonly prescribed for asthma: short-acting β_2-agonists, which generally work for 3-6 hours with immediate effect, and long-acting β_2-agonists, which

work for up to 18-24 hours. They are generally associated with few side effects, such as trembling, nervousness, and heart palpitation (72). However, there is some evidence that bronchodilator use can increase the risk of cardiovascular disease (73, 74).

Most asthma patients typically respond well to a combination of preventive and reliever medications. However, a small subset of individuals may not experience results with this standard treatment approach. Individuals with severe asthma that doesn't respond adequately to conventional therapy may become candidates for biologic therapy, such as monoclonal antibody treatment. Monoclonal antibody treatments target specific inflammatory pathways and slow down inflammatory processes in the airways (75). Examples of targets of monoclonal antibodies used in asthma therapy include immunoglobulin E (IgE), IL5, IL-4, and IL-13 (76), with anti-IgE in particular showing good results (77, 78).

2.1.5 Risk factors and protective factors

As described in Paragraph 2.1.2, there are variations in global trends and asthma prevalence worldwide. This observed geographical variation (30) suggests that there is a possibility that some populations may be exposed to different factors that protect against asthma; alternatively, there may be differences in exposures to risk factors in some populations. With regard to protective factors, there is evidence that exposure to microbes and/or differences in the airway microbiome may be important, with studies finding distinct differences in lower airway microbiome between asthmatics and healthy individuals (79, 80). Additionally, exposure to a rich microbial environment is thought to be protective against asthma development, while antibiotic use, especially in early life, is thought to be a risk factor (81). A study by Riedler et al. (82) showed that early exposure to stables and farm milk consumption was associated with a reduced risk of developing asthma and allergies, and extended farm exposure for the first five years of life showed the most significant reduction in asthma and

allergy frequency. The ISAAC phase II study showed an inverse correlation between endotoxin (or lipopolysaccharides (LPS), which make up most of the outside layer of gram-negative bacteria (83)) exposure and asthma and atopy (84), suggesting increased microbial exposure may be protective against asthma development. These findings support the "Hygiene Hypothesis", which posits that living in unhygienic or crowded conditions results in an increased risk of infections and exposure to micro-organisms and their components, which may lead to a lower prevalence of asthma, allergy, and atopy (85).

In the latter years of the 20th century, it became increasingly common to consider allergen exposure to be the major risk factor for asthma (86-88). This is because, in the early studies of asthma aetiology, allergens were found to play a significant role, with airborne house dust mites (86, 87) and indoor domestic allergens (such as pets, mites, and dust) (89) often associated with asthma symptoms. In particular, it was believed that exposure to airborne allergens in those with atopy resulted in allergic airway inflammation, with prolonged exposure causing asthma (87). However, even though there is consistent evidence that suggests that allergen exposure is involved in the exacerbation of asthma, there is a lack of evidence showing that allergen exposure has caused the global increase in asthma (90). In fact, the ISAAC phase II study showed most asthma cases in less developed countries were non-atopic, while asthma cases in more developed countries were more atopic (91).

With regard to identified asthma risk factors (other than atopy/allergy (discussed 2.2.1) that may be important in particular populations, a family history of allergy, sex, age, obesity, diet, respiratory infections, psychological stress, drugs (paracetamol or antibiotic use), vitamin D deficiency, pollution (both indoor and outdoor), and a range of other environmental exposures (such as fungal or bacterial exposures, pesticides, irritants or gases) have been found to be associated with an increased risk of asthma (reviewed in (92)). Low socioeconomic status is an identified risk factor, with low educational

levels and low-income being associated with increased asthma (93, 94). Smoking and exposure to air pollutants are also more common in lower socioeconomic groups and are associated with asthma, partly due to increased exposure to particulate matter (PM) present in both cigarette smoke and pollutants (95, 96). PM exposure is commonly used to measure air quality, and factors such as traffic-related air pollution, smoking, and indoor (household) pollution by burning biomass, coal, and kerosene for cooking and heating are all positively associated with an increase in asthma prevalence (97).

2.1.6 Asthma heterogeneity

A combination of differences in risk factors, protective factors, clinical characteristics, symptoms, patient characteristics, and even genetics (98) makes asthma incredibly variable and heterogeneous. In terms of clinical characteristics alone, there are significant differences in e.g. severity (99), age of onset (100), asthma control status, and asthma treatment response (7). This variability and multidimensionality are highlighted in recent studies using cluster analysis. This approach uses a combination of multiple variables to identify asthma subgroups (101). For example, one study by Moore et al. (102) identified 5 clusters based on 34 core variables and clinical characteristics. These were; (1) early onset atopic asthma with normal lung function; (2) early onset atopic asthma with preserved lung function; (3) late-onset non-atopic asthma primarily in obese women; and (4) and (5) severe airflow obstructed with a variety in age of asthma onset, gender, lung function, treatment response, and atopy status. However, there are some limitations with using cluster analysis; in particular, most studies to date have not included many variables associated with different aspects of asthma pathophysiology.

Although cluster analysis approaches can be useful, particularly for identifying clinical phenotypes or subtypes, the assessment of asthma inflammatory phenotypes is of significant interest for examining asthma pathophysiology. As discussed previously, in the early 20th century, asthma was thought to be primarily associated with, or caused by, atopy, allergies, and eosinophilic inflammation. However, numerous studies conducted over the last thirty years have shown that this is not the case, and only about half of asthma cases may be associated with allergic/eosinophilic pathways (9). Some asthma inflammatory phenotypes may be associated with increased airway neutrophils, a combination of eosinophils or neutrophils, or neither (18). This will be discussed in more detail in section 2.3.

2.2 Asthma Immunopathogenesis

2.2.1 Atopy, allergies, and TH2 inflammation

Atopy and allergies

Atopy and allergy are terms that are often confused. Atopy describes the propensity of an individual to produce IgE as a response to allergens (such as pollen, food allergens, and house dust mites), and the propensity to develop type 1 hypersensitivity (allergic) reactions (103). An allergy, however, is when this reaction leads to symptoms. As mentioned earlier, atopy is often identified through SPT. Atopy can also be measured by serum-specific IgE counts (104), with total IgE found to be increased and strongly associated with asthma (105). In several studies, asthma has been found to be associated with allergies, atopy, and allergic rhinitis (16, 53, 106).

Sensitisation

Allergic asthma develops in two stages: the sensitisation phase and the effector phase (107). During the sensitisation phase, dendritic cells ((DC); specialist antigen-presenting cells (APCs)) residing in the mucosal layer in the lungs are exposed to allergens. They then travel to lymph nodes and interact with naïve T cells, which differentiate into Th2 cells (108). These Th2 cells then interact with B-cells, through IL-4 and IL-13, to induce class-switch recombination, the process by which a B-cell changes antibody production from one type to another; in this case, changing to IgE antibody production (107). Eventually, these B-cells differentiate into plasma cells, producing antigen-specific IgE on a much larger scale. Plasma cells can secrete IgE over extended periods and can ultimately lead to sensitisation (107). Additionally, innate lymphatic cells (ILC2), which have been found to play an essential role in the development of allergic asthma, are triggered by alarmins released by epithelial cells to release cytokines such as IL-13 and IL-5 to help recruit other inflammatory cells, such as eosinophils (109) (**figure 1)**.

Th2 inflammation

After the sensitisation phase, mast cells increasingly present High-affinity IgE receptors (FcεRI) on the cell surface, which bind to specific IgE antibodies, leading to the secretion of numerous and various mediators (including cytokines and histamine), amplifying the inflammatory process (110). Basophils are also recruited and are coated with specific IgE, which can cross-link with IgE receptors on mast cells, also resulting in the release of various cytokines and mediators (111). The activity of these cells and mediators leads to the dilution of the blood vessels, bronchoconstriction, and overproduction of mucus (107). Alongside mast cells and basophils, eosinophils migrate to the airways by chemotaxis, predominantly due to IL-5 and IL-13 released by, but not only by, ILC2 (112). Thus, it is no surprise that multiple studies have shown significantly higher levels of airway eosinophils in allergic asthma (113-115). In the airways, eosinophils release various cytotoxic mediators, such as eosinophil derived neurotoxin (EDN), platelet activating factor (PAF), and eosinophil cationic protein (ECP), which induce microvascular leakage, airway inflammation, mucus secretion, and ASM contraction, resulting in AHR and bronchoconstriction (**figure 1)** (116).

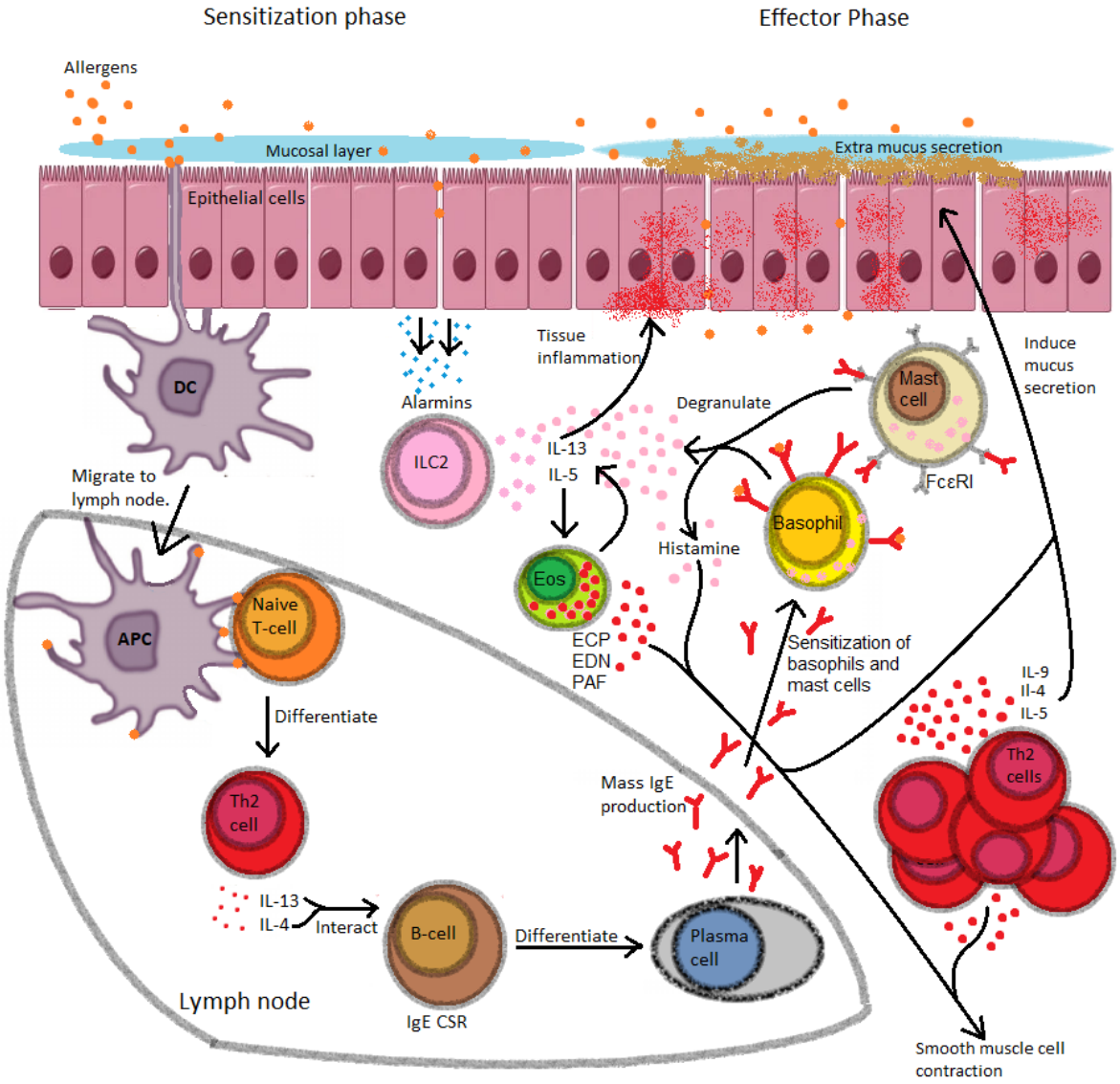

***Figure 1**: Overview of the initiation of the Th2 immune response. Sensitization phase: exposure to allergens results in antigen-presenting cells (APC) migrating to the lymph node where they interact with naïve T-cells, causing cell differentiation to Th2 cells. Il-13 and IL-4 mainly interact with B-cells, causing class-switch recombination and differentiating B-cells into plasma cells, producing specific IgE. Additionally, alarmins released by epithelial cells result in the secretion if IL-13 and IL-5 by Innate Lymphatic Cells (ILC2s), leading to eosinophil (Eos) migration. Effector phase: after establishing a Th2-immune response, Th2-cells migrate to the lungs, releasing Th2 cytokines such as IL-9, IL-4, and IL-5. IgE produced by plasma cells binds to FcεRI receptors on mast cells and basophils. This causes degranulation and release of mediators including IL-13, IL-5, and histamine, which results in eosinophil recruitment, as well as tissue inflammation, mucus secretion, and smooth muscle cell contraction.*

2.2.2 Innate immunity and asthma

Many studies have looked at the role of the adaptive immune system on asthma. However, evidence showing the lack of involvement of Th2-mediated inflammation in non-allergic asthma (or NEA) (9, 22) and the possible protective effect of microbial exposure on asthma (117) suggests that the innate immune system may be involved (9, 118). In support, a literature review by Douwes et al. (9) suggested that exposure to various environmental factors, such as endotoxins (119), particulate air pollutants (120), ozone (121), and micro-organisms (122), may result in a more active innate immune response, possibly due to the recruitment of neutrophils and the participation of toll-like receptors (TLRs), which may play a key role in the development of NEA (17).

Innate immunity

The innate immune system is a multi-component defence mechanism that quickly responds to invasive stimuli (123). Notable for its rapid and nonspecific nature, innate immunity contrasts the highly specific, yet somewhat slower, adaptive immune system. These systems work synergistically, offering comprehensive protection against pathogens (123). The innate immune system is particularly important in the lungs due to their continuous exposure to environmental particles and pathogens (22). Initiation of innate immune responses involves the recognition of distinct molecular patterns originating from both endogenous and exogenous sources, such as pathogen-associated molecular patterns (PAMPs) (22). PAMPs include LPS, foreign RNA/DNA, and endotoxins (124). Once in the lungs, PAMPs bind to pathogen recognition receptors (PRR), which are present on the surface of various leukocytes and structural/epithelial cells (125). PRR/PAMP ligation triggers signal transduction pathways leading to the expression of immune response genes and subsequent release of inflammatory cytokines, and co-stimulatory molecules (22). Various PRRs have been found to play a role in the pathogenesis of asthma, in particular, NEA (124, 126), with TLRs being particularly of interest.

TLRs and asthma pathogenesis

TLRs are a group of PRRs expressed both intra- and extracellularly (127). There are many sub-variants of TLRs. TLR2 and TLR4 are classified as lipid-recognising receptors and are activated by LPS, found in pathogenic gram-negative bacteria, and TLR3, and TLR7-9 are specific for foreign viral and bacterial DNA/RNA (128). These TLRs have been found to be associated with asthma immunopathogenesis (129-131). Some of this research suggests that as a result of TLR activation, a shift towards innate immune activation (Th1) as opposed to an allergic response (Th2) occurs (17). The underlying TLR-mediated mechanism leading to asthma is not fully understood; However, a study by Simpson et al. (132) found asthmatics with high levels of airway endotoxin were predominantly neutrophilic, had increased expression of TLR 2 and TLR4, as well as increased levels of pro-inflammatory cytokines such as IL-1β and IL-8. Taken together, this suggests that TLR activation may lead to increased innate-immune mediators in the airways, causing increased airway neutrophilia, which may lead to asthma (17).

2.2.3 Airway remodelling

What is airway remodelling

In asthma, structural changes to the airways are referred to as airway remodelling. Remodelling can affect larger and smaller airways (133, 134). Airway remodelling can result in ASM and subepithelial reticular basement membrane (RBM) thickening and angiogenesis (formation of new blood vessels), which may result in an irreversible loss in lung function from childhood to adulthood (135). The degree of remodelling is believed to impact disease outcomes, with increased ASM thickening found in more severe asthma cases (136).

In the airways, remodelling is also associated with changes in extracellular matrix (ECM) composition (137). The ECM consists of a network of proteins, such as elastin and collagen, and glycoproteins, such as fibronectin and laminin, that surround and support cells in tissue (138). In asthmatics, an increase of fibronectin and collagen proteins in airway walls was found to lead to increased mucus production, AHR, reduced elasticity, and altered lung mechanical properties (139). In the clinic, to determine if airway remodelling is present, invasive assessments such as biopsies can be done. These generally look at remodelling in the bronchial epithelium and ASM (135). However, although they provide useful information regarding airway remodelling and pathology, bronchial biopsies are very invasive and difficult procedures. A less invasive method to assess airway remodelling is using computerised tomography (CT) imaging (140).

Remodelling pathways

Both allergic (Th2-mediated) and innate inflammation have been shown to have the potential to induce airway remodelling (135, 141). Airway remodelling through the allergic pathways is thought to primarily impact ASM, as airway eosinophilia correlates significantly with increased ASM and airway wall thickness (133). One potential mechanism is through Th2 cytokine IL-13, which is believed to play a role in airway remodelling by interacting with the epithelial cells (142), stimulating the production of periostin, which can then stimulate eosinophil migration (143), and contribute to increased airway fibrosis and decreased airway flexibility (144). Chronic inflammation may lead to epithelial damage and angiogenesis (145), which is associated with increased levels of VEGF (146). Additionally, soluble ADAM33 (SADAM33) has been implicated in asthma and airway remodelling (147). Airway remodelling has also been shown in relation to innate immunity and neutrophils. Along with IL-8 and IL-1β, neutrophil elastase (NE) and MMP can also be released by neutrophils. These enzymes can modify the ECM and thus lead to airway remodelling (148), contributing to airway obstruction (17).

2.2.4 Neural involvement in asthma

Over the last few decades, evidence of the potential involvement of neural mechanisms in asthma development has been accumulating (149). Within the human airways there is a complex network of nerves consisting of efferent ‘’motor’’ nerves, transporting signals from the central nervous system (CNS) to the lungs, and afferent ‘’sensory’’ nerves, which transport signals from the lungs to the CNS (150). Together, they play a critical role in regulating breathing patterns, airway muscle tone, cough reflex, and pain transmission (151).

The efferent nerves, which are part of the autonomic nervous system, consist of three main types of nerve systems: the sympathetic adrenergic, parasympathetic cholinergic, and the non-adrenergic-non-cholinergic nervous systems (NANC) (152). The sympathetic nerves, involved in the body's ‘’fight or flight’’ mode, release epinephrine, which can interact with receptors on ASM, resulting in bronchodilation (153). The parasympathetic nerves signal via acetylcholine release, which can interact with receptors on ASM, resulting in ASM contraction (i.e. bronchoconstriction) and mucus release (154). Finally, the NANC nerves, which oppose cholinergic activity, regulate bronchodilation by releasing the neurotransmitters vasoactive intestinal peptide (VIP) and nitric oxide (NO) (152). In asthma, a dysfunction or disbalance between airway efferent nerves is thought to lead to more bronchoconstriction and mucus secretion (155).

The afferent sensory nerves are present throughout the lungs, including the airway epithelium, ASM, and bronchi, and play a vital role in the regulation of breathing patterns and the cough reflex (156). The sensory nerves in the airways consist of C-fibres and A-fibres, with C-fibres reacting to environmental and chemical stimuli (157, 158), and A-fibres responsible for rapid responses when change in lung volume, bronchoconstriction, or airway oedema occur (159). These fibres respond by

secreting neuropeptides such as substance P and NKA, which play an important role in inflammatory processes, ASM contraction, and mucus secretion (160).

The nervous system and immune system are thought to interact frequently and bidirectionally, and immune cells can be regulated by neuropeptides/transmitters from nerves, and proinflammatory mediators and cytokines from leukocytes can stimulate sensory nerves (149). Additionally, receptors for neuropeptides/transmitters can be found on the surface of leukocytes such as mast cells (161), and IgE receptor FcεRI (described in paragraph 2.2.1) and other Th2 receptors have previously been found on sensory nerves (162). Additionally, there is evidence suggesting that inflammatory cells can also produce neuropeptides or neurotransmitters (163) which can play a role in inflammatory processes (164, 165). For instance, epithelial cells and inflammatory cells can release acetylcholine that may interact with muscarinic receptors on ASM cells and inflammatory cells, potentially contributing to asthma symptoms (166). While neural-immune pathways are still relatively poorly understood, they are suspected to play a role in asthma, particularly in NEA (167).

2.3 Asthma phenotyping

As discussed, asthma immunopathogenesis is variable, complex, and multidimensional, and research has revealed many potential pathways leading to the development of asthma. This has led to an increased interest in phenotyping (or endotyping) asthma to assess underlying pathophysiology (18). Currently, phenotyping asthma based on airway inflammation is the most practical and commonly used approach. Harry Morrow Brown first showed in the 1950s that individuals with sputum eosinophilia responded to ICS (14). Subsequent research showed that eosinophils were present in endobronchial biopsies of many asthmatics, alongside an increased number of lymphocytes, mast cells, and macrophages (168). This eosinophilic airway inflammation is also associated with Th2-cell infiltration and increased secretion of allergic mediators such as IL-13 and IL-5 (168). This type of asthma is commonly referred to as EA or Th2-high asthma (169). On the other hand, in NEA or Th2-low asthma, there are no signs of eosinophilic airway inflammation and generally low levels of Th2 allergic mediators. However, at least in some NEA, there may be increased levels of Th1 mediators such as IL-1β, IL-6, and IL-8 and neutrophilic infiltration in the airways (9).

Early methods (such as bronchial biopsy) used to assess airway inflammation (in particular eosinophilia) were generally invasive, unpleasant, and difficult to conduct. The development and refinement of the sputum induction procedure (170) has provided an easier and more convenient alternative to assess airway inflammation. Sputum induction is a non-invasive, well-validated technique that generally involves the inhalation of a hypertonic saline solution using a nebuliser (170). This induces airway secretion of sputum (phlegm/mucus), which is then expectorated (coughed up), and collected for analysis (171); usually for leukocytes or mediators.

From the research examining IS in asthmatic populations conducted since the end of the 20th century, it appears that approximately 39%-48% of asthma cases are EA (18, 32, 167). In contrast, the remainder show no signs of airway eosinophilia and are often referred to as NEA (9, 32, 167). A groundbreaking study by Simpson et al (18) further divided these asthma groups (EA and NEA) into four phenotypes based on both eosinophil and neutrophil proportions in IS. Using this approach, the umbrella phenotype EA was divided into two groups: EA (>1.01% eosinophils and <61% neutrophils), MGA (>1.01% eosinophils, and >61% neutrophils). NEA was divided into NA (<1.01% eosinophils, and >61% neutrophils), PGA (<1.01% eosinophils, and <61% neutrophils) (**figure 2)**. While the eosinophil cut-off point used in this study was later revised and adjusted by Simpson et al. (172) to 3%, as this showed greater reproducibility, a cutoff of 2 - 3% for eosinophils (173, 174), and 60 - 70% for neutrophils (175) has been used to determine EA, MGA, NA, and PGA.

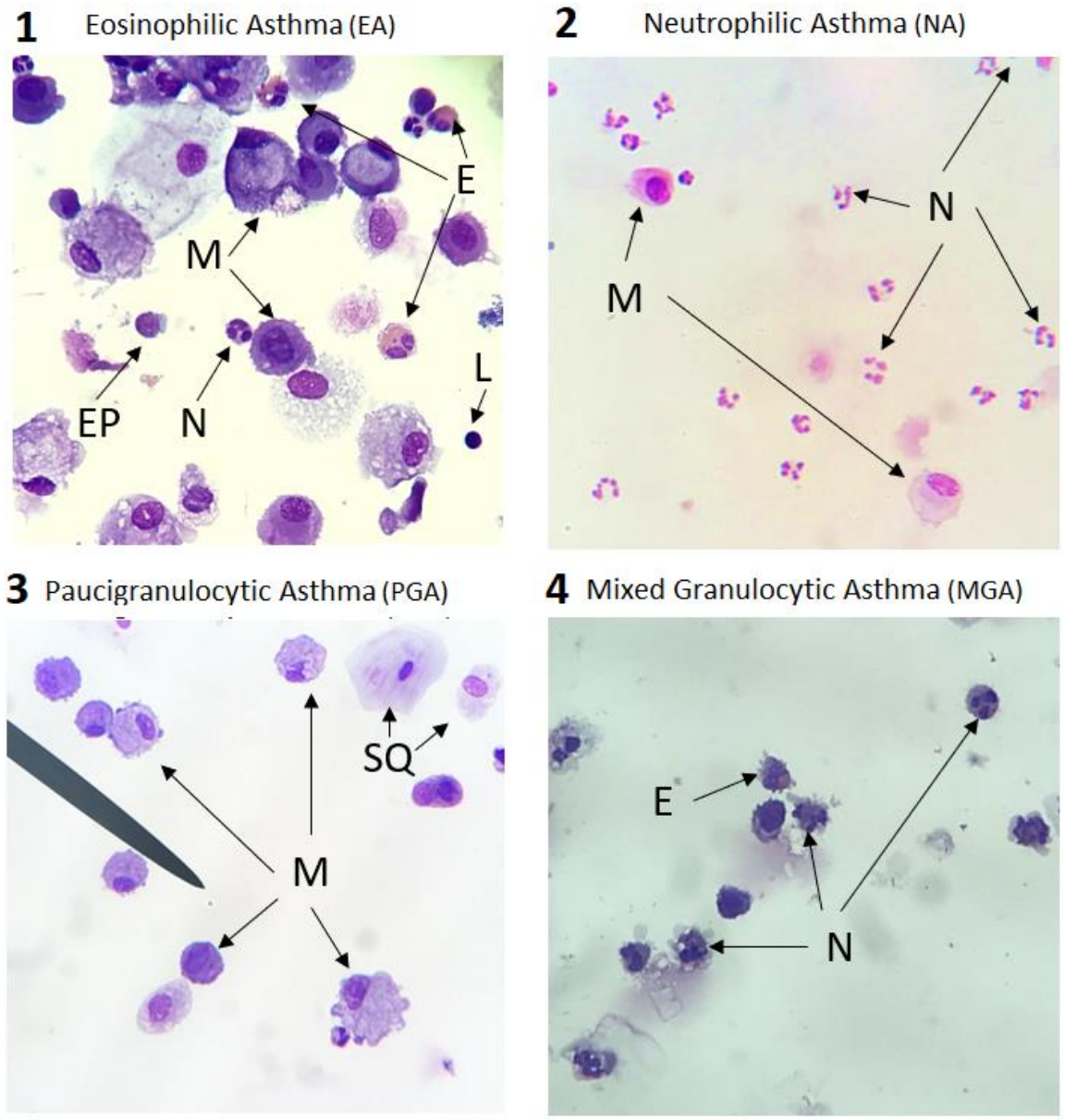

***Figure** 2: Sputum cell slides showing asthma inflammatory phenotypes as described by Simpson et al (18). 1: Eosinophilic Asthma (EA), 2: Neutrophilic Asthma (NA), 3: Paucigranulocytic Asthma (PGA), and 4: Mixed Granulocytic Asthma (MGA). M=macrophage, E=eosinophil, N=neutrophil, EP=epithelial cell, SQ=squamous cell, L=lymphocyte.*

2.3.1 Eosinophilic asthma

EA is well characterised and the most investigated of all the inflammatory phenotypes. As discussed in chapter 2.2.1, EA is predominantly associated with atopy, allergies, and Th2 inflammation, alongside eosinophilic infiltration (168). The prevalence of EA worldwide has been reported to be between 30 - 50% (18, 32, 176). However, this research has primarily taken place in HICs (31). EA has been associated with reduced FEV_1 and increased FeNO compared to NEA (177). Additionally, increased asthma severity (178), airway obstruction, and AHR (113) are associated with EA compared with NEA. However, EA appears to be more responsive to ICS, and it has been suggested that this responsiveness may lead to fewer hospitalisation visits compared to NEA (179). EA is also associated with increased expression of Th2 mediators such as IL-4 (180), IL-5, and IL-13 (181) (described in more detail in 2.3.5). IgE levels are also increased in EA (181).

2.3.2 Neutrophilic asthma

NA accounts for approximately 10-30% of asthma cases (18, 32, 176), although it can be undetectable in some populations (167).. NA is characterised by the infiltration of neutrophils in sputum, with a cutoff of ≥61% neutrophils often used (18). NA has been reported to be more common in older people (182), and sputum neutrophil levels are positively correlated to age (183). NA is also associated with some occupational-related asthma (184), and is more common in obese women (185), smokers (186), and in more severe asthma (182). NA appears to respond poorly to ICS, and it is possible that ICS treatment leads to higher levels of sputum neutrophils (175), as ICS inhibits neutrophil apoptosis and may drive neutrophilic activation (187). However, untreated patients also show signs of airway neutrophilia, suggesting NA can develop independently of ICS treatment (175). NA also appears to respond poorly to immunotherapies, such as those targeting anti-IL-8, IL-1β, IL-17, and IL-6 (188). NA is associated with an increased expression of Th1 mediators, such as IL-8 (20, 189), and IL-1β (190). Additionally, NA appears linked to various environmental exposures, like LPS, ozone, and PM (9).

Alongside these environmental exposures, increased expression of TLR2 and TLR4 has been associated with NA, suggesting the involvement of the innate immune system.

2.3.3 Mixed granulocytic asthma

MGA is the generally the rarest of asthma phenotypes, making up approximately 1 – 7% of asthma (18, 32, 191). MGA is associated with increased (>1.01%) eosinophilic and (>61%) neutrophilic infiltration in sputum and as such is believed to involve both Th1 and Th2 inflammation (192). MGA patients are more likely to have comorbidities and are more likely to be older than EA, NA, or PGA (193). MGA is also associated with more severe airflow obstruction and increased exacerbation frequency (194) compared to other asthma phenotypes. Poor asthma control and greater severity has also been reported in MGA when compared other inflammatory phenotypes (32). Treatment specifically for MGA is currently unavailable, but it has been shown that monoclonal antibody treatment targeting the IL-6 pathway may be beneficial (195). In MGA, both Th1 and Th2 mediators, such as IL-5 and IL-13 (181), IL-8 (20, 189), and IL-1β (190) are commonly increased (more on mediators in 2.3.5).

2.3.4 Paucigranulocytic asthma

PGA is one of the more common inflammatory phenotypes in asthma with a reported prevalence of between 31 - 50% (32, 196). Little is known about the underlying mechanisms, but PGA is characterised by reduced numbers of sputum neutrophils and eosinophils, with macrophages the most dominant sputum cell population (18). PGA is generally associated with stable (197), less severe (32) asthma, with better FEV_1 than the other phenotypes (191), although one study found that some patients with PGA have severe refractory asthma and poor asthma control (191). Although PGA is generally less severe, treatment response may be poor or non-existent (196). There are studies suggesting that in some cases, PGA may be misclassified EA due to positive ICS treatment response

(leading to a reduction in sputum eosinophils), but, a large group of PGA patients remain symptomatic without airway inflammation even without ICS treatment (196). While the pathophysiology underlying PGA remains unclear, a study by Elliot et al. (141) showed that some PGA patients had thickening of ASM and basement membrane, which was believed to be caused by airway remodelling independent of inflammation. Other studies have investigated the possible involvement of nerves, suggesting sensory nerves may play a role in PGA immunopathogenesis (198).

2.3.4 Inflammatory phenotype stability

Several studies have looked into the stability of inflammatory phenotypes in asthma and have observed mixed results (32, 199). For example, Simpson et al. (18) found that NEA was stable in both the short term (4 weeks) and long term (5 years). In the same study, EA, however, was found to be relatively unstable, as 86% changed from EA to NEA in the short term. In a more recent study, Pembrey et al. (32) showed that out of 623 asthmatics, 15% changed from EA to NEA, 17% changed from NEA to EA, and 67% remained stable over three months, suggesting that EA and NEA are more stable than first thought, with other studies supporting this (200, 201). Factors such as medications (179), exacerbations (202), allergen exposure (90), air pollution (126), and viral infections (203) have been shown to influence the increase or decrease of neutrophils and eosinophils in asthma, and may result in phenotype instability. ICS, in particular, is known to have a potential reducing effect on sputum eosinophils in asthmatics (67, 204), and a possible impact on increasing sputum neutrophils (175).

2.3.5 Measurement of soluble mediators: an alternative approach to assess asthma pathophysiology

As described above, sputum induction is commonly used to identify inflammatory phenotypes using leukocyte cell counts to assess asthma pathophysiology. There are other ways of looking at asthma pathophysiology, such as looking at sputum gene expression (169, 192). Additionally, other biomarkers in sputum, (proteins and cytokines often referred to as mediators), can be analysed to further investigate asthma pathophysiology (205). Enzyme-linked immunosorbent assay (ELISA) is a commonly used technique capable of analysing levels of specific mediators in a variety of samples (206). However, ELISAs can only measure only one mediator per sample. As an alternative, multiplex bead arrays (described in more detail in **appendix 4**) allow the measurement of multiple mediators in one sample (207). Alternatively, proteomics can be used to simultaneously assess hundreds of mediators in sputum (208, 209).

Most research assessing mediator levels in sputum has been done in a tertiary setting, generally in HICs, and in asthma in general, or in EA and NEA (31, 167, 205), rather than across the four inflammatory phenotypes described above. Examples of studies examining mediators across the four phenotypes include a study by Manise et al (181) which found increased levels of IL-5 and IL-13 in EA compared to healthy controls, NA and PGA, and a more recent study by Plavsic et al (210) which found increased levels of IL-8 in NA, and IL-17a in EA. **Table 1** provides examples of findings relating to specific mediators in the sputum of asthma patients.

***Table 1**: Examples of mediators assessed in the sputum of asthmatics.*

Mediator	Mechanism/Function	Finding in Asthma	References
IL-1β	Pro-inflammatory, pleiotropic, activates Th1/Th2 cells	↑ in severe asthma ↑ in EA ↑ in NEA ↑ in asthma associated with neutrophils	(167, 205, 211, 212)
IL-6	Pro-inflammatory, pleiotropic, activates Th1/Th2 cells	↑ in asthma associated with neutrophils	(213)
IL-8	Recruits and activates neutrophils	↑ NEA vs EA	(205, 214)
IL-5	Recruits eosinophils, promotes eosinophil survival	↑ poorly controlled asthma ↑ EA vs NEA	(181, 205, 215-217)
NE	Released by neutrophils, affects ECM	↑ in asthma associated with neutrophils	(218)
IFN-γ	Reduces Th2 and eosinophils, promotes neutrophilic inflammation	↓ in atopic asthma	(219, 220)
IL-13	Involved in B-cell class switch to IgE production, promotes allergic response, recruits eosinophils	↑ in asthma associated with eosinophils	(181, 221)
ECP	Cytotoxic, released by eosinophils	↑ asthma vs healthy controls ↑in EA	(167, 222)
TNF-α	Pro-inflammatory, pleiotropic, recruits Th1/Th2 cells, involved in innate response, induces AHR	↑ in asthma ↑ Neutrophils ↑ Severe asthma	(223, 224)
MMP-9	Involved in airway remodelling, restructures ECM	↑ NEA vs EA ↓ MMP-9/TIMP-1 ratio in severe asthma ↑ asthma vs healthy controls	(205, 225)
VEGF	Promotes vascular growth and angiogenic sprouting, suspected role in airway remodelling	↑ asthma vs healthy controls ↑ vascular density in asthmatics on CT imaging ↑ EA vs NEA	(167, 226)
TIMP-1	Inhibits MMPs	↓ TIMP-1 correlated to tissue remodelling in asthma	(26, 205)
Periostin	Restructures ECM through tube formation and MMP induction, recruits eosinophils	↑ in EA vs NEA in poorly controlled asthma	(167, 227-229)
Substance P	Involved in neurogenic inflammation, triggers release of inflammatory mediators	↑ asthma vs healthy controls Correlated with ↑ eosinophils	(28, 29, 230)
NKA	Involved in neurogenic inflammation, triggers release of inflammatory mediators	↑ asthma during acute exacerbation Correlated with ↑ eosinophils	(29, 230)
Nociceptin	Suspected anti-inflammatory, inhibits bronchodilation	↑ EA vs NEA ↑ severe asthmatics vs non-asthmatics	(167, 231)

3. Methods

An overview of the methodology and objectives of the WASP study has been published previously (31) and a summary of the methodology most relevant to this thesis is provided below. Briefly, the WASP study collected data in asthmatic and non-asthmatic children/adolescents (and adults in the UK) from five different research centres. These were located in areas with different levels of development (HICs and MLICs, based on gross national Income (GNI) per capita as previously determined in (91)), and asthma prevalence (high, medium, and low) as previously identified in the ISAAC study (30). The centres and countries involved were Wellington (NZ), Bristol (United Kingdom), Salvador (Brazil), Esmeraldas (Ecuador), and Entebbe (Uganda). **Table 2** provides an overview of the studies and centres.

***Table 2**: Centres and studies involved in the WASP study*

Study/Centre	National Income	Asthma Prevalence	Study description
The NZ Asthma and Allergy Cohort Study (NZA2CS); Wellington, NZ	High	High	NZAZCS birth cohort. New data collection at age 16–20 years. Further data from a cross-sectional study in children aged 12–16 years recruited from schools and the community.
Avon Longitudinal Study for Parents and Children (ALSPAC); Bristol, United Kingdom	High	High	Birth cohort study with extensive detailed longitudinal information. New data collection at age 26–27 years.
Social Change, Asthma and Allergy in Latin America (SCAALA); Salvador, Brazil	Middle	High	SCAALA cohort. New data collection at age 11–19 years. Cross-sectional study in children aged 11–19 years recruited from three schools.
Social Change, Asthma and allergy in Latin America (SCAALA); Esmeraldas, Ecuador	Middle	Medium	Population-based cohort. New data collection at age 8–12 years Cross-sectional study in children aged 12–16 years recruited from schools.
Entebbe Childhood asthma study; Entebbe, Uganda	Low	Low	Cross-sectional study in children aged 12–16 years recruited from schools.

Ethics: The overall WASP study was approved by the London School of Hygiene and Tropical Medicine (LSHTM) Ethics Committee (ref: 9776). The individual studies conducted in each centre were approved by local ethics bodies. In NZ, the study was approved by the Northern B Health and Disability Ethics Committee (16/NTB/64 & 15/NTB/2). All participants and caregivers/parents gave informed assent/consent.

Recruitment: Recruitment methods varied per country. Participants were generally recruited as part of ongoing cohort studies in NZ (232), the UK (233-235), Brazil, and Ecuador (236). In NZ, Ecuador, and Brazil, additional participants were recruited through the community via school surveys and local communities. In Uganda, participants were recruited as part of an extensive cross-sectional survey in schools (237). A total of 1355 (998 asthmatics, 357 controls) participants were recruited from Brazil (204 asthmatics, 40 controls), Ecuador (176 asthmatics, 68 controls), Uganda (207 asthmatics, 50 controls), NZ (235 asthmatics, 132 controls), and the UK (176 asthmatics, 67 controls) (32).

Recruitment criteria: The ages of individuals targeted for recruitment varied between countries. Individuals with chronic diseases (other than asthma) or who were pregnant were excluded from the study. As all studies required both asthmatic and non-asthmatic participants, asthma status was determined using the ISAAC phase II questionnaire (see below) and some additional questions regarding asthma control as described by Pembrey et al (32). Asthma was defined as a positive response to *wheezing/whistling in the chest* and/or *the use of asthma medication in the past 12 months* for children using the ISAAC phase II questionnaire (238, 239). Non-asthmatics were identified on the basis of a negative response to both these questions and with no previous history of asthma.

Screening and questionnaires: All participants or their caregivers completed a questionnaire largely based on the aforementioned ISAAC study phase II survey (238), which has been widely adopted and validated (61, 239). The questionnaire involved questions on wheeze, shortness of breath, cough,

chest tightness, eczema, and rhinitis. More detailed questions about asthma severity, medication use (asthma relievers and preventors) was also included. In addition, the Asthma ACQ was used to determine current asthma control status (240). An ACQ score of ≤1.5 was considered well-controlled, while an ACQ score >1.5 was considered poorly controlled.

Clinical tests

All participants underwent at least one clinical assessment. This involved a battery of tests including SPT, spirometry, FeNO, nasal lavage, and hypertonic saline sputum induction. Results for some of these tests were not available for the study described in this thesis, hence methods for blood collection, lung function, FeNO, and nasal lavage are not described below (these are described in the WASP study protocol document (31)).

Clinical test criteria: Clinical visit appointments were postponed if participants had acute asthma exacerbation or symptomatic respiratory infection in the four weeks prior to the clinical visit. In addition, participants were asked not to take any of the following asthma medication, if safe to do so, before the visit: anti-histamines (5 days prior), steroid nasal sprays (7 days prior), non-steroidal anti-inflammatories (NSAIDs) (6 hours prior), asthma medication (cromoglycate, nedocromil, short-acting β-agonists, and ipratropium bromide; 6 hours prior), and theophyllines (24-hours prior) (32).

Skin prick test: SPTs were done according to a well-definite protocol (238), using histamine and saline as positive and negative control. SPT was considered positive if the test showed a wheal size of at least 3mm (after subtraction of the negative control) for at least one allergen from a panel of eight allergens, consisting of: house dust mite (*Dermatophagoides pteronyssinus*), tree pollen mix, grass

pollen mix, cat and dog dander, mould mix (*Alternaria tenuis*, Penicillium mix), plus locally relevant allergens (Cladosprium for NZ (31)).

Sputum induction: Sputum induction was conducted using a standardised, well-validated protocol as previously described (241), following ERS guidelines (171). Briefly, a hypertonic saline solution (4.5% w/v for all centres except the UK (which used 5% w/v due to availability)) was aerosolised using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000, Langen, Germany) and administered orally through a mouth piece (Hans-Rudolph Inc, Kansas City, USA) in intervals from 30 seconds up to 4 minutes, up to a total of 15.5 minutes. For safety, between intervals, spirometry (methods described in (31)) was conducted, and 200mg salbutamol was administered if the FEV_1 dropped to 75% predicted or less. At the end of the test, the participant was encouraged to produce a sputum sample, which was collected in a sterile plastic container, and processed as described below. A second appointment was scheduled if an insufficient sputum sample was produced at the first visit.

Sputum processing: Sputum processing was conducted by laboratory technicians who had received standardised training in sample processing and handling specifically for the WASP study. The protocol used is well-established (241). In all countries, samples were processed within 2 hours of collection. Samples were first transferred to a sterile plastic petri-dish. Sputum plugs were visually selected and isolated using tweezers. Whenever possible, a minimum of 100µL sputum plugs were transferred into a 15ml centrifuge tube using a displacement pipette. Four times the selected sputum volume of 10x diluted dithiothreitol (DTT) (0.01g/mL DTT) (Sputasol, Oxoid Ltd, Basingstoke, Hampshire, England) was then added. The sputasol/sputum plug mixture was then incubated for 30 minutes on a rotary mixer to disperse sputum plugs and break down mucus. After 30 minutes, phosphate-buffered saline (PBS) (Sigma-Aldrich, Auckland, NZ) was added (4x volume of the selected plugs). The sample suspension was then filtered through a 60µm filter (Millipore, County Cork, Ireland). A small volume

of cell suspension was mixed with trypan blue and used to determine Total Cell Count (TCC) (only in NZ and UK) and viability (%). The cell suspension was then centrifuged at 1600 RPM for 8 minutes. Sputum supernatants were aliquoted and stored at -80˚C for biomarker analysis. The cell pellet was resuspended in PBS to a concentration of $1x10^6$ cells/mL. 30 to 100µL of this suspension was added to the cytospin apparatus and centrifuged at 44xg for 5 minutes to produce cytospin slides. Adequate slide quality was determined visually. Slides were air-dried for up to 4 days prior to staining. A more detailed protocol for sputum sample processing can be found in **Appendix 1.**

Sputum cell slide staining and differential cell count (DCC): Cell slides were stained using the Diff-Quik® or Giemsa fixative/stain set (Dade Behring, Deerfield, IL). After drying, slides were covered using DPX mounting medium (Australian Scientific, Kotara, NSW) and a coverslip, air-dried, and stored in a dark place at room temperature. A detailed protocol can be found in **Appendix 2.** Stained cell slides from each country were transported to NZ for assessment except for slides from Brazil, which could not be sent overseas due to ethical restrictions. Cell slides were assessed using a light microscope. Using a 40x objective, >400 non-squamous cells per slide were counted. Specific cell populations (macrophages, eosinophils, neutrophils, lymphocytes, bronchial epithelial cells) were identified on the basis of characteristics described in **Table 3**. The percentage of squamous epithelial cells of total cells was also determined. Slide quality was considered adequate for inflammatory phenotyping if the total number of squamous cells was <80%, with at least 400 non-squamous cells counted.

***Table 3**: Cell characteristics -Diff-quik stained slides.*

	CELL size/shape	NUCLEUS size/shape/ chromatin	CYTOPLASM colour/granules	COMMENT
Bronchial or columnar epithelial cell	10-20µm oval or columnar	8µm single, round, purple, loose chromatin pattern	blue – light purple	Often cilia visible, "treetrunk" like appearance
Squamous cell	40-60µm polygonal	5-10µm single, round, purple, loose	blue – pink, non-complex, bacteria often evident	Very large
Macrophage	20-40µm oval Large to small	12-15µm round 1 or more purple, dense	blue / grey foamy ≅ (white vacuoles)	smokers have black / purple dots
Neutrophil	16µm oval	3-5 lobes purple, tight	pink / blue granules, blue cytoplasm	
Lymphocyte	9-12µm oval	1 round purple fills cell, tight	sky blue thin rim	Large lymphocytes may look like small monocytes
Eosinophil	16µm oval	2 lobes purple, dense	brick red granules, pink cytoplasm	Often have "sunburnt man with sunglasses" appearance
Mast cell	15-24µm slightly irregular	small, round, purple. Can be obscured by granules	blue / purple with numerous dark granules	Rare, don't count

In the case of the Brazilian slides, phenotyping was conducted in Brazil by a trained researcher, and quality control was conducted remotely in NZ via the assessment of microscope images. Quality control and confirmation of phenotyping by the laboratory technician (Jeroen Burmanje) in NZ was overseen by an experienced researcher. A detailed protocol for phenotyping cell-slides can be found in **appendix 3.**

Definition of asthma sub-groups: EA and NEA were defined using a cut-off of ≥2.5% eosinophils in DCC (32, 241). In this study, when referring to ''asthma sub-groups'', it is referring to the EA and NEA groups.

Definition of asthma inflammatory phenotypes: Four inflammatory phenotypes were identified using cutoffs of ≥2.5% eosinophils and ≥61% neutrophils in DCC (18, 241). In this study, when referring to ''asthma phenotypes'', it is referring to these 4 asthma inflammatory phenotypes.

Asthma sub-group EA consists of phenotypes:

- **Eosinophilic asthma (EA):** ≥2.5% eosinophils and <61% neutrophils.
- **Mixed granulocytic asthma (MGA):** ≥2.5% eosinophils and ≥61% neutrophils.

Asthma sub-group NEA consists of phenotypes:

- **Neutrophilic asthma (NA):** <2.5% eosinophils and ≥61% neutrophils.
- **Paucigranulocytic asthma (PGA**): <2.5% eosinophils and <61% neutrophils.

Sputum mediator analysis: After thawing, sputum supernatants were assessed for a range of 20 biomarkers. For convenience, in this thesis, they have been categorised into three groups as follows: inflammatory mediators (IL-1β, IL-6, IL-8, NE, IL-13, ECP, prostaglandin D2 (PGD-2), and histamine), neural mediators (neural growth factor β (NGF-β), NKA, substance P, nociceptin, and brain derived neurotrophic factor (BDNF)), and remodelling mediators (MMP-1, MMP-9, tissue inhibitor of metalloproteinase (TIMP-1), VEGF-α, periostin, elastin, and sADAM33).

IL-1β, IL-6, IL-8, IL-13, NGF-b, BDNF, MMP-1, MMP-9, TIMP-1, VEGF-a were assessed using procartaplex assays (Invitrogen, CA, USA) on the MAGPIX platform utilising xMAP technology (Luminex Corporation, TX, USA; see **appendix 4** for more detail). All other mediators were analysed using commercial ELISA as follows: ECP (MBL International, MA, USA), PGD-2 (Cayman Chemical, MI, USA), NE and periostin (Merck Millipore, MA, USA), histamine and substance P (Abcam, Cambridge, United Kingdom), NKA (RayBiotech. Inc, GA, USA), nociceptin (Creative Diagnostics, NY, USA), elastin and sADAM33 (Cusabio, Wuhan, China). All ELISAs were analysed using the TS800 microplate reader

(BioTech®, VT, USA). All assays were conducted according to manufacturer protocols in Massey University's Research Centre for Hauora and Health, Wellington, NZ. Raw data were processed using Milliplex Analyst (Merck Millipore, MA, USA) for MAGPIX analysis and Gen5 (BioTek, VT, USA) for ELISA analysis. The effect of DTT on sputum mediator analyses was determined as described previously (242), and was negligible for the mediators of interest (data not shown). In the case of some analytes (i.e. PGD-2 for Brazilian samples and ECP, PGD-2, and histamine for Ecuadorian samples), analyses were not conducted due to limited sample availability.

Data analysis: Population, clinical, inflammatory characteristics, and mediator levels were compared between asthmatics and controls, asthma sub-groups (EA/NEA), and asthma phenotypes (EA, NA, MGA, and PGA). Analyses were conducted for all participants combined (overall), stratified per country, and comparing specific phenotypes between countries. Analyses were conducted using chi-square, t-tests, Mann-Whitney U-test, or Kruskal-Wallis test (with Dunn's post-comparison test) as appropriate. Data are presented as median (interquartile range (IQR)), mean (min – max), or number/ frequency (%) as appropriate.

Mediators with lower than 15% detectability overall were treated as categorical variables i.e. detectable vs non-detectable. Mediator levels that fell below the lowest limit of detection were assigned ½ of the assay's lowest limit of detection (243). Associations between demographics (age, sex, atopy, etc.) and mediator levels overall were assessed using linear regression or Mann-Whitney U test.

Correlations between leukocytes and mediators in asthma, EA, and NEA, and correlations between mediators in asthmatics overall were determined using Spearman correlation analysis with results presented as Spearman correlation coefficients (r). Additionally, analyses between groups stratified

on the basis of medication use (preventer users, reliever users, or non-users overall, as well as stratified per country) were done using Mann-Whitney U test or Chi-Square test, with data presented as median (IQR) or detectability (%). All univariate analyses were conducted using GraphPad PRISM 5 (Graphpad Software Inc, La Jolla, CA, USA).

Multivariate analysis was performed using STATA version 11 (STATA Corp, College Station, TX, USA). Mediator levels between asthmatics and controls, asthma sub-groups (EA/NEA) and controls, and phenotypes (EA, NA, MGA, PGA) were analysed in each country. Linear regression was used for continuous variables and logistic regression for dichotomous variables, adjusting for age and sex (32). Data were presented as regression coefficients (95% confidence interval) or odds-ratios (OR) (95% confidence interval) as appropriate. For within country phenotype analysis, EA was used as the reference group due to low NA and MGA prevalence. Comparisons between countries was conducted comparing specific phenotypes only.

4. Results

4.1 Participant characteristics

A total of 1225 participants who took part in the WASP study provided a sputum sample as previously described (32). Demographic, clinical, and inflammatory characteristics are shown in **table 4.** A total of 718 (58.6%; 527 asthmatics, 191 controls) participants had slides that were considered of adequate quality for inflammatory phenotyping and had a sample with sufficient supernatant for soluble mediator analysis. When comparing population characteristics of the same group across countries, there were significant differences in sex, age, and atopy (Kruskal-Wallis $p<0.01$) for both asthmatics and controls (**table 4**). In particular, the oldest participants were based in the UK (mean age 26 years), and the youngest in Ecuador (mean age 12 years). Significant differences in sex ($p<0.01$) were observed, with, for example, the percentage of females ranging from 74.0% in asthmatics in the UK to 28.2% in controls from Ecuador. As expected, atopy prevalence was higher in asthmatics compared to controls across all countries, with the highest prevalence observed in NZ and Brazil (80.8% and 84.6%, respectively). Although atopy prevalence was significantly ($p<0.05$) lower amongst controls (ranging from 6% in Uganda to 39% in NZ), atopy prevalence was relatively high in Brazilian controls (73%). Amongst asthmatics, the prevalence of poorly controlled asthma ranged from 3% in Ecuador to 29% in Uganda. All participants from the UK and almost all in NZ (94.2%) reported asthma medication use (either reliever or preventer) in the past 12 months; 67.7% of Ugandan and 70.9% of Ecuadorian asthmatics used no medication.

When examining DCC overall, squamous cell contamination was approximately 10% in all countries (**table 4**). Macrophages accounted for the majority of non-squamous sputum cells across all countries, ranging from 68.0% in the United Kingdom to 86.1% in Ecuador; the exception to this was Uganda where levels of macrophages were considerably lower i.e. 33.4%. Lymphocytes and epithelial cells

were detected at very low levels across all countries, with no significant differences between asthmatics and controls. Of all groups, the highest median sputum neutrophil percentages were found in Ugandan asthmatics and controls (60.5% and 65.5%, respectively) (**table 4**). Amongst asthmatics, the highest median sputum eosinophil percentages were observed in NZ (2.3%) and the lowest in Uganda (0.5%). Eosinophils were generally detected at very low levels (median 0%) in controls across all countries.

When asthma was stratified into the asthma sub-groups, EA and NEA **(table 5)**, differences were observed across countries. EA prevalence varied from 50% of asthma cases in NZ to 32% in the UK, with NEA thus being the more dominant asthma sub-group. When comparing population characteristics between the same sub-groups across countries, there were significant differences in age, gender, and atopy between countries age; $p<0.01$, gender: $p<0.05$, atopy: $p<0.01$). Atopy was more prevalent in EA in all countries, although, atopy prevalence in EA in Ecuador was 20 – 30% lower than other countries.

When asthma was stratified into the four inflammatory phenotypes, EA, NA, MGA, and PGA (**table 6**), differences in the prevalence of these asthma phenotypes across countries were observed. In particular, the proportion of EA varied from 48.6% in NZ to 22.1% in Uganda. The highest prevalence of NA and MGA was observed in Uganda (representing 38.2% and 10.3% of cases, respectively). All other countries had relatively low prevalence of NA/MGA (≤12% of asthma cases per country). PGA was the most prevalent asthma phenotype across all countries, representing over half of all asthma in the United Kingdom (56.0%), Brazil (60.4%), and Ecuador (61.2%). When comparing population characteristics of each phenotype with the same phenotype from different countries, significant differences in age, sex, and atopy (age: $p<0.01$, gender: $p<0.05$, atopy: $p<0.05$) were observed. Asthma control was higher in NA across all countries, but this was based on relatively small numbers of NA.

Asthma control was poorest among MGA across all countries, although again, this was based on relatively small numbers of MGA cases.

***Table 4**: Demographic, clinical, and inflammatory characteristics of asthmatics and controls per country*

	New Zealand (Wellington)		United Kingdom (Bristol)		Brazil (Salvador)		Uganda (Entebbe)		Ecuador (Esmeraldas)		Total	
Participants who provide:	**Asthmatics**	**Controls**	**Asthmatics**	**Controls**	**Asthmatics**	**Controls**	**Asthmatics**	**Controls**	**Asthmatics**	**Controls**	**Asthmatics**	**Controls**
Total sputum samples (n)	350		229		181		221		244		1225	
Total countable sputum slides (n)	332		111		137		118		183		881	
a high-quality slide for mediator analysis (n)	204	103	50	22	91	11	68	16	116	39	527	191
Female (n, %)	99 (48.5%)	61 (59.3%)	37 (74.0%)	12 (54.5%)	62 (68.1%)	8 (72.7%)	50 (73.5%)	10 (62.5%)	55 (47.4%)	11 (28.2%)	303 (57.5%)	102 (53.4%)
Age (mean, min-max)	14.3 (9 – 20)	14.9 (9 – 19)	26 (25 – 27)	26.1 (25 – 27)	18.2 (13 – 24)	20.3 (18 – 23)	15.3 (12 – 18)	15.8 (13 – 19)	12.2 (10 – 17)	11.7 (11 – 12)	15.7 (9.0 – 27.0)	16.0 (9.0 – 27.0)
Atopy (n, %)	165 (80.8%)	40 (38.8%)	31 (62.0%)	8 (36.4%)	77 (84.6%)	8 (72.7%)	37 (54.4%)	1 (6.3%)	38 (32.8%)	7 (17.9%)	348 (66.0%)	64 (33.5%)
Asthma control (ACQ6) (n)	182	-	50	-	90	-	67	-	116	-	505	-
Well-controlled	155 (85.2)	-	43 (86.0%)	-	66 (73.3%)	-	47 (70.1%)	-	112 (96.6%)	-	423 (83.8%)	-
Poorly controlled	27 (14.8)	-	7 (14%)	-	24 (26.7%)	-	20 (29.9%)	-	4 (3.4%)	-	82 (26.2%)	-
Asthma medication use †												
None	12 (5.8%)	-	0 (0.0%)	-	33 (37.9%)	-	44 (67.7%)	-	51 (70.9%)	-	140 (36.5%)	-
Reliever	182 (89.2%)	-	45 (97.8%)	-	51 (57.9%)	-	21 (31.3%)	-	18 (24.6%)	-	317 (60.2%)	-
Preventer	140 (68.6%)	-	33 (75.0%)	-	17 (19.5%)	-	5 (7.7%)	-	6 (8.2%)	-	201 (35.8%	-
% cells (median/IQR)												
% Neutrophils	16.0 (6.0 – 33.5)	21.5 (9.9 – 43.0)	26.6 (13.2 – 52.9)	34.7 (20.8 – 52.9)	6.0 (1.3 – 22.0)	28.0 (4.5 – 62.5)	60.5 (28.3 – 82.9)	65.5 (32.4 – 78.7)	10.2 (4.6 – 22.7)	8.8 (3.0 – 19.5)	26.5 (5.2 – 41.7)	21.5 (7.8 – 46.3)
% Eosinophils	2.3 (0.5 – 10.3)	0.0 (0.0 – 0.0)	1.3 (0.3 – 3.2)	0.0 (0.0 – 0.3)	1.0 (0.0 – 4.8)	0.0 (0.0 – 0.8)	0.5 (0.0 – 3.7)	0.4 (0.0 – 1.6)	0.7 (0.0 – 3.9)	0.0 (0.0 – 0.3)	1.4 (0.0 – 5.9)	0.0 (0.0 – 0.8)
% Macrophages	75.4 (53.2 – 86.9)	75.7 (53.7 – 87.5)	68.0 (37.4 – 85.5)	64.1 (46.8 – 76.9)	85.5 (66.5 – 95.3)	58.5 (37.3 – 92.3)	33.4 (15.2 – 55.0)	32.4 (20.8 – 64.4)	86.1 (65.7 – 92.6)	89.3 (78.8 – 96.0)	75.8 (48.1 – 89.6)	75.8 (51.2 – 88.8)
% Lymphocytes	0.0 (0.0 – 0.5)	0.22 (0.0 – 0.64)	0.3 (0.0 – 0.7)	0.2 (0.0 – 0.3)	1.5 (0.8 – 2.5)	1.0 (0.5 – 1.5)	0.25 (0.0 – 0.5)	0.4 (0.3 – 0.7)	0.0 (0.0 – 0.0)	0.2 (0.0 – 0.7)	0.25 (0.0 – 1.0)	0.3 (0.0 – 0.7)
% Epithelial cells	0.0 (0.0 – 0.6)	0.0 (0.0 – 0.25)	0.3 (0.0 – 0.7)	0.0 (0.0 – 0.3)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.3)	0.0 (0.0 – 0.3)	0.0 (0.0 – 0.2)	0.0 (0.0 – 0.3)	0.0 (0.0 – 0.3)	0.0 (0.0 – 0.3)
% Squamous cells	13.0 (5.1 – 42.0)	13.0 (3.8 – 32.0)	9.6 (4.1 – 17.0)	11.6 (4.5 – 26.5)	10.7 (5.2 – 16.7)	17.2 (4.1 – 21.3)	10.5 (1.5 – 18.5)	12.5 (6.5 – 19.3)	3.9 (1.5 – 8.7)	2.9 (1.2 – 8.9)	9.0 (3.4 – 18.8)	10.7 (2.9 – 23.5)

†: Asthma medication use in the last 12 months (participants may use reliever/preventer concurrently). **‡:** Based on ACQ6 score: Well-controlled asthma = ≤1.5, poorly controlled asthma = >1.5.

***Table 5**: Demographic, clinical, and Inflammatory characteristics of asthma sub-groups (EA and NEA) per country.*

Asthma sub-groups	NZ		UK		Brazil		Uganda		Ecuador		Total	
	EA	NEA	EA	NEA	EA	NEA	EA	NEA	EA	NEA	EA	NEA
Cases (n, % of asthmatics)	101 (50.0%)	101 (50.0%)	16 (32.0%)	34 (68.0%)	31 (34.1%)	60 (65.9%)	22 (32.4%)	46 (67.6%)	38 (32.8%)	78 (67.2%)	208 (39.5%)	319 (60.5%)
Female n (%)	44 (43.6%)	55 (54.5%)	11 (68.8%)	26 (76.5%)	20 (64.5%)	42 (70.0%)	14 (63.6%)	36 (78.3%)	12 (31.6%)	43 (55.1%)	101 (48.5%)	202 (63.3%)
Age (mean (min-max))	13.9 (9.0 – 16.0)	15.0 (9.0 – 20.0)	26.0 (25.0 – 27.0)	26.1 (25.0 – 27.0)	18.1 (13.0 – 24.0)	18..2 (14.0 – 23.0)	15.1 (13.0 – 17.0)	15.4 (12.0 – 18.0)	12.6 (11.0 – 17.0)	11.9 (10.0 – 17.0)	15.4 (9.0 – 27.0)	16.0 (9.0 – 27.0)
Atopy	85 (84.2%)	78 (77.2%)	12 (75.0%)	19 (55.9%)	29 (93.5%)	48 (80.0%)	17 (77.3%)	20 (43.5%)	21 (55.2%)	17 (21.8%)	164 (78.8%)	182 (57.1%)
Asthma control ‡	90	89	16	34	31	59	21	46	38	78	196	306
Well controlled (score ≤1.5)	72 (80.0%)	83 (91.2%)	14 (87.5%)	29 (85.3%)	22 (71.0%)	44 (74.6%)	14 (66.7%)	33 (71.7%)	34 (89.5%)	78 (100.0%)	156 (79.6%)	267 (87.3%)
Poorly controlled (score >1.5)	18 (20.0%)	8 (8.8%)	2 (12.5%)	5 (14.7%)	9 (29.0%)	15 (25.4%)	7 (33.3%)	13 (28.3%)	4 (10.5%)	0 (0.0%)	42 (21.4%)	41 (13.4%)
Asthma medication use †												
None	2 (1.9%)	10 (9.9%)	0 (0.0%)	0 (0.0%)	12 (40.0%)	21 (36.8%)	11 (52.3%)	33 (75.0%)	20 (71.4%)	31 (70.5%)	45 (33.1%)	95 (38.4%)
Reliever	93 (92.1%)	87 (86.1%)	16 (100.0%)	29 (96.6%)	16 (51.6%)	35 (61.4%)	11 (50.0%)	10 (22.2%)	8 (27.6%)	10 (22.7%)	144 (64.3%)	171 (57.8%)
Preventer	74 (73.6%)	64 (63.3%)	12 (75.0%)	21 (75.0%)	7 (23.3%)	10 (17.5%)	4 (19.0%)	1 (2.2%)	0 (0.0%)	6 (13.6%)	97 (38.2%)	102 (34.3%)

†: Asthma medication use in the last 12 months (participants may use reliever/preventer concurrently). **‡:** Based on ACQ6 score: Well-controlled asthma = ≤1.5, poorly controlled asthma = >1.5. Asthma sub-group EA consists of asthma phenotypes EA and MGA, while sub-group NEA consists of phenotype NA and PGA.

***Table 6**: Demographic, clinical, and inflammatory characteristics of asthma phenotypes (EA/NA/MGA/PGA) per country.*

	NZ	UK	Brazil	Uganda	Ecuador	Total
Eosinophilic asthma (EA)						
Cases (n, % of asthmatics)	98 (48.6%)	14 (28.0%)	29 (31.9%)	15 (22.1%)	33 28.4%)	189 (35.9%)
Female n (%)	44 (44.9%)	9 (64.5%)	18 (62.1%)	9 (60.0%)	10 (30.3%)	90 (47.6%)
Age (mean (min-max))	13.9 (9.0 – 20.0)	25.9 (25.0 – 27.0)	18.1 (13.0 – 24.0)	14.9 (13.0 – 17.0)	12.4 (11.0 – 16.0)	15.3 (9.0 – 27.0)
Atopy	83 (84.7%)	10 (90.9%)	28 (96.6%)	13 (86.7%)	19 (57.6%)	153 (82.7%)
Asthma Control ‡	87	14	29	14	33	177
Well controlled	70 (80.5%)	12 (85.7%)	21 (72.4%)	10 (71.4%)	30 (90.9%)	143 (80.8%)
Poorly controlled	17 (19.5%)	2 (14.3%)	8 (27.6%)	4 (28.6%)	3 (9.1%)	34 (19.2%)
Asthma Medication (n) †						
None	2 (2.0%)	0 (0.0%)	12 (41.3%)	8 (57.1%)	17 (70.8%)	39 (21.9%)
Reliever	90 (91.8%)	14 (100.0%)	15 (51.7%)	7 (46.6%)	7 (28.0%)	133 (70.4%)
Preventer	71 (72.4%)	10 (71.4%)	6 (21.4%)	2 (14.3%)	0 (0.0%)	89 (50.0%)
Neutrophilic asthma (NA)						
Cases (n, % of asthmatics)	14 (6.9%)	6 (12.0%)	5 (5.5%)	26 (38.2%)	7 (6.0%)	58 (11.0%)
Female n (%)	10 (71.4%)	5 (83.3%)	4 (80.0%)	19 (73.1%)	3 (42.9%)	41 (70.7%)
Age (mean (min-max))	11.9 (9.0 – 16.0)	26.0 (25.0 – 27.0)	17.4 (16.0 – 19.0)	15.3 (12.0 – 18.0)	12.1 (10.0 – 15.0)	15.4 (9.0 – 27.0)
Atopy	11 (78.5%)	3 (50.0%)	4 (80.0%)	14 (53.8%)	2 (28.6%)	34 (58.6%)
Asthma Control ‡	14	6	4	26	7	57
Well controlled	14 (100.0%)	6 (100.0%)	4 (100.0%)	18 (69.2%)	7 (100.0%)	49 (86.0%)
Poorly controlled	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (30.8%)	0 (0.0%)	8 (14.0%)
Asthma Medication (n) †						
None	0 (0.0%)	0 (0.0%)	3 (60.0%)	20 (76.9%)	6 (100.0%)	29 (50.0%)
Reliever	14 (100.0%)	6 (100.0%)	2 (40.0%)	4 (15.4%)	0 (0.0%)	26 (45.6%)
Preventer	9 (64.3%)	5 (83.3%)	0 (0.0%)	1 (4.0%)	0 (0.0%)	15 (26.8%)
Mixed Granulocytic asthma (MGA)						
Cases (n, % of asthmatics)	3 (1.5%)	2 (4.0%)	2 (2.2%)	7 (10.3%)	5 (4.3%)	19 (3.6%)
Female n (%)	0 (0.0%)	2 (100.0%)	2 (100%)	5 (71.4%)	2 (40.0%)	11 (57.9%)
Age (mean (min-max))	14 (11.0 – 16.0)	26 (26.0 – 26.0)	18.5 (18.0 – 19.0)	15.4 (13.0 – 17.0)	14.0 (12.0 – 17.0)	16.2 (11.0 – 26.0)
Atopy	2 (66.7%)	2 (100.0%)	1 (50.0%)	4 (57.1%)	2 (40.0%)	11 (57.9%)
Asthma Control ‡	3	2	2	7	5	19
Well controlled	2 (67.7%)	2 (100.0%)	1 (50.0%)	4 (57.1%)	4 (80.0%)	13 (68.4%)
Poorly controlled	1 (33.3%)	0 (0.0%)	1 (50.0%)	3 (45.9%)	1 (20.0%)	6 (31.5%)
Asthma Medication (n) †						
None	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (42.9%)	3 (60.0%)	6 (31.5%)
Reliever	3 (100.0%)	2 (100.0%)	1 (50.0%)	4 (57.1%)	1 (25.0%)	11 (61.1%)
Preventer	3 (100.0%)	2 (100.0%)	1 (50.0%)	2 (28.6%)	0 (0.0%)	8 (42.1%)
Paucigranulocytic asthma (PGA)						
Cases (n, % of asthmatics)	87 (43.0%)	28 (56.0%)	55 (60.4%)	20 (29.4%)	71 (61.2%)	261 (49.5%)
Female n (%)	45 (51.7%)	21 (75.0%)	38 (59.9%)	17 (85.0%)	40 (56.3%)	161 (61.7%)
Age (mean (min-max))	15.2 (9.0 – 20.0)	26.1 (25.0 – 27.0)	18.3 (14.0 – 23.0)	15.5 (12.0 – 18.0)	11.9 (10.0 – 17.0)	16.1 (9.0 – 27.0)
Atopy	67 (77.0%)	16 (76.2%)	44 (80.0%)	6 (30.0%)	15 (21.1%)	148 (56.7%)
Asthma Control ‡	77	28	55	20	71	251
Well controlled	69 (89.6%)	23 (82.1%)	40 (72.7%)	15 (75.0%)	71 (100.0%)	218 (86.9%)
Poorly controlled	8 (10.4%)	5 (17.9%)	19 (34.5%)	5 (25.0%)	0 (0.0%)	37 (14.7%)
Asthma Medication (n) †						
None	10 (11.5%)	0 (0.0%)	18 (34.6%)	13 (68.4%)	25 (65.8%)	66 (30.3%)
Reliever	73 (83.4%)	23 (95.8%)	33 (63.5%)	6 (31.6%)	10 (26.3%)	145 (66.5%)
Preventer	55 (63.1%)	16 (72.7%)	10 (19.2%)	0 (0.0%)	6 (15.8%)	87 (39.9%)

†: asthma medication usage in the last 12 months (participants may use reliever/preventer simultaneously). **‡:** Based on ACQ6 score: ≤1.5= Well controlled asthma, >1.5 = poorly controlled asthma.

4.2 Detectability of sputum mediators

16/20 mediators were detectable in over 50% of all samples (**Table 7**). However, IL-13 was detectable in only 5.6%, NGF-β in 7.9%, BDNF in 15.6%, and substance P in 47.2% of all samples. For a full overview of the detectability per country and phenotype, see **appendix 5.**

***Table 7**: Detectability of sputum mediators across the different countries in asthmatics and controls.*

	Biomarker	Detectability per country (%)										Mean detection (%)
		NZ		UK		Brazil		Uganda		Ecuador		
		Asthma	Control	Asthma	Control	Asthma	Control	Asthma	Control	Asthma	Control	
Inflammatory	IL-1β	93.5	87.4	94.0	90.9	96.7	100.0	98.5	93.8	95.5	90.0	94.0
	IL-6	63.3	73.5	64.0	54.5	65.9	80.0	52.9	50.0	70.1	63.3	63.8
	IL-8	98.9	98.9	98.0	100.0	100.0	100.0	100.0	100.0	98.2	90.0	98.4
	IL-13	9.7	8.2	2.0	0.0	2.1	9.1	8.8	31.3	2.7	0.0	7.4
	NE	99.3	97.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7
	ECP	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	–	–	100.0
	PGD-2	97.7	100.0	100.0	100.0	–	–	100.0	100.0	–	–	99.6
	Histamine	84.0	70.0	100.0	100.0	55.8	50.0	100.0	100.0	–	–	82.5
Neurogenic	NKA	100.0	100.0	93.8	100.0	82.3	100.0	95.6	100.0	76.8	100.0	94.9
	Substance P	21.9	21.1	68.7	60.0	43.5	60.0	67.6	62.5	37.6	33.3	47.6
	Nociceptin	100.0	100.0	97.9	100.0	54.5	30.0	100.0	93.8	73.0	58.8	80.8
	NGF-β	40.7	30.0	0.0	0.0	0.0	0.0	0.0	12.5	0.0	0.0	8.3
	BDNF	2.0	0.9	16.0	0.0	27.4	9.1	26.5	6.3	18.1	13.3	11.9
Remodelling	MMP-1	20.3	15.0	88.0	68.2	48.4	54.5	92.6	87.5	31.8	36.6	54.3
	MMP-9	99.5	96.9	98.0	100.0	100.0	100.0	100.0	100.0	98.2	90.0	98.3
	TIMP-1	99.5	100.0	98.0	100.0	93.4	100.0	100.0	100.0	98.2	90.0	97.9
	VEGF	91.3	84.7	94.0	90.9	98.9	100.0	92.6	100.0	96.4	90.0	93.9
	Periostin	83.2	72.6	96.0	95.5	67.7	36.6	85.3	93.8	57.0	50.0	73.8
	Elastin	99.3	91.3	100.0	100.0	77.1	60.0	100.0	93.8	91.6	85.7	89.9
	SADAM33	77.0	81.1	94.0	95.2	71.8	44.4	77.2	75.0	91.5	85.7	79.3

–: data not available.

4.3 Associations between population demographics and sputum mediator levels for all countries combined

When examining associations between demographics and mediator levels for participants from all countries combined, BDNF, MMP-1, substance P, SADAM33, and PGD-2 were positively associated with age ($p<0.05$) (data not shown). Additionally, IL-6, VEGF, and PGD-2 were higher in males ($p<0.05$) and NE and substance P higher in females ($p<0.05$) (data not shown). Periostin, ECP and PGD-2 were higher in atopic participants ($p<0.001$) (data not shown), and IL-1b and IL-8 were higher in non-atopic vs atopic asthmatics ($p<0.01$).

4.4 Combined mediator analysis

Sputum mediator levels in asthma and controls (for all countries combined).

Overall, asthmatics had significantly higher levels of ECP (median 853.9 pg/mL vs 189.4 pg/mL), VEGF (median 1174 pg/mL vs 663.6 pg/mL; both $p<0.001$), IL-1β (120.2pg/mL vs 96.1 pg/mL; $p<0.01$), PGD-2 (919.0 pg/mL vs 692.8 pg/mL), periostin (3420 pg/mL vs 2025 pg/mL; both $p<0.05$) when compared with controls (**appendix 6**)**.** Although not reaching statistical significance, nociceptin was higher in asthmatics than controls (79.0 ng/mL vs 31.5 ng/mL; $p<0.1$).

Sputum mediator levels in asthma sub-groups and controls (for all countries combined)

When comparing EA, NEA and controls overall, significant differences were found for IL-1β (NEA vs controls: 143.2 pg/mL vs 96.1pg/mL; $p<0.01$), IL-8 (NEA vs EA: 1083 pg/mL vs 897.4pg/mL; $p<0.01$), NE (NEA vs EA: 1590 ng/mL vs 1100 ng/mL; $p<0.05$), ECP (EA vs NEA vs controls: 1300 ng/mL vs 444.8 ng/mL vs 188.9 ng/mL; $p<0.001$) BDNF (EA and NEA vs controls, 15.4% and 13.7% vs 3.8% detectability;

p<0.05), VEGF (EA vs NEA vs controls: 1379 pg/mL vs 1089 pg/mL vs 663.6 pg/mL; p<0.05) and periostin (EA vs NEA vs controls: 6120 pg/mL vs 2520 pg/mL and 2025 pg/mL; p<0.001) (**appendix 7**).

Sputum mediator levels in asthma phenotypes and controls (for all countries combined)

When comparing EA, NA, MGA, PGA, and controls, significant differences were found for levels of 16/20 mediators (all p<0.05) (**table 8)**. For example, IL-1β, NE, substance P, and both MMP-1 & 9 levels were significantly higher in NA and MGA, and ECP and periostin levels were significantly higher (p<0.05) in EA vs PGA and controls. All mediator levels were generally lower in PGA and controls compared to MGA and NA, although this was not always statistically significant.

***Table 8**: Mediator levels in inflammatory phenotypes (EA/NA/MGA/PGA) for all countries combined.*

	Analyte	EA (n=189)	NA (n=58)	MGA (n=19)	PGA (n=261)	Controls (n=191)	Kruskal-Wallis P-value
Inflammatory	IL-1β	92.6 (39.9 – 205.9) † ‡	367.6 (71.6 – 751.2) # $	466.8 (204.8 – 1258) # $	120.2 (46.2 – 339.6)	96.1 (37.8 – 234.6)	<0.0001
	IL-6	62.4 (41.3 – 143) ‡	129.5 (42.1 – 283)	183.4 (110.4 – 643.1) # $	63.4 (41.3 – 223.7)	69.3 (41.3 – 158.5)	0.0004
	IL-8	785.7 (371.8 – 1354) † ‡ #	1386 (629 – 3575)	1751 (1256 – 2658)	1008 (544.7 – 2437)	909.6 (417.6 – 1848)	<0.0001
	Neutrophil Elastase (ng/mL)	946.8 (325.2 – 2400) † ‡	3480 (1870 – 7090) # $	4320 (2680 – 9000) # $	1340 (516.8 – 3000)	1221 (547.7 – 3112)	<0.0001
	ECP (ng/mL)	1230 (325.8 – 3590) # $	947.4 (203.5 – 1580)	2560 (1250 – 3560) # $	273.9 (91.8 – 1650)	188.9 (53.9 – 976.1)	0.0002
	PGD-2	990 (619.2 – 2064)	674.7 (449.3 – 1987)	1403 (828.8 – 2090)	863.0 (482.9 – 1703)	692.8 (439.5 – 1101)	0.4013
	Histamine	5193 (2763 – 7369)	5958 (3807 – 8316)	8415 (4343 – 10134)	3555 (441.0 – 7191)	5297 (992.3 – 8678)	0.0021
	IL-13	10/182 (5.5%)	5/58 (8.6%)	2/19 (10.5%)	11/255 (4.3%)	14/187 (7.5%)	0.4364
Neural	Neurokinin A	271 (64.08 – 696.8)	464.9 (216.7 – 828.9)	948.0 (286.5 – 8509) #	236.7 (61.7 – 680.1)	290.6 (72.3 – 857.7)	0.0051
	Substance P	43.9 (43.9 – 191.1) † ‡	429.1 (43.9 – 1069) # $	505.3 (43.9 – 1464) $	43.9 (43.9 – 284.1)	43.9 (43.9 – 271.7)	<0.0001
	Nociceptin (ng/mL)	62.8 (10.4 – 302.6) ‡	149.9 (21.1 – 544.4)	477.3 (29.9 – 1201)	75.9 (9.9 – 349.2)	31.5 (8.64 – 221.6)	0.0651
	NGF-β	18/134 (13.4%) †	0/45 (0.0%) # $	1/17 (5.9%)	25/230 (10.9%)	18/157 (11.5%)	0.0715
	BDNF	21/187 (11.2%) ‡	10/58 (17.2%) ‡	11/19 (57.9%) # $	33/257 (12.8%)	7/180 (3.8%)	<0.0001
Remodelling	MMP-1	44.4 (44.4 – 129.7) † ‡	129.2 (44.4 – 327.5) # $	372.8 (55.7 – 1011) # $	44.4 (44.4 – 193.8)	44.4 (44.4 – 119.6)	<0.0001
	MMP-9	1361 (571.6 – 3385) † ‡	4255 (1046 – 10490) #	4446 (1967 – 16533) #	1431 (581.7 – 3787)	1809 (678.4 – 4562)	<0.0001
	TIMP-1 (ng/mL)	71.8 (20.4 – 191.7) ‡	116.4 (34.1 – 746.8)	292.7 (27.5 – 1200)	87.0 (24.6 – 573.7)	78.6 (33.2 – 469.5)	0.0056
	VEGF	1151 (440.8 – 2567) ‡	1064 (295.4 – 3615)	3629 (1490 – 6870) $	1094 (367.3 – 3875) $	663.6 (209.7 – 2218)	0.0250
	Periostin	6300 (1170 – 24840) # $	4770 (877.5 – 14580)	4590 (877.5 – 14400)	2475 (877.5 – 8550)	2025 (877.5 – 8640)	0.0008
	Elastin	12994 (5784 – 24345) ‡	18426 (11701 – 29807)	66744 (23670 – 176317) # $	14540 (6751 – 25940)	14580 (7525 – 20033)	0.0002
	SADAM33	6670 (140.6 – 27832) † ‡	26925 (6311 – 58276)	59144 (16064 – 150462) $	15479 (4144 – 41140)	10611 (2528 – 26562)	<0.0001

Data presented as median IQR in pg/mL or detectability (%) unless indicated otherwise. P-value calculated with Kruskal-Wallis, and P-value between phenotypes calculated using Dunn's test or Chi-Square test. **†:** P<0.05 compared to NA, **‡:** p<0.05 compared to MGA, **#:** P<0.05 compared to PGA, **$:** P<0.05 compared to controls.

4.5 Sputum mediator comparisons within, and between countries

For each country, sputum mediator levels are plotted in a dot-plot for asthmatics/controls, asthma sub-groups (EA and NEA), and asthma phenotypes (EA, NA, MGA, and PGA) (**figure 3 – 22**). Each plot shows the median and adjusted p-values of significant findings from multivariate analyses, with regression coefficients (95% CI) and odds ratios (95% CI) shown in **appendix 8.** Multivariate analysis, focusing at differences in levels of mediators between the same phenotypes (EA, NA, MGA, and PGA) across countries (also referred to as country vs country analyses), are described below. An example of such analysis is one that compares levels of ECP in NZ EA vs Ecuador EA. Regression-coefficients (95% CI) and odds-ratios (95% CI) of the country vs country analysis can be found in **appendix 9**.

4.5.1 Inflammatory mediators

Il-1β

Multivariate analyses within each country showed significant differences between various groups, particularly in sub-group NEA and phenotype NA **(figure 3)**. While no differences were observed between asthmatics and controls in each country, in asthma sub-groups (EA and NEA) higher levels of IL-1β were observed in NEA vs controls in the UK ($p<0.1$), and NEA vs EA in Brazil ($p<0.1$). When comparing different phenotypes (EA, NA, MGA, PGA), IL-1β was shown to be higher in NA vs EA in NZ and Ecuador (both $p<0.001$), and higher in MGA vs EA in Uganda and PGA vs EA in the UK (both $p<0.1$).

Multivariate analyses comparing the same asthma phenotypes across different countries showed higher levels of IL-1β in EA in Uganda vs NZ (113.1 vs 69.4 pg/mL; $p<0.05$), in NA in Ecuador vs Uganda (573.7 vs 319.0 pg/mL; $p<0.01$), and in PGA in Uganda and Ecuador vs NZ (186.0 and 199.5 vs 54.3 pg/mL; both $p<0.05$) (**appendix 9)**.

IL-6

Multivariate analyses within each country showed higher levels of IL-6 in UK asthmatics vs controls ($p<0.1$). When comparing sub-groups (EA and NEA) higher levels of IL-6 were observed in UK NEA vs controls and EA (both $p<0.05$); also, higher levels were observed in controls vs EA in Brazil ($p<0.1$) (**figure 4)**. When comparing phenotypes, IL-6 was higher in NA vs EA in Brazil ($p<0.05$) and Ecuador ($p<0.001$) and it was also higher in PGA vs EA in the UK ($p<0.1$).

Multivariate analyses comparing the same asthma phenotypes across different countries showed higher levels of IL-6 in NA in Ecuador vs NZ (399.7 vs 136.7 pg/mL; $p<0.01$) **(appendix 9)**.

Il-8

Multivariate analyses within each country showed no significant differences between asthmatics and controls **(figure 5)**. However, when comparing sub-groups, higher levels of IL-8 were found in NEA and controls vs EA in NZ ($p<0.1$ and $p<0.05$ respectively). When comparing phenotypes, higher levels of IL-8 were found in NA vs EA in NZ, Brazil, and Uganda (all $p<0.05$), as well as MGA vs EA in Uganda ($p<0.1$) and Ecuador ($p<0.001$).

Multivariate analyses comparing the same asthma phenotypes across different countries showed higher levels of IL-8 in EA in Ecuador vs NZ (1443 vs 616.3 pg/mL; $p<0.01$), and Brazil and Ecuador vs UK (both $p<0.01$) (**appendix 9)**. In NA, higher levels of IL-8 were found in Brazil and Ecuador vs NZ (7906 and 3216 vs 702.5 pg/mL; $p<001$ and $p<0.05$ respectively) and higher levels in Brazil vs Uganda and Ecuador (7906 vs 1348 and 3216 pg/mL; both $p<0.01$). In PGA, IL-8 levels were higher in Ecuador vs NZ, Brazil, and Uganda (2033 vs 573.6, 1556, and 934.9 pg/mL; $p<0.05$ for all).

Neutrophil Elastase

Multivariate analyses comparing NE levels within each country showed no differences between asthmatics and controls and EA and NEA **(figure 6)**. However, comparisons between phenotypes showed higher levels of NE in NA vs EA in NZ, Ecuador, and Uganda ($p<0.001$ for NZ and Ecuador, and $p<0.1$ for Uganda). Additionally, NE was higher in MGA vs EA in Uganda and Ecuador ($p<0.001$ and $p<0.05$ respectively).

Multivariate analyses comparing the same asthma phenotypes across different countries showed higher levels of NE in PGA in the UK vs NZ and Brazil (4800 vs 2630 and 1100 ng/mL; both $p<0.05$) (**appendix 9)**.

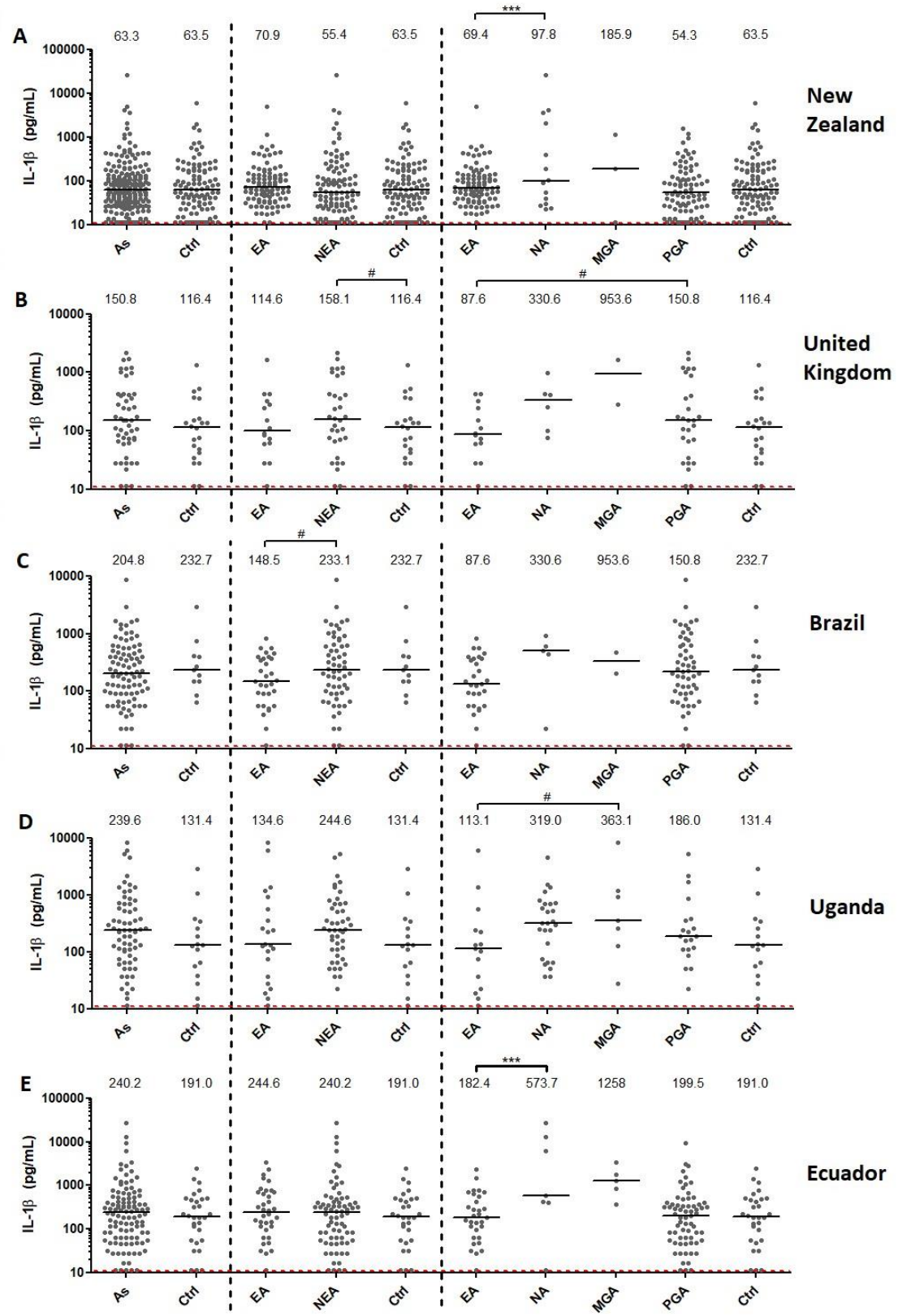

***Figure 3**: **IL-1β levels in different groups in different countries**. A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

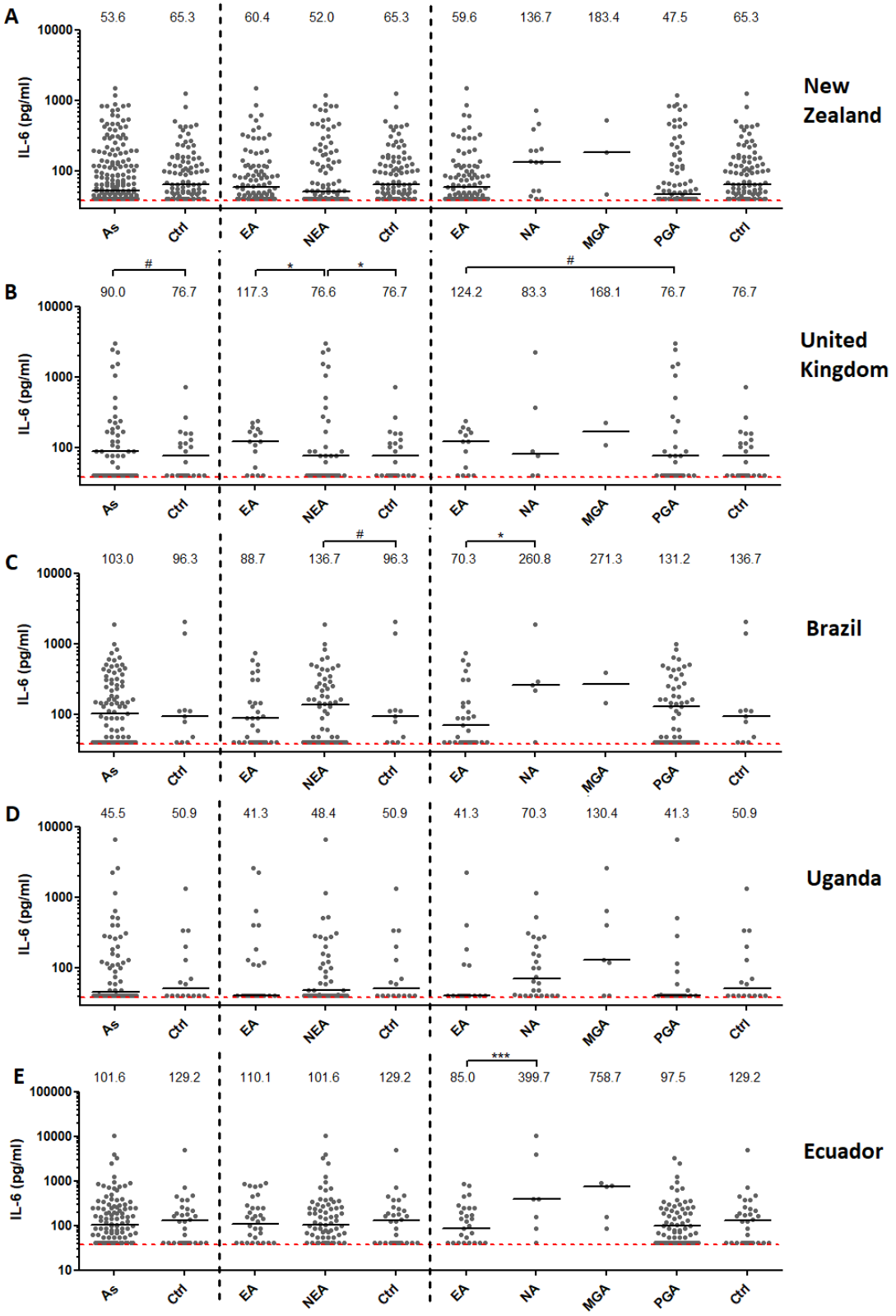

Figure 4: IL-6 levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

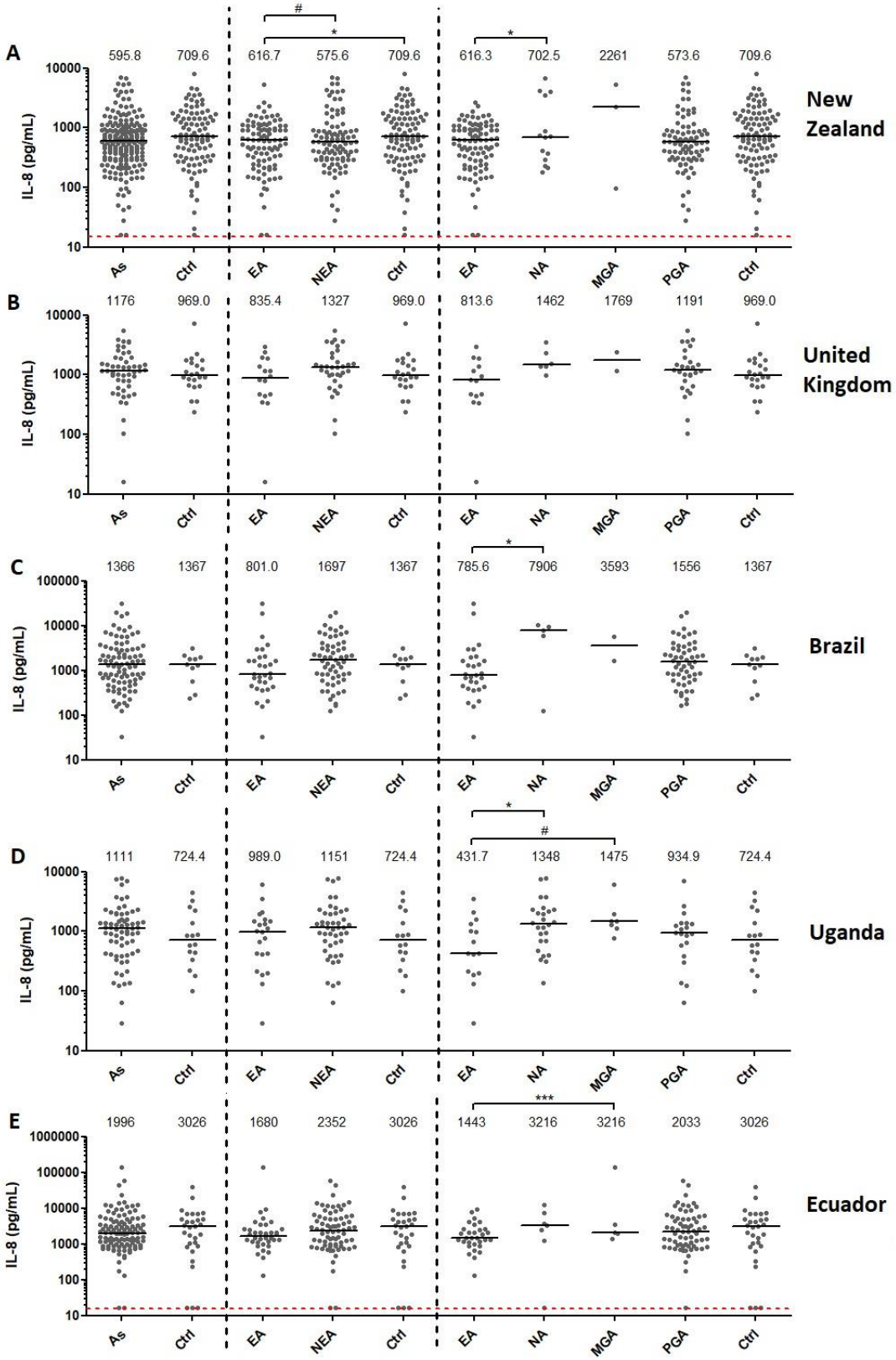

Figure 5: IL-8 levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

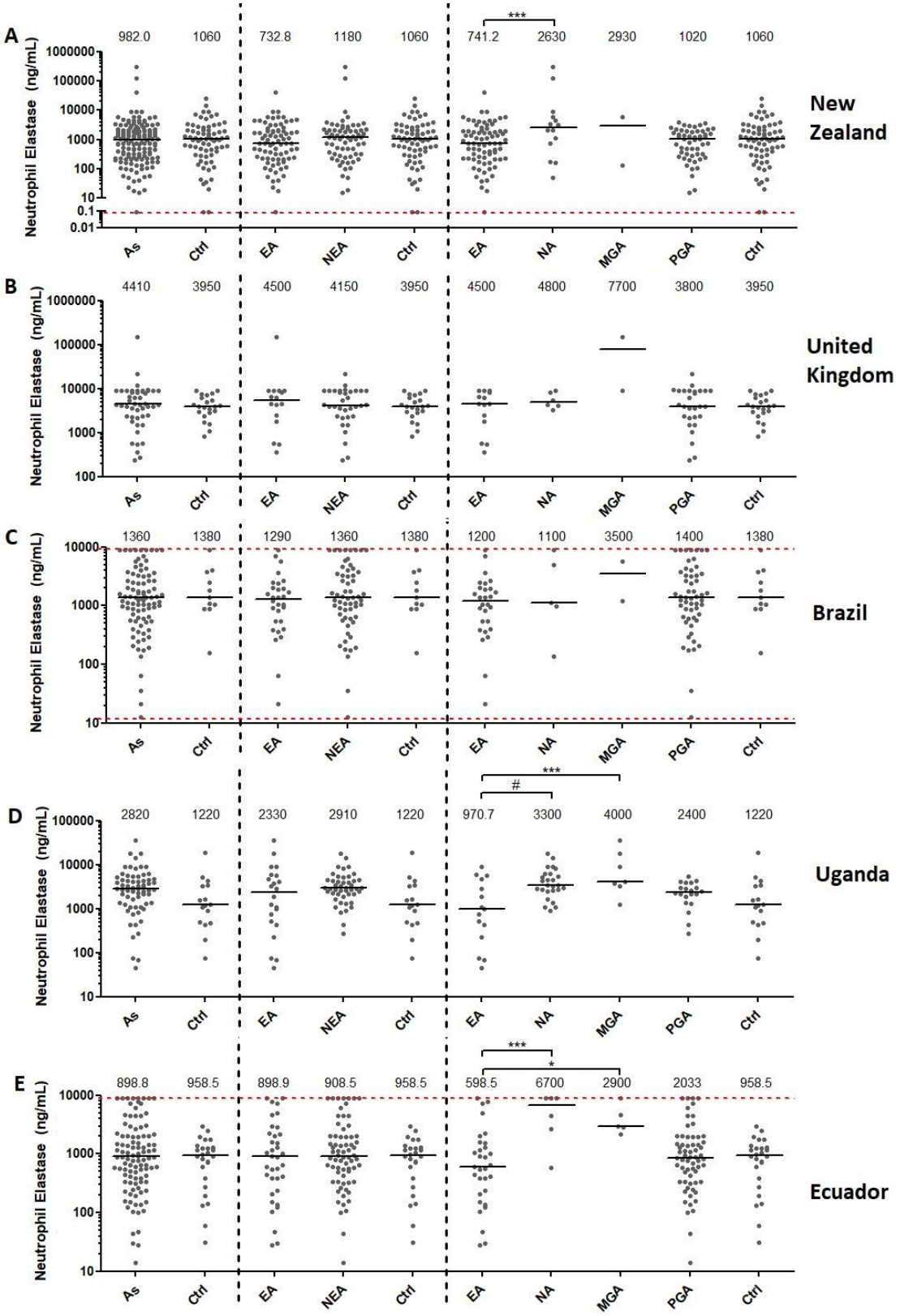

Figure 6: Neutrophil elastase levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

ECP

Multivariate analyses within each country showed higher levels of ECP in Brazilian asthmatics vs controls ($p<0.1$) **(figure 7)**. When comparing sub-groups (EA and NEA), ECP was higher in EA vs NEA and controls in Brazil and Uganda ($p<0.01$ for all in Brazil, and $p<0.05$ for all in Uganda). When comparing phenotypes, ECP was higher in EA vs PGA in the UK and Brazil ($p<0.001$, and $p<0.1$ respectively).

Multivariate analyses comparing the same asthma phenotype across different countries showed higher levels of ECP in NA in Brazil vs Uganda (3600 vs 628.8 ng/mL; $p<0.01$) (**appendix 9**).

Histamine

No significant differences were found in asthmatics/controls, asthma sub-groups, and phenotypes within each country in multivariate analysis **(figure 8)**.

Multivariate analyses comparing the same asthma phenotype across different countries showed higher levels of histamine in EA in NZ and Uganda vs UK and Brazil (all $p<0.05$) (**appendix 9**). For NA, histamine levels were higher in NZ and Uganda vs Brazil (5873 and 6813 vs 441.0 pg/mL; both $p<0.05$). For MGA, histamine levels were higher in Uganda vs Brazil ($p<0.05$), and for PGA, higher histamine levels were found in Uganda vs NZ and Brazil (8613 vs 3573 and 441.0 pg/mL; both $p<0.05$); higher levels were also found in the UK vs NZ and Brazil (7259 vs 3573 and 441.0 pg/mL; $p<0.05$ vs NZ, and $p<0.001$ vs Brazil).

PGD-2

Multivariate analyses within each country showed higher PGD-2 levels in asthma vs controls ($p<0.001$) in NZ, and when comparing sub-groups, PGD-2 levels were higher in EA vs controls in NZ and the UK (both $p<0.05$) **(figure 9)**.

Multivariate analyses comparing the same phenotype across countries showed higher levels of PGD-2 for EA in UK vs Uganda (911.1 vs 675.6 pg/mL; $p<0.05$) (**appendix 9**).

IL-13

Multivariate analyses within each country showed no significant differences in IL-13 detectability between asthmatics/controls, asthma sub-groups, and phenotypes **(figure 10)**.

Multivariate analyses comparing the same phenotype across countries showed higher detectability in PGA in Uganda vs UK and Brazil (both $p<0.05$) (**appendix 9**).

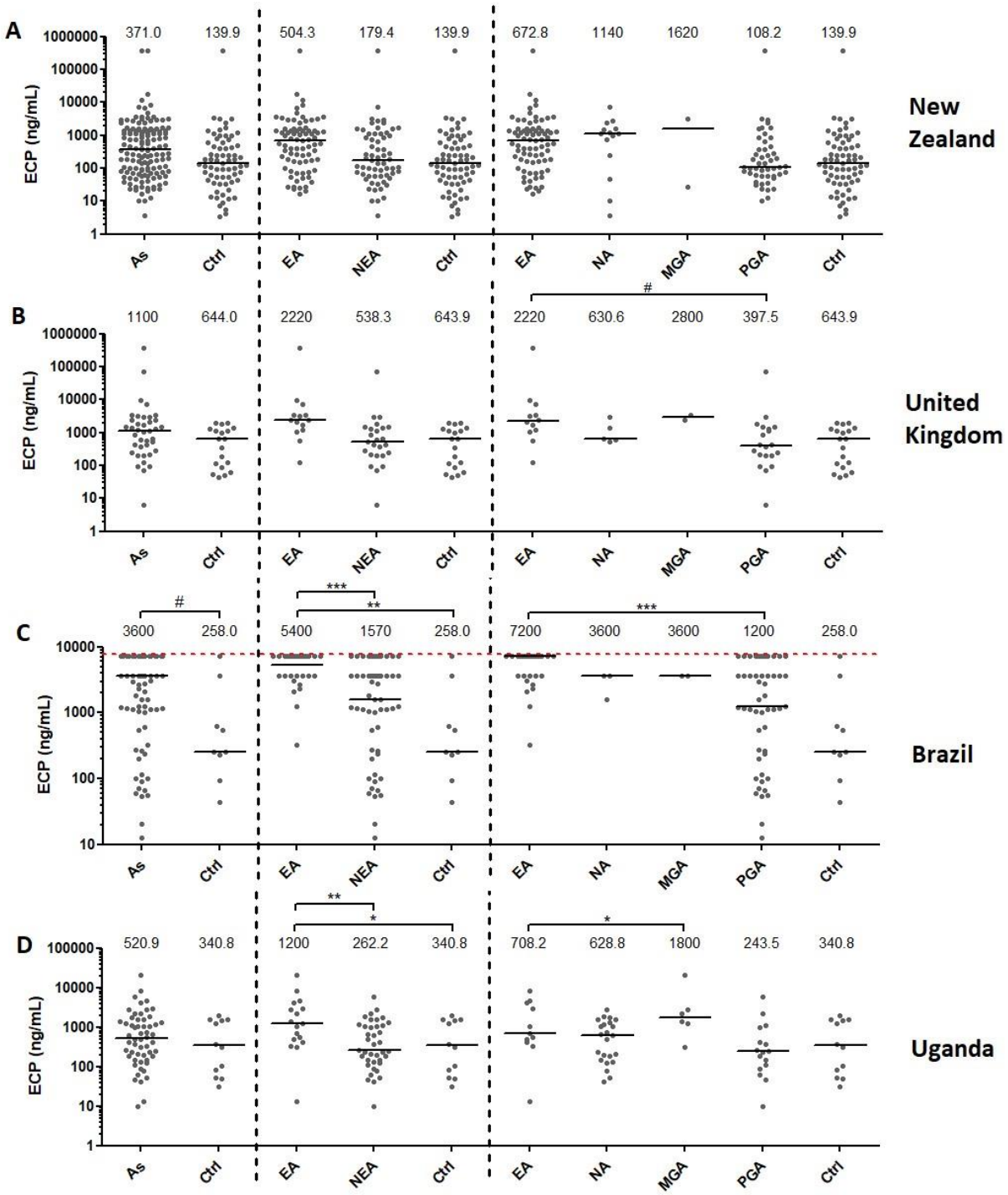

Figure 7: ECP levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, and D: Uganda. Ecuador data not available. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

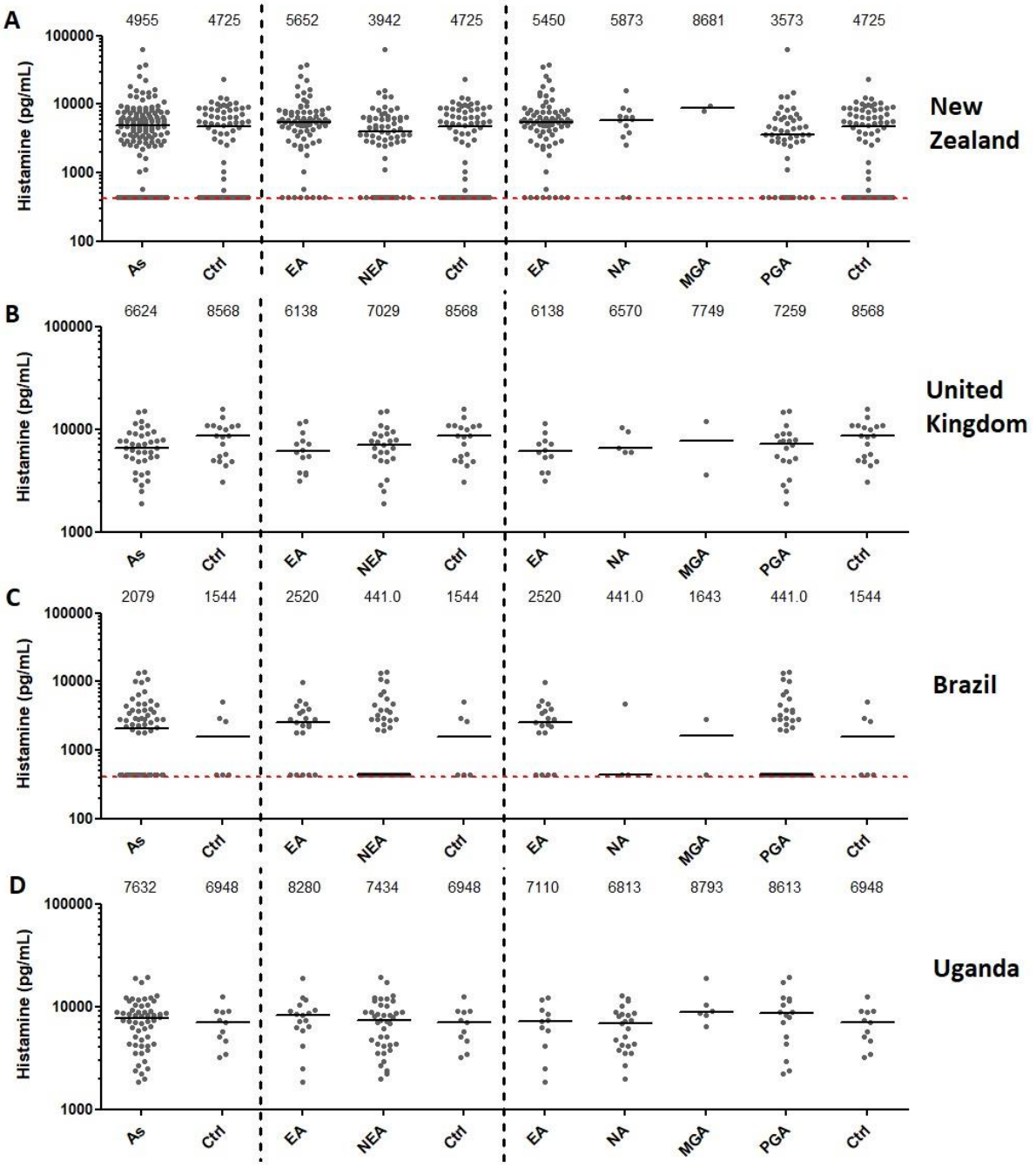

Figure 8: Histamine levels in different groups in different countries*. A: New Zealand, B: UK, C: Brazil, and D: Uganda. Ecuador data not available. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

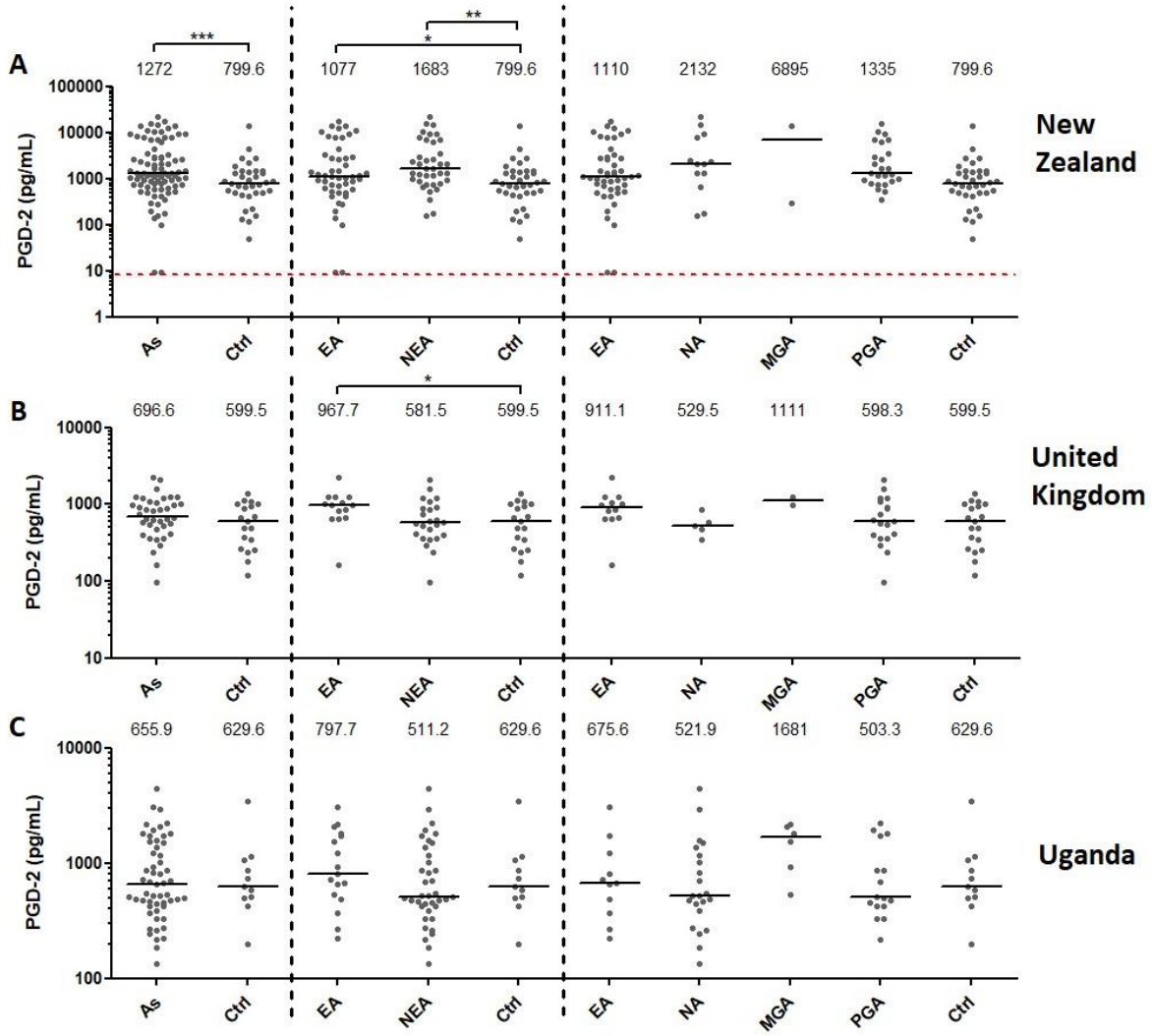

Figure 9: PGD-2 levels in different groups in different countries. *A: New Zealand, B: UK, and C: Uganda. Brazil and Ecuador data not available. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

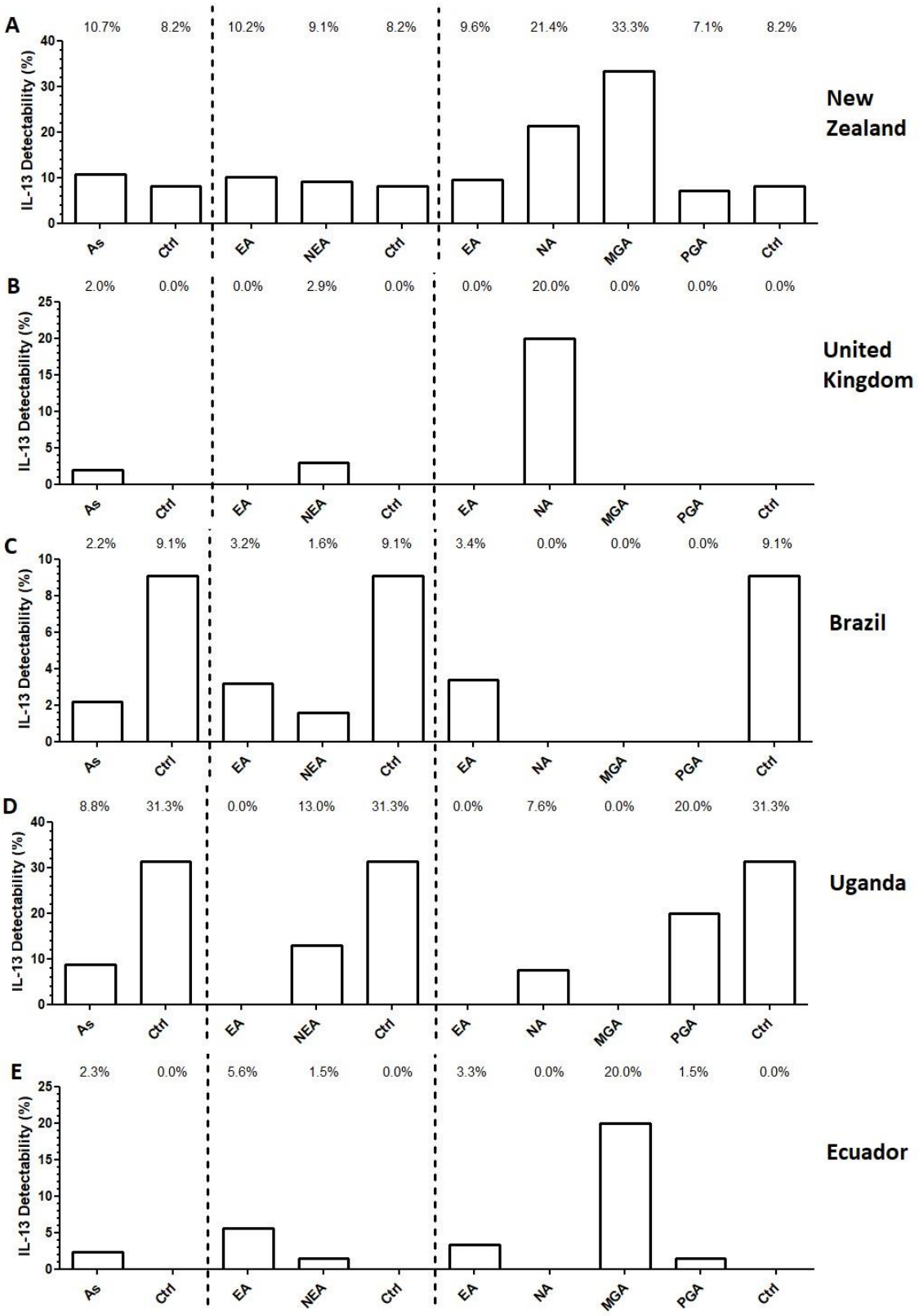

Figure 10: IL-13 detectability in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. P-value determined through multivariate analyses using logistic regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

4.5.2 Neural mediators

Neurokinin A

Multivariate analysis within each country showed higher levels of NKA in controls vs asthmatics in NZ ($p<0.1$) **(figure 11)**. Between sub-groups (EA and NEA), NKA was significantly higher in EA vs controls and NEA in the UK ($p<0.01$, and $p<0.05$, respectively). Additionally, NKA was higher in NEA vs controls in NZ ($p<0.1$). Between asthma phenotypes, NKA was significantly higher in MGA vs EA in Ecuador ($p<0.001$).

Multivariate analyses comparing the same asthma phenotype across different countries showed higher levels of PGA in Uganda vs NZ and Ecuador (500.0 vs 113.0, and 231.8 pg/mL; $p<0.05$ and $p<0.01$, respectively) (**appendix 9).**

Substance P

Multivariate analyses withing countries showed that substance P levels were higher in asthmatics vs controls in the UK ($p<0.1$), and comparisons between asthma sub-groups (EA and NEA) showed higher levels of substance P in NEA vs controls in the UK ($p<0.1$) **(figure 12)**. When comparing phenotypes, substance P was higher in MGA vs EA in Uganda and Ecuador ($p<0.1$ for Uganda, and $p<0.01$ for Ecuador).

Multivariate analyses comparing the same asthma phenotype across countries showed higher levels of substance P in EA in Uganda vs NZ, Brazil, and Ecuador (300.0 vs 43.9, 43.9, and 43.9 pg/mL; $p<0.001$ respectively), with similar findings for PGA in Uganda vs NZ, Brazil, and Ecuador (**appendix 9)**. In PGA, substance P was higher in Ecuador vs NZ ($p<0.05$).

Nociceptin

Multivariate analyses within each country revealed higher levels of nociceptin in asthmatics vs controls in the UK and Brazil ($p<0.05$, and $p<0.1$, respectively) (**figure 13**). Comparisons between asthma sub-groups (EA, and NEA) showed that levels of nociceptin were higher in EA vs controls in NZ, and UK ($p<0.1$, and $p<0.01$ respectively), higher in EA vs NEA in the UK, and Brazil ($p<0.05$, and $p<0.1$ respectively), and higher in NEA vs controls in Brazil ($p<0.05$). When comparing phenotypes (EA, NA, MGA, and PGA), it was shown that nociceptin was higher in EA vs PGA in the UK, and Brazil (both $p<0.1$), higher in EA vs NA in the UK ($p<0.05$), and higher in MGA vs EA in Uganda ($p<0.01$).

Multivariate analyses comparing the same asthma phenotype across countries showed higher nociceptin levels in EA in Uganda vs NZ and Brazil (304.9 vs 45.3, and 16.7 ng/mL; all $p<0.05$) (**appendix 9)**. Additionally, in MGA, nociceptin levels were higher in Uganda vs Ecuador (1200 vs 61.6 ng/mL; $p<0.05$).

NGF-β

There were no significant differences in the detectability of NGF-β within (**figure 14)** and across countries for all groups **(appendix 9)**.

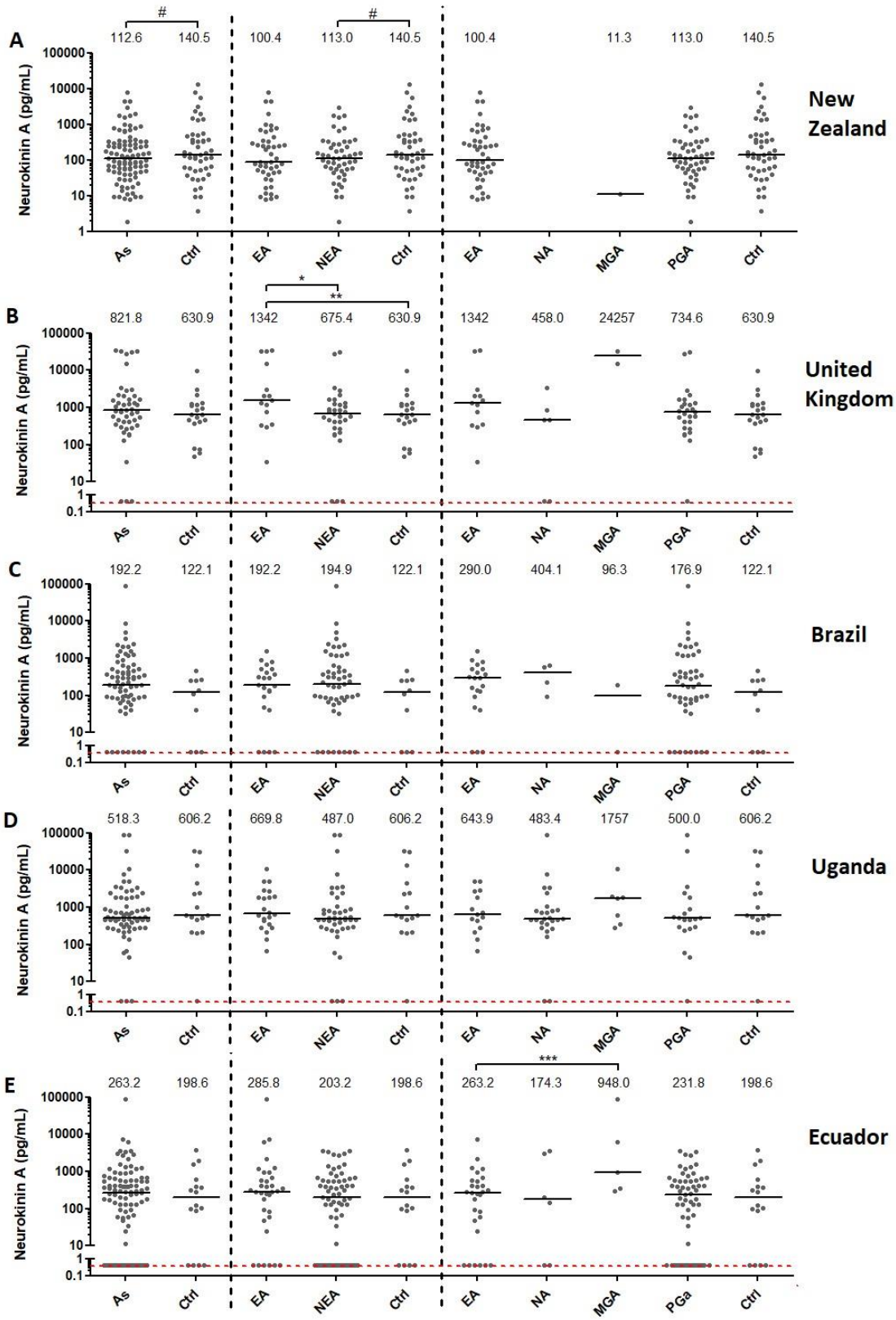

Figure 11: Neurokinin A levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

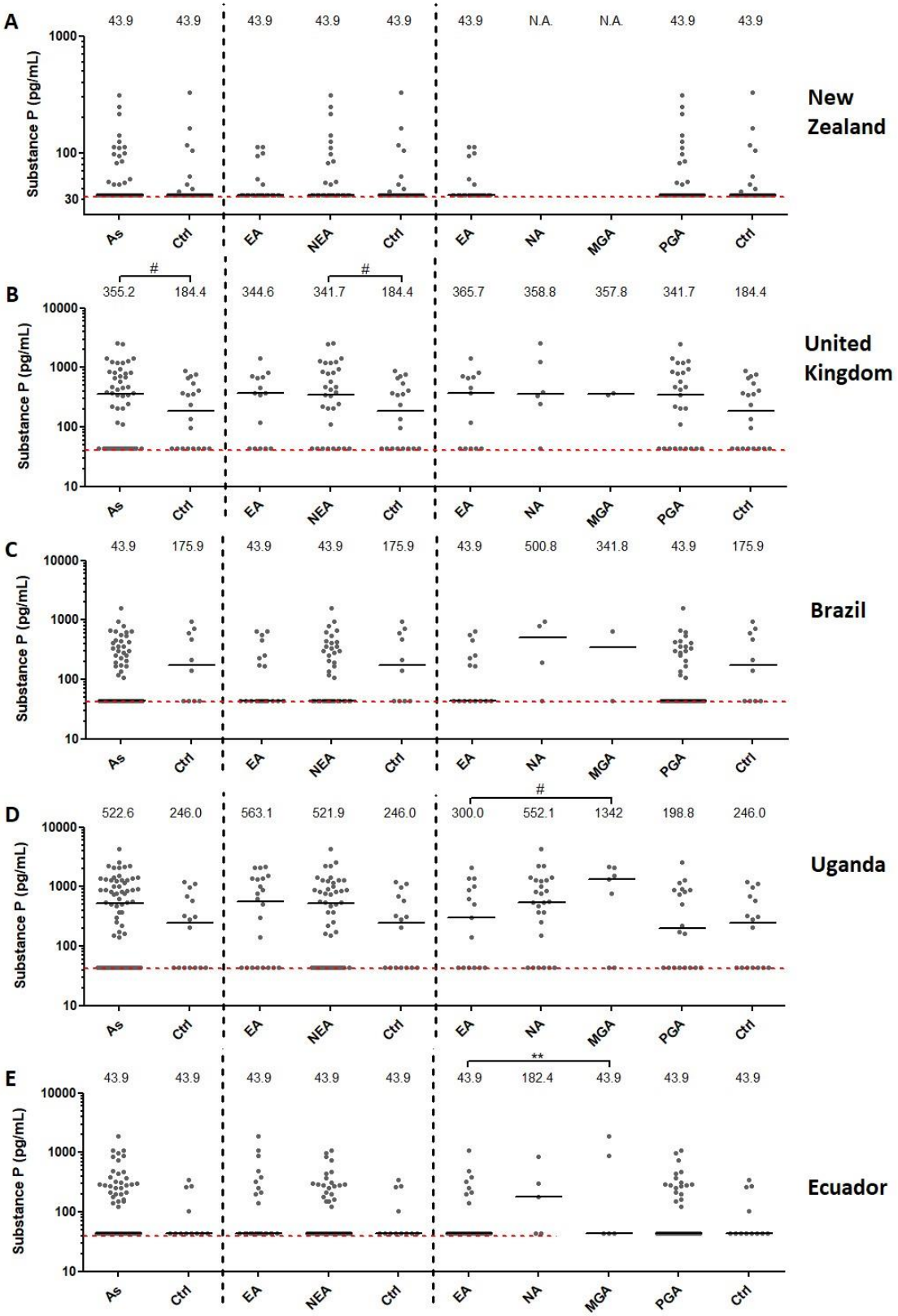

Figure 12: Substance P levels in different groups in different countries*. A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

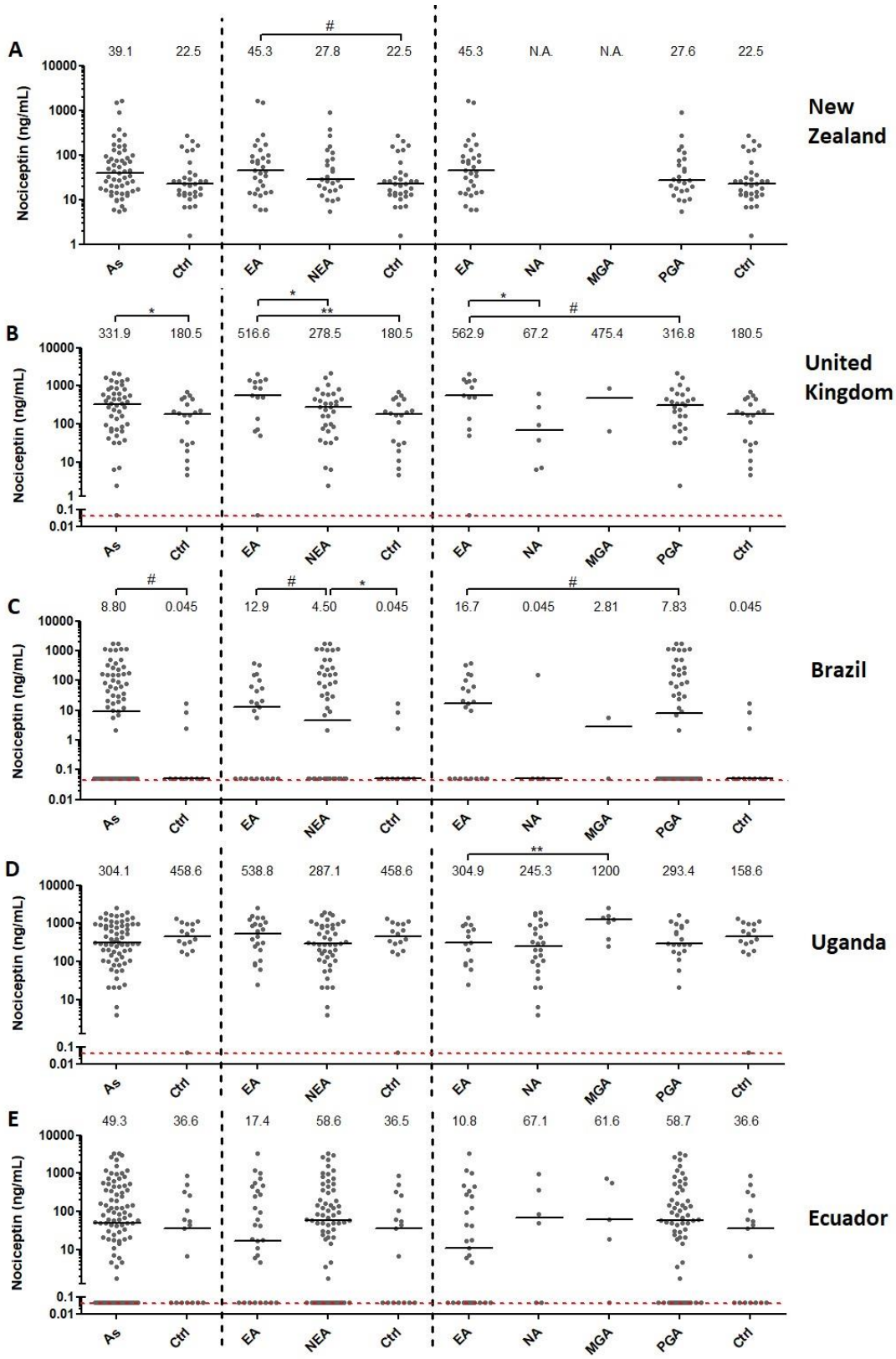

Figure 13: Nociceptin levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

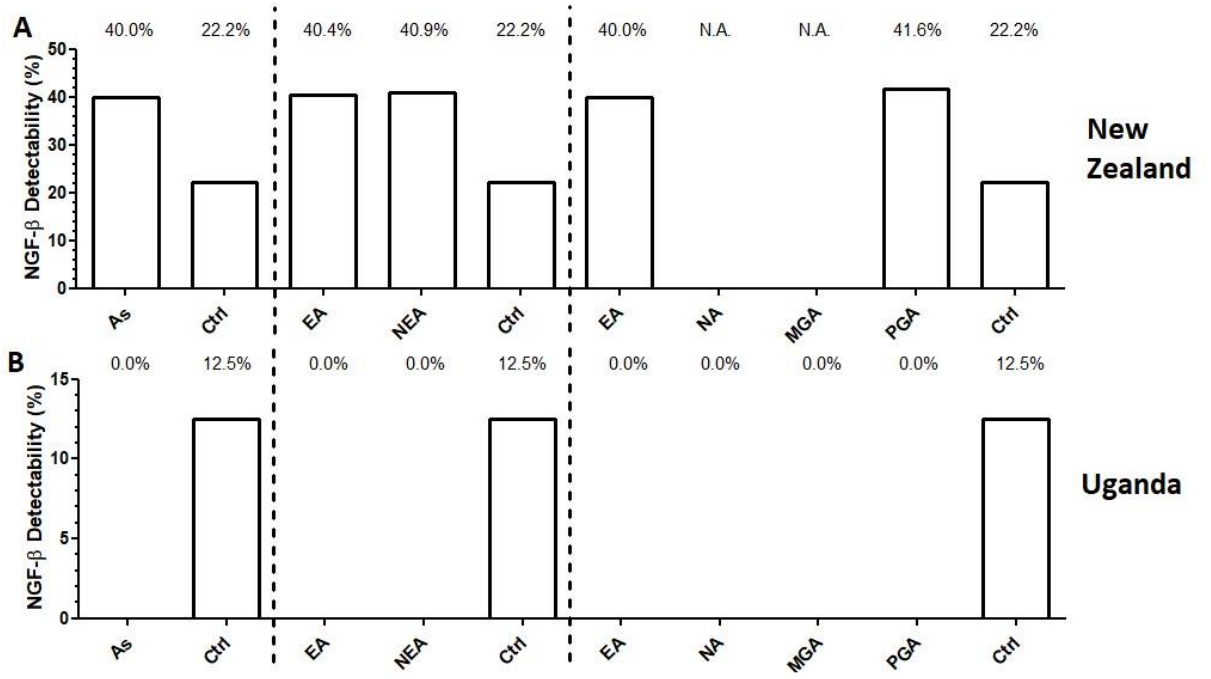

Figure 14: NGF-β detectability in different groups in different countries*. A: New Zealand, and B: Uganda. UK, Brazil and Ecuador data not available. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. P-value determined through multivariate analyses using logistic regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

BDNF

Multivariate analyses within each country showed no differences in BDNF detectability between asthmatics and controls. However, when comparing sub-groups, BDNF was detected more frequently in EA vs NEA in Uganda (31.8% detectability, vs 21.7%; p<0.1) (**figure 15)**. Additionally, when comparing asthma phenotypes, BDNF was more frequently detectable in MGA compared to EA in Uganda and Ecuador (both p<0.05).

Multivariate analyses comparing the same asthma phenotype across countries showed higher levels of detection of BDNF in PGA in Brazil vs UK (9.1% vs 0.0%; p<0.05) (**appendix 9).**

4.5.3 Remodelling mediators

MMP-1

Multivariate analyses within countries showed increased levels of MMP-1 in controls vs asthmatics in Ecuador ($p<0.1$) (**figure 16**)**.** When comparing asthma sub-groups, MMP-1 levels were found to be higher in EA vs controls in Uganda ($p<0.1$), and when comparing phenotypes, MMP-1 was found to be higher in NA vs EA in the UK ($p<0.05$), and in MGA vs EA in Ecuador ($p<0.05$).

Multivariate analyses comparing the same phenotype across countries showed higher levels of MMP-1 in EA in the UK, Brazil, and Uganda vs NZ (medians of 201.9, 44.4, and 167.6 vs 44.4 pg/mL; $p<0.05$ for all). In PGA, higher levels of MMP-1 were present in Uganda and Brazil vs NZ (all $p<0.05$) (**appendix 9).**

MMP-9

Multivariate analyses within each country showed no significant differences in levels of MMP-9 between asthmatics/controls and asthma sub-groups (**figure 17)**. However, when phenotypes were compared, it was found that MMP-9 levels were higher in NA vs EA in NZ, Brazil, and Uganda ($p<0.1$ for all). Additionally, MMP-9 levels were higher in MGA vs EA in Ecuador ($p<0.001$). Multivariate analsysis comparing the same phenotype across countries showed no differences.

TIMP-1

Multivariate analyses within each country showed higher levels of TIMP-1 in controls vs asthmatics in NZ and Ecuador ($p<0.1$, and $p<0.05$ respectively) (**figure 18)**. Between sub-group comparisons showed that TIMP-1 was higher in controls vs EA in NZ and Ecuador ($p<0.1$ for NZ, and $p<0.05$ for Ecuador), and higher in controls vs NEA in Ecuador ($p<0.05$). When comparing phenotypes, TIMP-1 was found

to be higher in NA vs EA in NZ ($p<0.05$); it was also higher in MGA vs EA in Uganda ($p<0.05$) and in EA vs NA in Ecuador ($p<0.05$).

Multivariate analyses comparing the same phenotype across countries showed higher levels of TIMP-1 for EA in Uganda vs NZ (93.6 vs 72.2 ng/mL; $p<0.05$) (**appendix 9).** TIMP-1 levels were also higher for PGA in UK and Ecuador vs NZ (169.8, and 1200, vs 54.2 ng/mL; $p<0.001$ and $p<0.05$ respectively), and higher for PGA in the UK vs Brazil (169.8 vs 46.7 ng/mL; $p<0.05$).

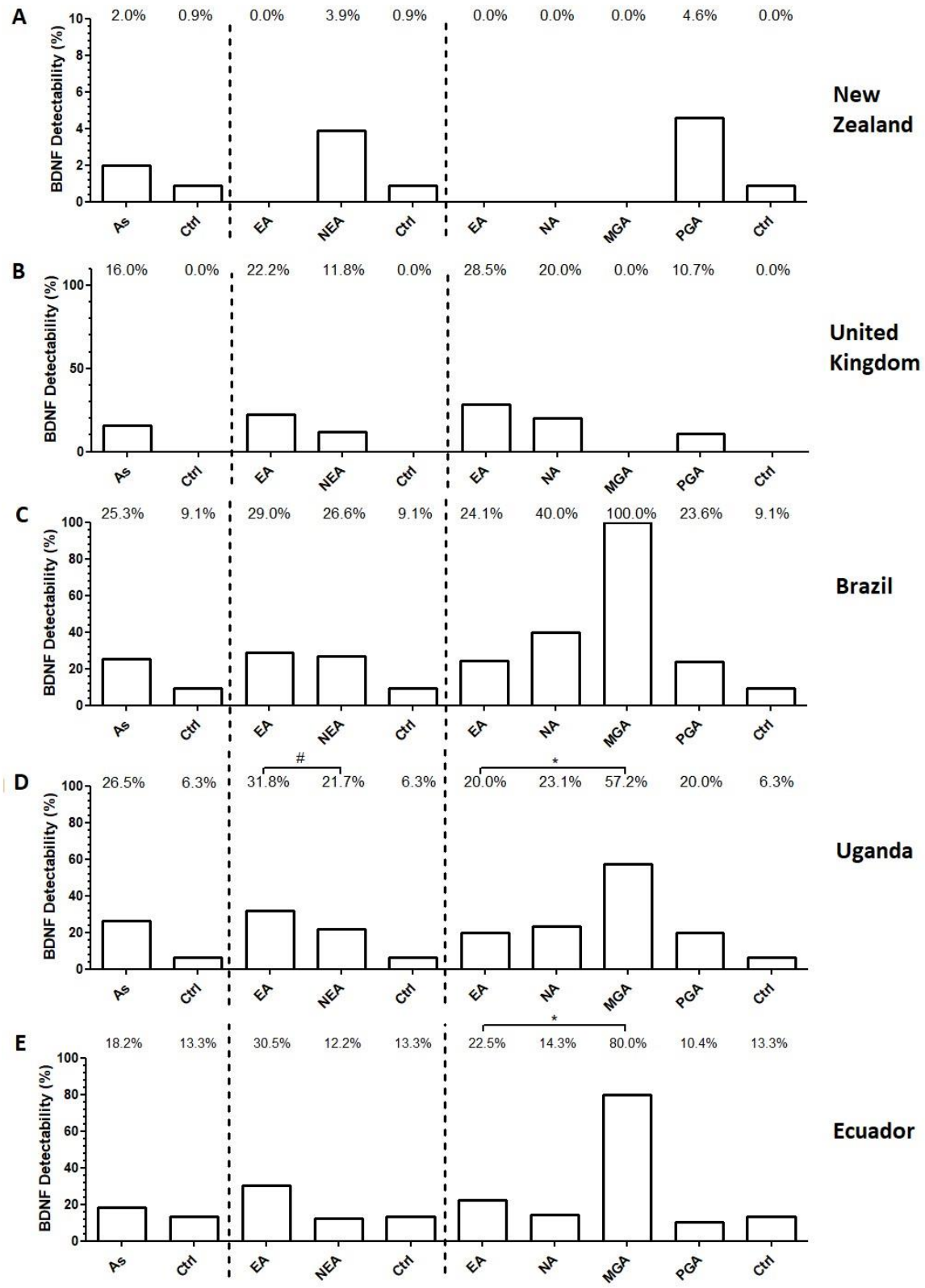

Figure 15: BDNF detectability in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. P-value determined through multivariate analyses using logistic regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

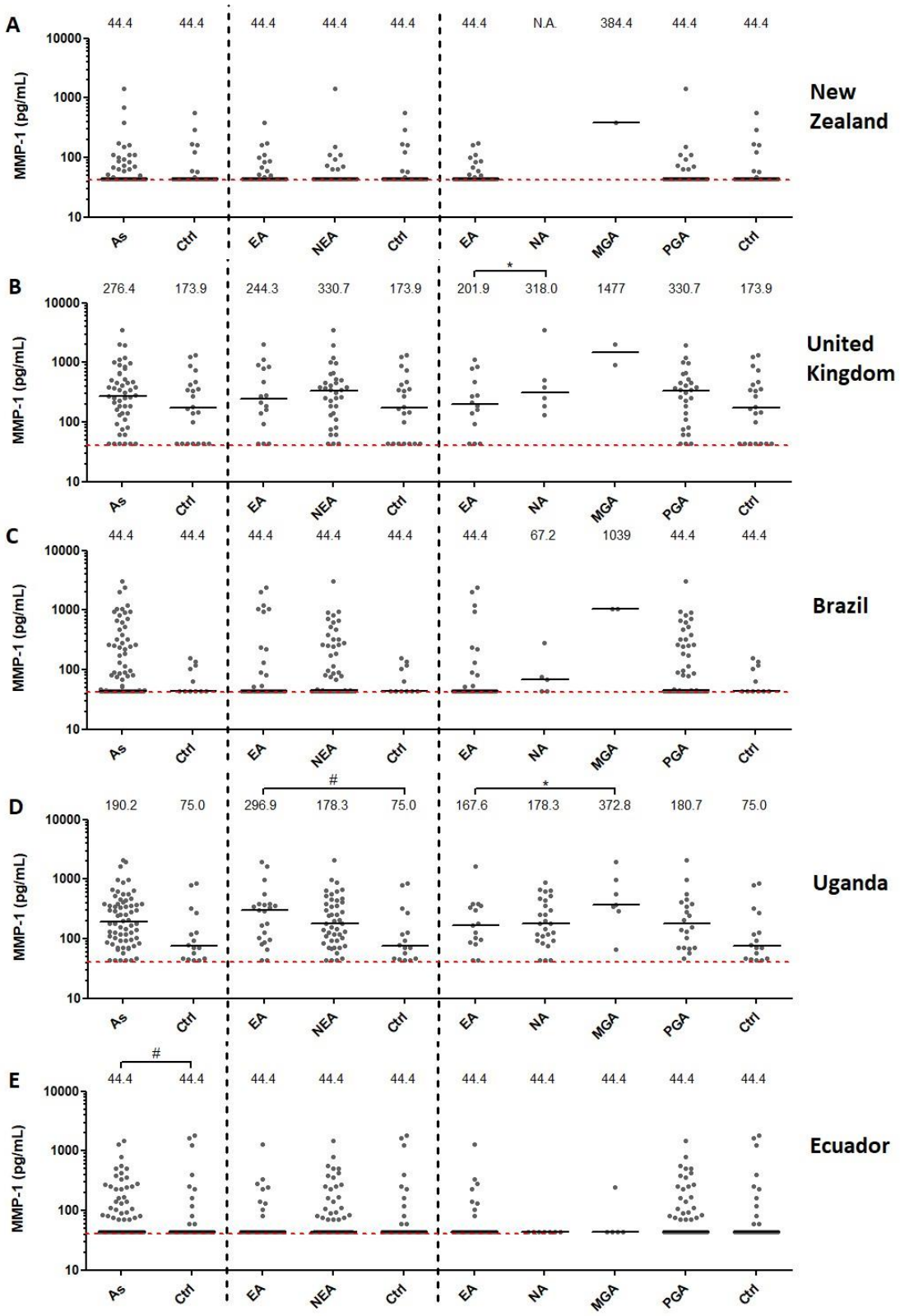

Figure 16: MMP-1 levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

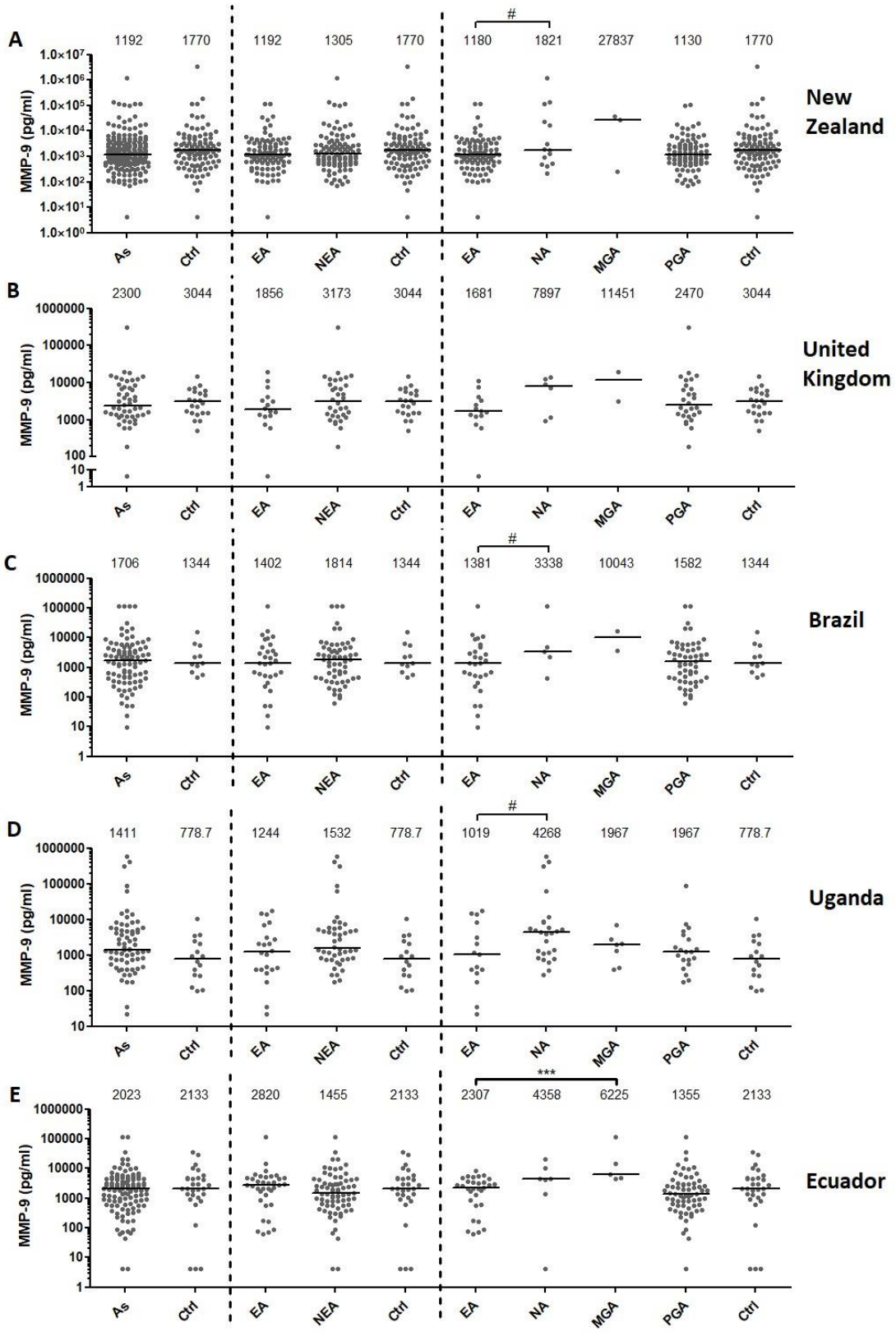

Figure 17: MMP-9 levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

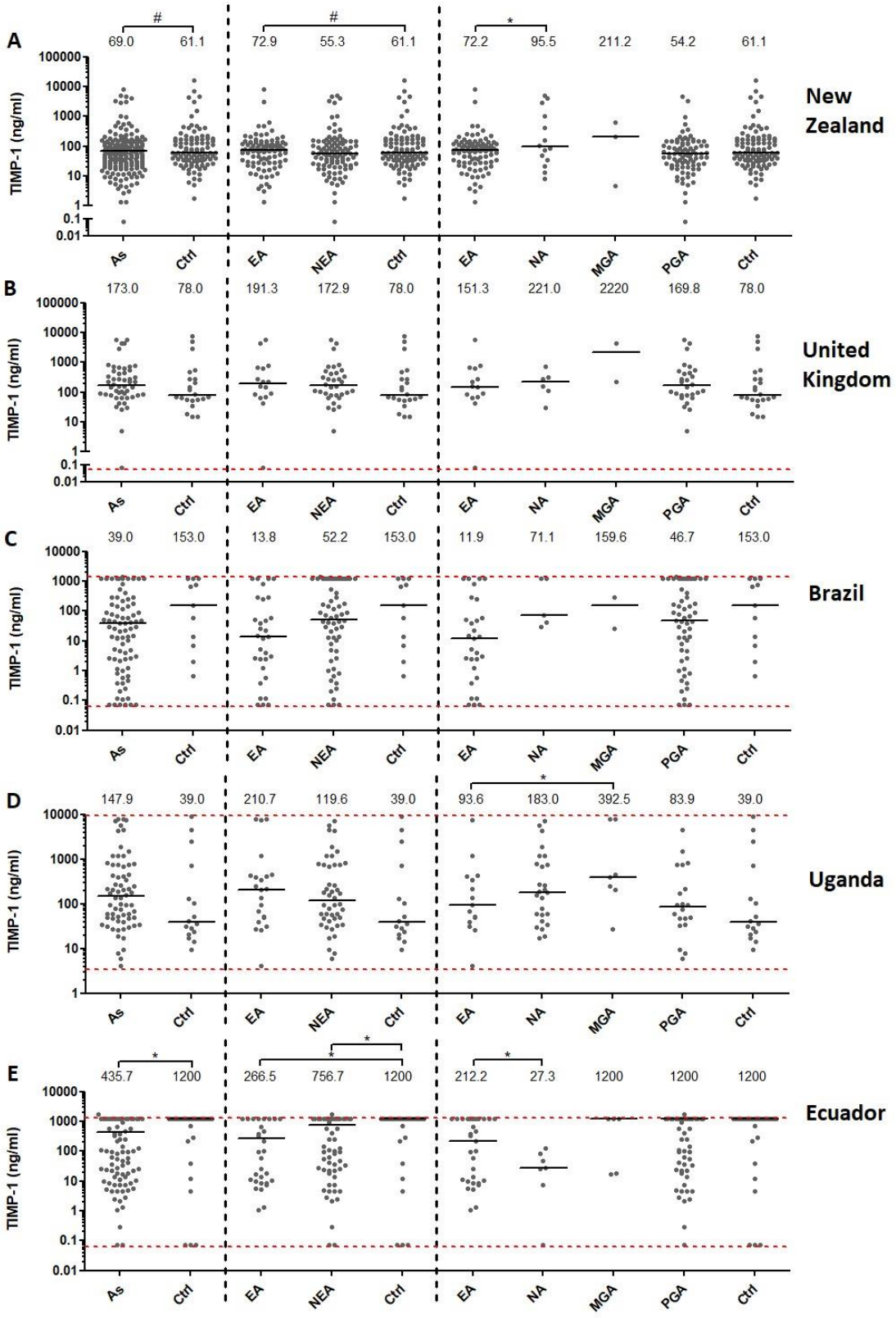

Figure 18: TIMP-1 levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

VEGF

Multivariate analyses within each country showed no differences in levels of VEGF between asthmatics and controls (**figure 19)**. However, between asthma sub-groups, levels of VEGF were higher in EA vs controls in the UK ($p<0.1$), and when phenotypes were compared, it was found that VEGF was higher in NA vs EA in NZ ($p<0.01$), and it was also higher in MGA vs EA in Uganda and Ecuador (both $p<0.1$).

Multivariate analyses comparing the same phenotype across countries showed higher levels of VEGF in EA in Brazil and Ecuador vs NZ (1622, and 3479 vs 739.8 pg/mL; all $p<0.001$); it was also higher in EA in Brazil vs Uganda (1622 vs 914.0 pg/mL; $p<0.05$) (**appendix 9)**. For MGA, VEGF was higher in Ecuador vs NZ and Uganda (10368 vs 1789, and 3629 pg/mL; both $p<0.05$), and for PGA, Ecuador had higher levels of VEGF vs NZ, UK, and Uganda (3095 vs 590.0, 1052, and 617.8 pg/mL; all $p<0.05$); higher levels were also observed in Brazil vs NZ (3432 vs 590.0 pg/mL; $p<0.01$).

Periostin

Multivariate analyses within each country showed higher levels of periostin in asthmatics vs controls in NZ ($p<0.05$) (**figure 20**). Between asthma sub-group comparisons showed that periostin levels were higher in EA vs NEA in NZ, Uganda (both $p<0.1$), and the UK ($p<0.05$), and it was also higher in EA vs controls in NZ ($p<0.001$), the UK ($P<0.05$), Brazil, and Ecuador (both $p<0.1$). When comparing phenotypes (EA, NA, MGA and PGA) it was found that periostin was present in higher levels in EA vs PGA in NZ, the UK (both $p<0.05$), Uganda, and Ecuador (both $p<0.1$). Additionally, in Uganda, periostin was higher in EA vs NA ($p<0.05$) and MGA vs NA ($p<0.1$).

Multivariate analyses comparing the same phenotype across countries showed higher levels in EA in NZ vs Ecuador (10260 vs 1170 pg/mL; p<0.05) and in EA in UK vs Brazil and Ecuador (23895 vs 3780, and 1170 pg/mL; both p<0.05) (**appendix 9).** For NA, periostin was also higher in NZ vs Brazil and Uganda (10935 vs 877.5, and 4545; both p<0.05). For PGA, higher levels of periostin were found in NZ, the UK, Brazil, and Uganda vs Ecuador (4815, 10980, 2520, and 5130 vs 990.0 pg/mL; all p<0.05); it was also higher in the UK vs Brazil (10980 vs 2520 pg/mL; p<0.05).

Elastin

Multivariate analyses within each country showed no differences between asthmatics and controls in any country (**figure 21).** However, between sub-group comparisons showed that elastin was higher in NEA vs controls in Brazil (p<0.1). When comparing phenotypes, higher levels were observed in MGA vs EA in Uganda (p<0.01).

Multivariate analyses comparing the same phenotype across countries showed higher levels of elastin in EA in Uganda vs NZ, UK, Brazil, and Ecuador (18312 vs 10618, 24901, 18262, and 10406 pg/mL ,all p<0.05), and the same trend was shown for PGA (20826 vs 9001, 17919, 14309, and 17617, all p<0.05) (**appendix 9**). Additionally, higher levels of elastin were found for MGA in Uganda vs Brazil (194121 vs 21889 pg/mL; p<0.05).

SADAM33

Multivariate analyses within each country showed no differences between asthmaticsand controls, and between sub-groups (EA, and NEA) (**figure 22)**. Between phenotype comparisons showed that SADAM33 was higher in NA vs EA in NZ, and Uganda (both p<0.05), and higher in MGA vs EA in Uganda (p<0.05).

Multivariate analyses comparing the same phenotype across countries showed higher levels of SADAM33 in EA in Ecuador vs NZ and Uganda (21782 vs 5172, and 140.6 pg/mL; both $p<0.05$) (**appendix 9)**.

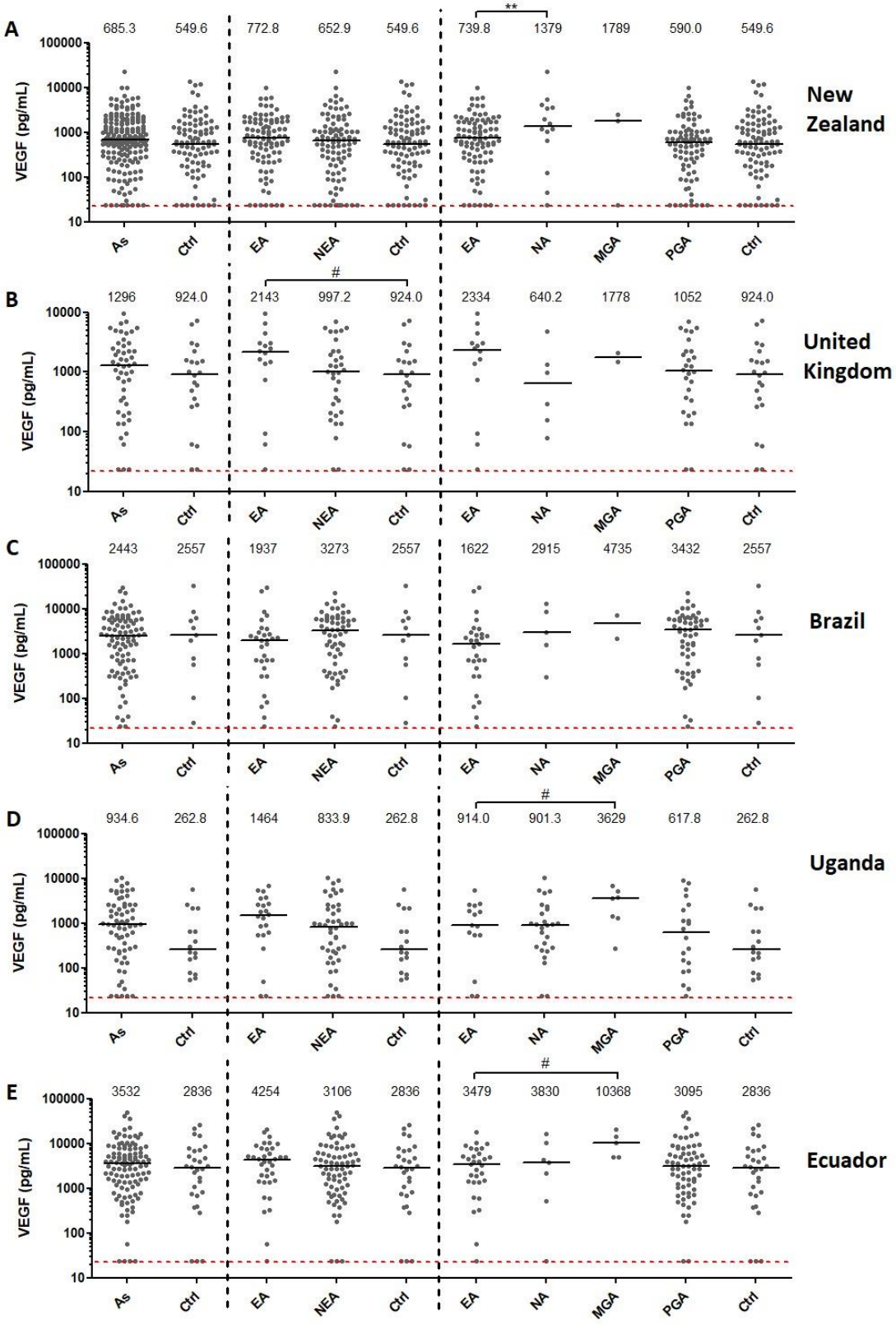

Figure 19: VEGF levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

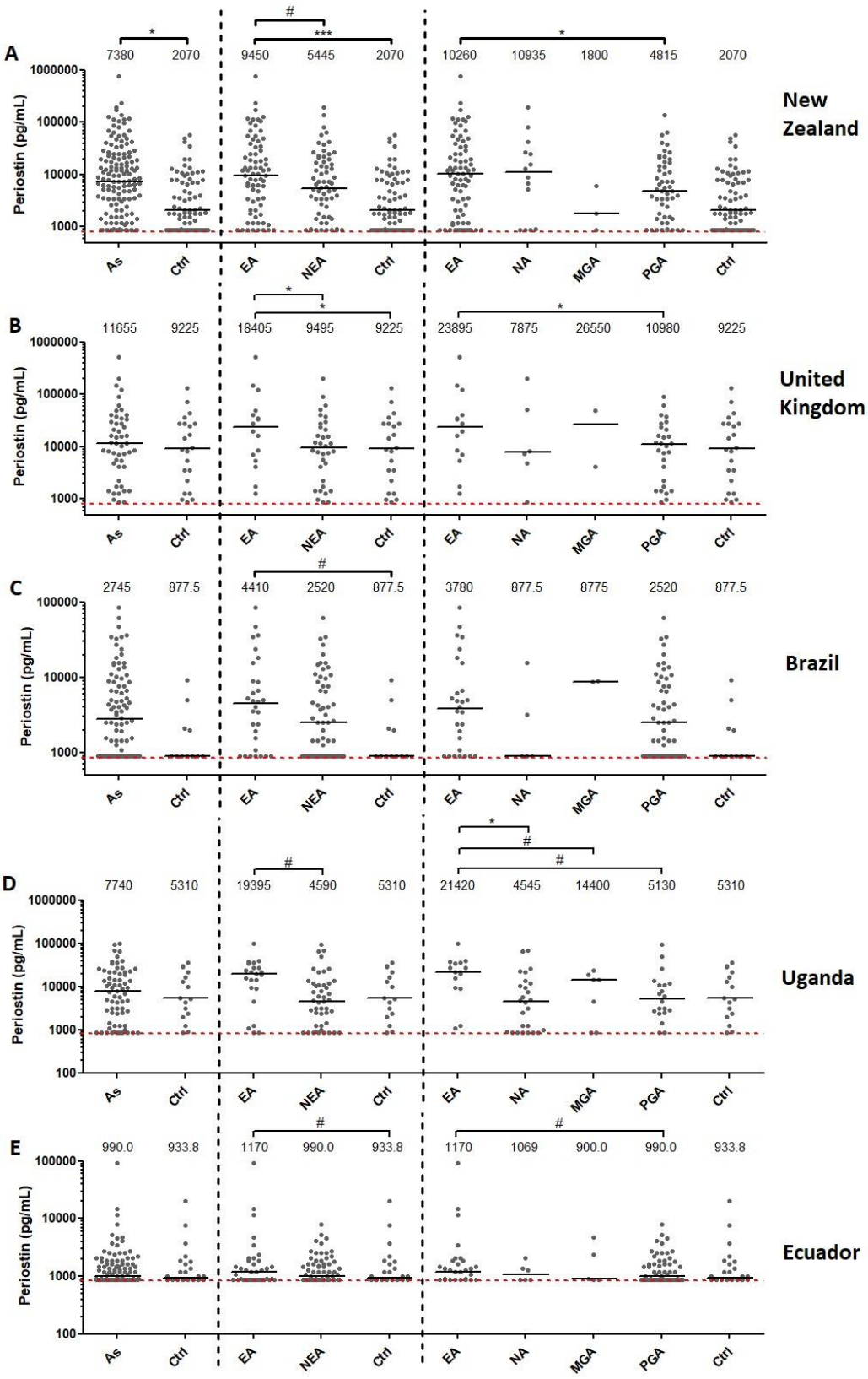

Figure 20: Periostin levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001*

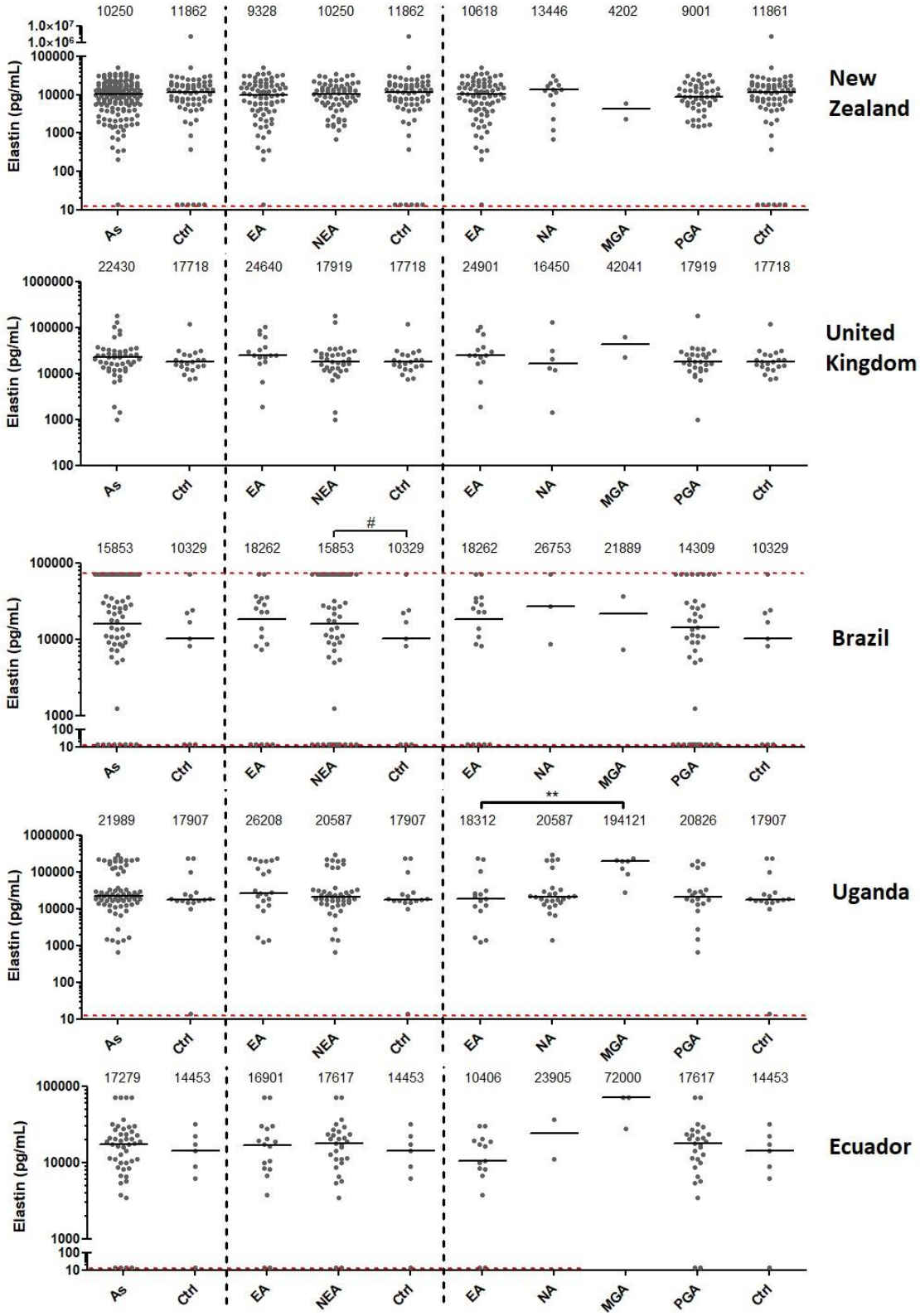

Figure 21: Elastin levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

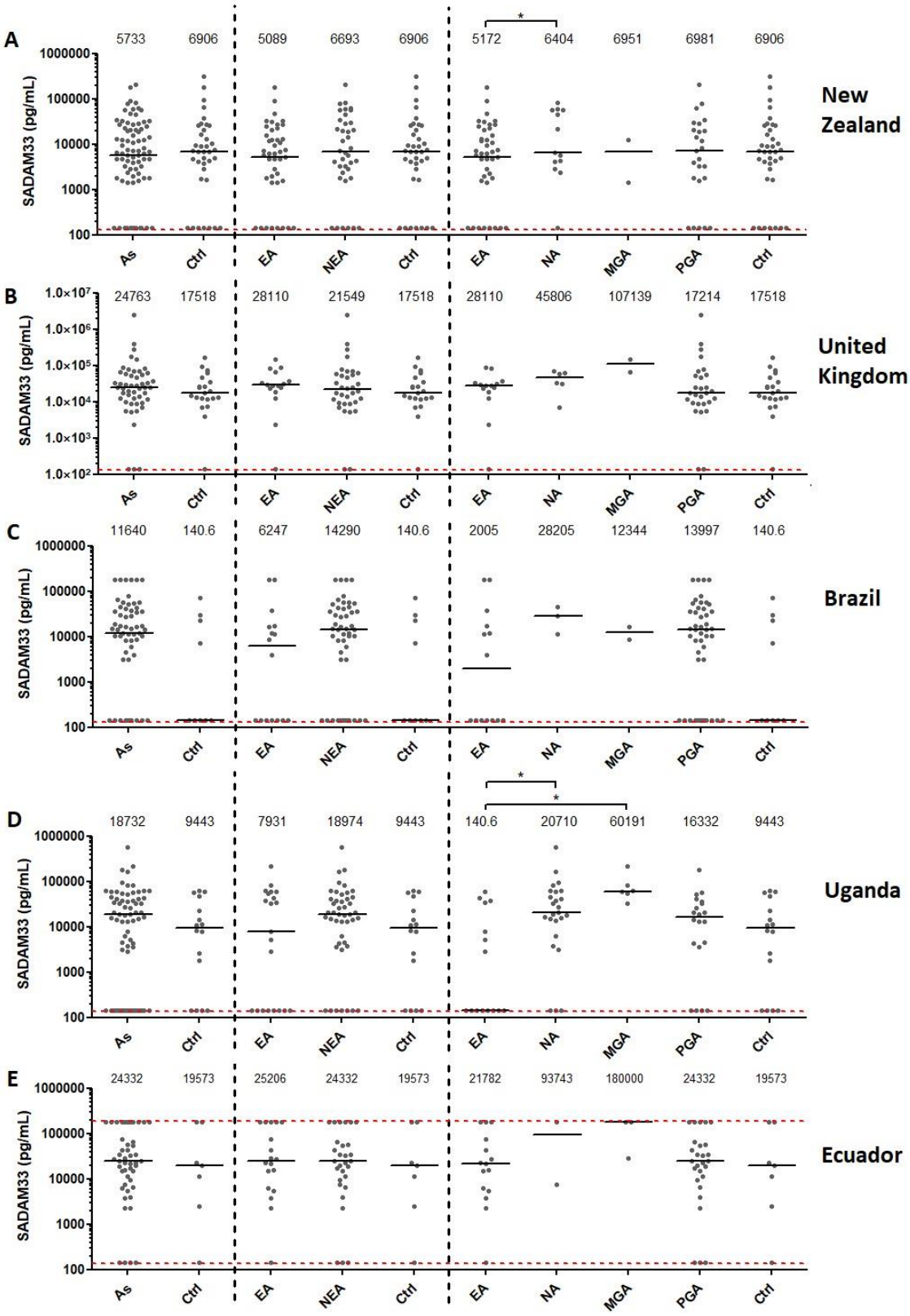

Figure 22: SADAM33 levels in different groups in different countries. *A: New Zealand, B: UK, C: Brazil, D: Uganda and E: Ecuador. As: asthma, Ctrl: Controls, EA: Eosinophilic Asthma sub-group (consisting of phenotypes EA + MGA), NEA: Non-Eosinophilic Asthma sub-group (consisting of phenotypes NA + PGA), NA: Neutrophilic asthma, MGA: Mixed Granulocytic asthma, PGA: Paucigranulocytic asthma. Median - black bar and number above each plot. Limit of detection (LOD) - red dotted line (where applicable). P-value determined through multivariate analyses using linear regression, adjusted for age and gender. #: p<0,1, *:p<0.05, **: p<0.01, ***:p<0.001.*

4.6 Correlation between mediators and cells

When examining correlations between sputum leukocytes and mediators, several cell types significantly correlated with mediator levels (r=-0.46 to 0.47, p<0.05 – 0.001) (appendix 11). For example, in asthma, macrophage percentages were significantly correlated with 15/20 mediators (all p<0.01), eosinophils with 4/20 mediators (including ECP and periostin; all p<0.05), and neutrophils with 16/20 mediators (including NE, IL-8, and IL-1β; all p<0.05). Lymphocytes and epithelial cells were only weakly (r=0.1 – 0.3) correlated with 7/30 mediators (p<0.05).

4.7 Correlation between mediators

Between mediators, many strong (r=0.5 – 0.75) correlations were found (appendix 12). Examples of some of the strongest correlations include: IL-1β with IL-8 (r=0.7; p<0.001) and MMP-9 (r=0.69; p<0.001); IL-6 with IL-8 (r=0.62; p<0.001) and VEGF (r=0.63; p<0.001); and NE with MMP-9 (r=0.63; p<0.001), and sADAM33 (r=0.66; p<0.001).

5. Discussion

5.1 Summary of main findings

This research examined differences in sputum mediator levels between asthma sub-groups and inflammatory phenotypes and compared phenotypes across various geographical locations that encompass different levels of socioeconomic development, lifestyles, environmental exposures, and ethnicities. In general, and as expected, neutrophil-associated sputum mediators were found to be higher in neutrophil-associated asthma phenotypes (NA and MGA), while eosinophil-related mediators were higher in eosinophil-associated asthma phenotypes (EA and MGA), irrespective of geographic location. Overall, the highest levels of inflammatory, remodelling, and neural mediators were found in MGA. Although higher mediator levels associated with eosinophils or neutrophils were found in EA, NA, or MGA across countries, there were significant differences in mediator levels when making comparisons between countries. For example, periostin levels in EA in the UK were significantly higher than periostin levels in EA in Brazil and Ecuador. With regards to neural mediators, there was little evidence of differences between phenotypes or centres; however, nociceptin levels were significantly higher in asthmatics, particularly in EA, in several countries. While several mediators were associated with specific phenotypes (in particular EA, NA, and MGA), no associations were found with PGA in any centre, and the pathophysiological mechanisms underlying PGA, therefore, remain unclear.

5.2 Differences in mediator levels between phenotypes in each country

Overall, and when analysing data within each country, distinct patterns of sputum mediator expression were observed when comparing inflammatory phenotypes. In general, phenotypes characterised by elevated neutrophils (NA, and MGA) or eosinophils (EA, and MGA) were associated with correspondingly higher neutrophil and eosinophil-related mediators, irrespective of centre. For

example, in all countries, elevated levels of IL-1β, IL-6, IL-8, NE, and MMP-9 were observed in NA and MGA (and to a lesser extend in NEA, which encompasses NA and MGA). However, some findings were not statistically significant; this is possibly due to limited power, as the prevalence of NA and MGA was low in some centres (e.g. 5.5% NA, and 2.5% MGA in Brazil) (32). Similarly, higher levels of eosinophil markers – in particular ECP, periostin, and to a lesser extent, PGD-2 - were observed in EA across all countries, and particularly in phenotypes EA and MGA in the UK, Brazil and Uganda, although, as with the neutrophil-associated findings, statistical significance was not consistently reached, probably for the same reasons (i.e. limited power in phenotypes with low prevalence). Ultimately, it is not entirely surprising that levels of neutrophil-associated mediators are predominantly associated with neutrophilic and eosinophil-associated mediators with eosinophilic phenotypes, as previous studies have established strong associations between airway levels of IL-8, NE, IL-1β, IL-6, MMP-9, and neutrophils (189, 205, 218, 244-246), and between ECP and periostin and eosinophils in asthma (167, 227, 245, 247). However, to the author's knowledge, this has not previously been studied across such a diverse range of geographical locations. The consistency of the results observed in this study with previous research strongly suggests that findings are generalisable across diverse populations and geographical locations i.e. irrespective of factors such as location, environmental exposure, and socioeconomic status, pathology of eosinophil and neutrophil-driven phenotypes appears highly compatible across different geographical locations.

5.3 Differences in mediator levels between different countries in the same phenotypes

When comparing sputum mediator levels in specific asthma phenotypes between centres, many significant differences were found i.e. differences were observed in levels of all (20/20) of the mediators measured. For example, IL-1β levels were higher in Uganda EA than NZ EA, and NE levels

were higher in UK PGA than NZ and Brazilian PGA. The reasons for the observed differences are unclear, but it may be due to either methodological or population-specific factors.

With regards to methodology, as sputum supernatants were analysed in one central lab and by the same laboratory technician (with exception of the samples from Brazil), differences are unlikely due to analytical variance. However, although sputum processing training was standardised across the centres, there were variations in level of expertise, equipment availability, and time constraints when collecting and handling samples, which may have led to variations in sputum supernatant yield and quality. Additionally, differences in cell density/number, which is strongly correlated with sputum mediator levels (248) and may occur with variations in sample handling, may explain at least some of the differences in mediator levels observed. As TCC/mL of sputum data was not collected in several centres (and therefore was not included in this thesis) results could not be adjusted for this.

With regards to population-specific factors, differences in population characteristics may explain the observed differences. For example, differences in medication-use between centres could have played a role, as e.g. corticosteroid use has previously been shown to impact the levels of mediators in sputum (249, 250). Therefore, in a sub-analysis, the impact of asthma medication on sputum mediator levels was examined (results are shown in appendix 12). This found reduced sputum levels of IL-1β, IL-8, substance P, VEGF, and periostin in asthmatics using preventers and/or relievers compared to non-medication users. However, a stratified analysis per country did not find significant differences in mediator levels between users of preventers, relievers, or non-medication users (data not shown), suggesting that the impact of different medication-use on mediator levels between centres was likely to be minor.

Differences in asthma control or severity in the same phenotype in different centres may also possibly explain differences in mediator levels, as studies suggest that poorly controlled asthma may be

associated with increased sputum eosinophils (251, 252), which could potentially affect inflammatory mediator levels (227, 245, 246). However, this pattern was not observed, as centres with the highest percentage of poorly controlled asthma (Brazil and Uganda) showed no increased proportion of EA (31.9 and 22.1% respectively, with an average proportion of EA to be between 30-40% (18, 32, 176)). An evaluation of the association between asthma severity and mediator levels found showed higher levels of TIMP-1 in well-controlled asthmatics compared to poorly controlled asthmatics, but no other associations were observed (data not shown). This suggests that the effect of asthma severity on mediator levels is minimal.

Finally, it is possible that factors such as environmental exposures (e.g. air pollution), lifestyle, stress, or previous infections may have played a role as each is known (or suspected) to affect airway inflammation (253).

5.4 Increased nociceptin in asthma and EA

Whilst previous studies have reported associations between neural mediators and asthma (28, 29, 254) this study did not observe a difference between asthmatics and non-asthmatics and within sub-categories of asthmatics for most neural mediators. The exception was nociceptin, which showed higher levels in 3/5 countries (UK, Brazil, and NZ (although not significant)) in asthmatics vs. controls; significantly higher levels were also observed in 4/5 countries (NZ, UK, Brazil, and Uganda) across eosinophil-driven asthma groups (sub-group EA, and phenotypes EA and MGA). Nociceptin, often referred to as nociceptin/orphanin FQ (N/OFQ), is an endogenous, non-opioid peptide that, together with the N/OFQ receptor (NOP receptor), has been suggested to play a role in asthma (231). Whilst classified as a neural mediator in this study, it remains unclear exactly how nociceptin is involved in asthma immunopathogenesis. However, on the basis of animal studies, there are reports that it might inhibit capsaicin-induced bronchoconstriction and airway inflammation (255), increase airway

compliance, reduce bronchial wall thickness, and cause abnormal growth and proliferation of ASM cells (256). In humans, a previous study by Ali et al. (167) in 111 young (14-21yo) asthmatics found increased nociceptin in asthmatics vs controls, particularly in EA vs controls ($p<0.05$). Similar findings were reported by Singh et al. (231) in 55 older (>50 yo) asthmatics. Thus, this study provides further evidence that nociceptin may be involved in asthma, particularly in eosinophil-associated asthma. It also extends these findings to include LMIC countries in South America and Africa, suggesting that findings may be generalisable across diverse populations and geographical locations. If confirmed in other studies, then the nociceptin and its receptor may be a promising new target for novel treatment strategies for allergic asthma or EA in particular, as also suggested Singh et al. (231).

5.5 Increased neutrophils in Uganda

As reported previously, the WASP study found that Uganda has a high prevalence of NA (32). Compared to this earlier paper, the current study had some slight variations in population characteristics, due to differences in the availability of sputum samples considered adequate for mediator analyses, but Uganda still had the highest percentage of sputum neutrophils in asthma (60.5%) and controls (65.5%) compared to other countries (6.0 – 26.6% and 8.8 – 34.7%, respectively) (**table 4**). Additionally, in Uganda, higher levels of NE, IL-8, IL-6, and IL-1β were found in the sub-group NEA, and phenotypes NA and MGA. The reasons why neutrophils and neutrophil-associated mediators are increased in the airways in children from Uganda is unclear. Neutrophils are actively involved in innate immune responses (22), and there is strong evidence that certain environmental exposures, such as endotoxins (119), PM (120, 257), and viral infections (258) can lead to innate immune activation through TLRs, which may ultimately lead to neutrophil activation and migration (132). Interestingly, as Ugandan controls also had higher levels of neutrophils, it is possible that such innate immune activation may not be associated with asthma pathology and instead be related to shared (between asthmatics and non-asthmatics) environmental exposures, including those referred to

above. Future research investigating different aspects of specific environmental exposures in these different countries (e.g. sampling and assessing irritant exposure in participant households or their local area) may help clarify this.

5.6 PGA immunopathology remains unclear

NEA, previously reported to account for over 50% of asthmatics (9), was slightly more prevalent in this study, with an average percentage across the five countries of 60.5%, with NA only representing a small fraction of this (11%) and PGA representing the remainder (49.5%). This finding aligns with previous studies including one by Brooks et al. (241) who reported that 54% of adolescent asthmatics were NEA; the majority of which were PG with very little evidence of NA, and a study by Simpson et al (18) who reported 51% NEA with a majority being PGA. Previous research has characterised PGA as a phenotype with little or much lower-grade inflammation and generally good control compared with EA (191, 259). This research showed similar findings, with a high percentage of PGA being well-controlled, and with levels of sputum inflammatory cells and mediators not much different from healthy controls.

In the absence of overt inflammation, previous studies have suggested that other non-inflammatory mechanisms could play a role; for example, neural involvement or structural changes (remodelling) (260). However, in this study, increased levels of mediators associated with these pathways were not found in PGA, and therefore there was no clear evidence that these pathways are involved in the immunopathogenesis of PGA in any of the centres involved in the WASP study. This corresponds with earlier findings of a study conducted in Wellington, which found no clear involvement of neural and remodelling mediators in NEA (167). The immunopathogenesis of PGA in the participating Centres therefore remains unclear. Despite this, it is possible that measuring mediators or inflammatory cells in sputum is inadequate for the assessment of neural or remodelling pathways, or that PGA may

include some participants incorrectly identified as asthmatics. Regarding the former, previous studies have found evidence of previous remodelling in the absence of current inflammation or ongoing remodelling (141), and a recent study by Ali et al (27) found that airway sensory nerve reactivity, which would not be detectable using mediator analysis, was increased in young adults with NEA versus EA. Regarding the latter, although misclassification of asthma status could explain the high prevalence of PGA observed across centres, the use of the ISAAC asthma questionnaire in this study, known for its high sensitivity and specificity compared to physician-diagnosed asthma (261, 262), makes it unlikely that this would be the case for the majority of PGA.

5.7 Other findings

In addition to the findings discussed above, several other findings emerged from the analysis of sputum mediators. For example, TIMP-1 was higher in controls vs asthmatics, particularly in NZ and Ecuador; it was also higher in EA, NA, and MGA in several centres. Also, NKA was higher in NZ controls, and higher in the asthma sub-group EA, in the UK. The reasons for these findings are unclear. In a previous study, TIMP-1 was found to be higher in asthmatics vs healthy participants, particularly in atopic asthmatics, (225), however, this was not observed in this study. Furthermore, while an association between NKA and eosinophils in asthmatics was found in the UK, an association between NKA and eosinophils was not observed (appendix 10). Another finding was that NGF-β was detected significantly more in EA and PGA vs controls in NZ, and was barely detectable in other countries. The reasons for this are also unclear, but it may be due to variations in sample quality, with NZ possibly having higher quality samples (the centre in NZ had the most experience dealing with sputum samples). Finally, it is possible that some of these findings were due to chance. Data for this study were derived from analysing 20 mediators in sputum from multiple groups involving four inflammatory phenotypes across five countries, resulting in many comparisons. When considering the

use of p=0.05 to indicate statistical significance, or p<0.1 to indicate trends, there is a 1/20, or 1/10, chance of randomly finding a significant finding.

5.8 Importance and meaning of this research

When discussing ‘’*different geographical locations’’*, and ‘’ *HICs and LMICs*’’, it entails more than physical location and income. Differences in geographical locations and HICs and LMICs also mean differences in: socio-economic status, ethnicity, culture, lifestyle, diet, healthcare, ethnicity, genetics, occupation, environmental exposure, etc. Each of these factors may affect the associations described in this study, particularly as previous research has shown associations with asthma and socio-economic status (94), ethnicity/race (263, 264), various aspects of lifestyle (265), genetics (266), environmental factors such as air pollution (95, 97), exposure to biodiverse environments (267) and farm exposures (82). Nonetheless, even when considering these differences, several clear patterns were observed between mediators in different asthma classifications. This suggests, as noted earlier, that findings may be applicable to a wide range of social and geographical settings independent of environmental exposures. This is important as novel treatments targeting any of the inflammatory, neural, or remodelling pathways/mediators described in this thesis may therefore be effective across an equally wide range of social and geographical settings, although this remains speculative at this stage.

5.9 Study strengths

A notable strength of this research and the WASP study overall is the multicentre international collaboration, and the large population size recruited from all the centres involved. To the best of the author's knowledge, there have been no prior studies assessing sputum mediator levels across diverse geographical locations in relation to asthma inflammatory phenotypes. Although previous studies

have examined inflammatory phenotypes and/or mediator levels within one centre (usually representing a Western or HIC) there is a lack of comparative studies between HIC and MLIC with respect to sputum mediator levels and asthma phenotypes. While comparisons between asthma clinical characteristics in different geographical locations and between phenotypes have been studied in the past (30, 32), the involvement of assessing sputum inflammatory, neural, and remodelling mediators in this study allows a unique perspective on asthma immunopathology in these different geographical locations. Finally, this study is one of the few studies that are conducted in the general population, rather than in tertiary care, that has attempted to use clear and standardised approaches to asthma phenotyping across several centres spanning four continents using sputum mediator levels.

5.10 Limitations

Despite the strengths, this study had several limitations. Firstly, although standardisation of laboratory procedures, particularly sputum processing, was attempted (with standardised training provided at the start of the study and support available throughout) this proved difficult to achieve in reality. Sputum slide quality varied widely across the centres (table 3), with the percentage of participants with both high-quality sputum slides and supernatant for mediator analysis ranging from as high as 87.7% in NZ to as low as 31.4% in the UK. Secondly, this study had no standardised data on ethnicity available. The ISAAC Phase III study has previously demonstrated that there are longitudinal variations in asthma prevalence, which although high, appear to be declining in English-speaking nations, and from much lower prevalence, rising in Africa, Latin America, and Asia (30). Considering the potential effect ethnicity may have on asthma (268), exploring the association between ethnicity and sputum mediators and asthma phenotypes may have been helpful. However, demographic data were in some centres derived from prior studies with various approaches to defining ethnicity (e.g. (232, 235-237)), meaning no standardised data were available, preventing adjustment for ethnicity or potential exploration of its effects on sputum mediator levels. Finally, the assessment of mediators was

generally conducted at a single point in time. As previously reported, sputum mediator levels are likely to be affected by many factors that vary over time (199, 269). Therefore, a single measure in time may not provide a sufficiently accurate picture of typical mediator levels. This is despite earlier evidence that asthma phenotypes are relatively stable over time (18, 172, 201).

5.11 Future studies

Considering the findings of this study and the limitations described above, there are several potential approaches to future research. With five different countries, four asthma phenotypes, twenty sputum mediators, and a large number of other variables, a study like this is complex, multidimensional, and produces a significant amount of data, with potential for confounding and multicollinearity. Cluster analysis is a potentially useful approach to help identify subgroups, or clusters, within a complex dataset, and may be particularly well-suited for handling such complexity by identifying non-obvious patterns or relationships. Previously, cluster analysis has been used in asthma research to identify five asthma phenotypes based on clinical characteristics (102). Using this type of analysis, it would be interesting to see if clusters can be identified on the basis of a combination of clinical characteristics, inflammatory cells, and mediator levels; this may be particularly useful when considering how little is understood about PGA. Additionally, sensory nerve reactivity could be further investigated, as previously shown by Ali et al. (27), and the use of CT scanning (which has previously been shown to help assess airway remodelling) (140) in the different centres to better understand the involvement of neural pathways and airway remodelling in PGA. Another potential avenue of future research could be to look at the association between specific environmental exposures (such as air pollution, microbial exposure (117) or green space/biodiversity (267)) and asthma phenotypes and sputum mediators in the different centres. While a limited number of studies have looked at the relationship between environment (117), and biodiversity (270) and asthma characteristics, this has not been done in a setting where sputum mediators are analysed, or been conducted across such a diverse range of

geographical settings. A more detailed assessment of specific environmental exposures of participants in the individual centres and subsequent examination of their relationship with asthma phenotypes and sputum mediator levels could provide more insights into the causal exposures responsible for asthma and lead to more tailored asthma prevention, treatment, and management strategies. Lastly, while 20 sputum mediators were analysed, BDNF, NGF-β and IL-13 showed poor detectability. Other methods of detection could be used to gather more information about the mediators in sputum in relation to inflammatory phenotypes. Additionally, other important mediators that have previously been associated with asthma, such as tumour necrosis factor alpha (TNF-α) (271), and interferon-γ (IFN-γ) (196), but also the expression of various TLRs on the cell surface in relation to inflammatory phenotypes in different settings can be interesting to look at.

5.12 Conclusion

In conclusion, this research shows that differences in levels of sputum mediators, particularly those associated with eosinophils and neutrophils, are present between asthma phenotypes in all countries with similar patterns. This highlights that, regardless of variables such as different geographical locations, socioeconomic status, exposures, and ethnicity, similar associations between specific mediators and asthma phenotypes are present globally, emphasising the robustness of these findings and the potential for generalised asthma treatments and interventions. Additionally, differences in levels of sputum mediators within specific phenotypes were present between all countries, highlighting the need for further exploration into methodological and population-specific factors, and how they impact sputum mediator levels in asthma. Additionally, while PGA is the most common asthma phenotype, it is still poorly understood and further research is required. Elevated nociceptin levels in asthma, particularly in EA, suggests a potential role in asthma immunopathogenesis. Despite limitations, such as variations in laboratory procedures and a lack of standardised ethnicity data, the research contributes significantly to the understanding of asthma heterogeneity worldwide.

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Appendix 1: Induced Sputum processing protocol

Purpose

To describe the procedure to be followed when processing saline-induced sputum or spontaneous sputum samples for asthma studies at CPHR

Equipment

- Observation base plate (black)
- Forceps
- Scissors
- Inverted microscope (if necessary)
- Pipettes (positive displacement pipette, 2-20μL adjustable auto pipette [yellow top with yellow tips], 20-200μL adjustable auto pipette [yellow top with yellow tips], 100 – 1000μL adjustable auto pipette [blue top with blue tips], and Easypet pipette [trigger handle with glass pipettes) and tips
- Centrifuge (Heraeus Labofuge 400)
- Millipore nylon filter (60μm) and filter holders
- Haemocytometer and cover slip
- Microscope (to 40x; e.g. Leika DME)
- Cytocentrifuge adaptors for a standard centrifuge or e.g. Shandon Cytospin
- Cytocentrifuge apparatus (filter cards, sample holders, and metal clips)

MATERIALS

- Sputasol (Oxoid, Cat# SR0233A)
- Trypan blue (Sigma Cat no: T8154)
- Sterile distilled water (Baxter Cat# AHF7114)
- PBS (Phosphate Buffered Saline) (Sigma: SIGP4417/50TABS) [M019]
- Petri dish (Global Science: 6729923401) [M075] or alternative
- Glass slides (Global Science: 852700100W) [M054] or alternative
- Disposable pipettes (Global Science: 7468432) [M064] or alternative
- 15mL tubes (polypropylene) (Global Science: 672553041) [M089] or alternative
- Filters (Millipore 60um Cat#NY600250)
- 2.5, 10mL and 20ml syringes (DMB Cat no: S500 and S520) or alternative
- Mixing cannula (DMB Cat no: C010) or Fairmont Medical (MIX1001)
- 1.5mL Eppendorf tubes (Global Science: 620CT175C [M087]) or alternative

Set-Up

- Photocopy worksheets for the day
- Remove sputasol from the fridge and allow it to equilibrate to room temperature.
- Make up diluted Sputasol by adding 750ul to 9.25mL of sterile distilled water in a 15mL Falcon tube. Label tube with date made and initials. This is stable for 48 hours at 4°C.
- Ensure tweezers/forceps are clean prior to sample processing.
- Prepare a waste pot with a small volume of 10% trigene solution for disinfecting labware

Protocol

1. Label a 15mL tube with patient number
2. Fill out the sputum worksheet details for visit number and collection details.
3. Invert the sample jar into a clean open petri dish.
4. Evaluate the quality of sputum (fill out a worksheet for sputum volume, color [i.e. opaque - looks like jelly, yellow, green], is sample largely saliva, etc).NOTE: small white space 1.5 x 3mm on observation base plate can be used as a guide for the size of one mucus clump, although this may vary considerably between samples. If it is uncertain whether the sample contains lower respiratory clumps, inspect the sample in petri dish using an inverted microscope (trying to find non-squamous cell areas) if available.
5. Use clean tweezers/forceps (preferably two sets) to separate out mucus clumps (plugs) from saliva bubbles.
6. Collect plugs by pinching with 1 set of tweezers whilst pulling with the other and transfer to the other part of the Petri dish (either lid or base).
7. Pipette all the suitable mucus clumps (minimum of 100μL, maximum of 1ml) into the labeled 15ml tube using a positive displacement pipette and clean tip. Record the actual volume of plugs selected on the worksheet. (In some samples, the plugs will be dispersed and difficult to select. In these cases, process the whole sample and note this on the worksheet)
8. Add four times the volume of diluted Sputasol to the sample in the tube. Mix up and down with a disposable pipette or Pasteur pipette (for example, if 300μL of sputum is used, add 1.2mL of Sputasol).
9. Cap tube and place in rotating mixer (set at slowest speed) for 30 minutes to disperse the sputum plug sample and dissolve mucus. NOTE: this can be extended up to 60 minutes if required when there is incomplete dispersion. If the sample has not dispersed after a maximum of 1hr, continue as below.
10. During this 30-minute incubation period, prepare a filter apparatus and syringe. Carefully place a filter in cannula base using tweezers, add O-ring, then screw on top. Fill a syringe with PBS (at least 5 mls), attach to a cannula, and "prewet" filter by running PBS through. Detach syringe for sample collection (5.14). Prepare mixing cannula for use with the syringe for a sample collection from the 15ml tube.
11. After rotation mixing, add the same volume of PBS as Sputasol (i.e. 4 times the volume of the selected sputum sample). Mix with a disposable pipette or Pasteur pipette and put the tube back on the rotating mixer for 1-2 minutes (adequate to homogenise the sample).
12. Collect all the homogenised samples using a syringe/mixing cannula MIX1001. Remove the mixing cannula, attach to the cannula containing the filter, and filter the sputum/sputasol mixture through the nylon filter apparatus into a fresh, labeled 15ml tube. NOTE: insert 2.5, 5 or 10mL syringe, depending on volume to be filtered, into mixing cannula, use pipette/ syringe to draw up sample then slowly, i.e. one drop every 1-2 seconds, filter sputum and sputasol mixture through the nylon mesh filter and down the side of the tube.
13. Record post-filtered volume on the data sheet (to be used for Total Cell Count [TCC]).
14. Use an autopipette to place 15μL of Trypan blue (neat) into a clean Eppendorf tube, then add 15μL of homogenised sputum sample. Mix well by slowly drawing up sample into the pipette and then releasing back into Eppendorf tube. Repeat this several times to ensure that the sputum sample and Trypan blue are mixed thoroughly.
15. Use an autopipette to pipette sputum and Trypan blue solution onto the Haemocytometer gently.
16. Before conducting count, centrifuge the tube containing the remaining cell suspension at 1600rpm for 8 minutes (no brake, rotor 8172). NOTE: 2300rpm = 1000g on rotor 8172.
17. Use a light microscope to perform a TCC. Count at least 2 diagonally opposite corner quadrants on the 40x objective. See Figure 5.1. (top left and bottom right). As yields are often low, if possible also duplicate count (bottom left, top right)

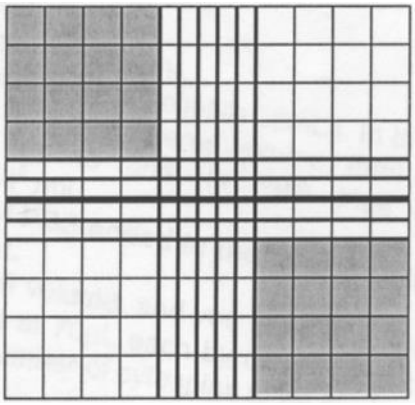


Diagram of Haemocytometer

(Highlighted is the area to be counted)

Calculate TCC and viability using the formula below:

$$TCC = \frac{Inflammatory\ cell\ count}{Number\ of\ quadrants} \times 0.02$$

$$= x.xx \times \frac{10^6}{mL}(C_1)$$

$$\%\,Viability = \left[\frac{Number\ of\ live\ cells}{Total\ number\ of\ cells}\right] \times 100$$

18. Report the TCC and viability on the worksheet

19. After centrifugation, evenly pipette the supernatant into Eppendorf tubes labelled with (study I.D., SPUTUM S/N and data on main label, and study I.D. number and "S/N" on the top lid label) 1.6ml Eppendorf tube store in the fridge until completion of the remainder of the protocol, then and freeze store in labelled boxes at -70 to -80°C. Store as many aliquots as possible – a minimum of 5 will be required—aliquots of a minimum of 225 (i.e. between 250-500Ul) for ELISA.

20. Resuspend the remaining cell pellet (after removal of all supernatant) to a final concentration of 1x10^6/ml in a volume of PBS determined by the following equation:

$$Volume\,(V_2) = C_1 \times Post\ \ filtered\ volume\,(V_1)$$

Sputum cell slide protocol

For each participant, at least one good quality slide is required for an adequate differential cell count. Based on the amount of squamous contamination, this usually involves the use of between 25ul (high squamous contamination) and 75ul (little squamous contamination) of cell suspension at 1x10^6/ml in PBS.

1. Assemble cytospin apparatus (labeled slide (with lab number and date), filter paper, sample bucket, and metal clip), making sure that the filter card circle lines up with the filter cup circle by observing the reverse side of the metal clip when assembled. Add cytospin tube to the appropriate hole, and place complete cytospin apparatus in centrifuge, ensuring that it is appropriately balanced. See cytospin instructions if you are unsure.

2. Using disposable pipette, add the required volume (V_2) of PBS to the dry cell pellet, and mix up and down a few times to ensure the sample is completely dispersed (sample now at 1 x 10^6 cells per ml).
3. Add 30 to 40ul of PBS to the cytospin tube, and press quick spin. Allow the centrifuge to get to >1000rpm, and then release. This step dampens the filter paper in preparation for the sample and generally improves the quality of the slide.
4. Add 25-100μL of the sample (see above) to the cytospin sample bucket, and centrifuge sample at 44xg for 5 minutes (rotor 8176), no brake.
5. Carefully and quickly remove cytospins from the apparatus, flipping off the filter paper and not affecting the actual cytospin area.
6. Quickly assess the quality of the cytospin under the microscope; if the slide looks very dense, then repeat the process using a reduced volume of cell suspension until a suitable slide density is achieved. The slide can be left to air dry for 1-4 days, before fixing, staining with Dif-quik, and mounting (using Entellan). Diff-Quik® fixative/stain set (Dade Behring, Deerfield, IL).

Appendix 2: Cell Slide Staining Protocol

Purpose: To describe the procedure to be followed when staining slides with the May Grunwald - Giemsa stain.

Equipment

- Coplin Jars
- 100mm Glass Funnel
- Tripod
- 50-100mL Glass Measuring Cylinder
- 200mL Glass Beaker
- Filter Paper - Whatmans Grade 1 (Global Science: 9001001042) [M072]
- Tweezers
- Microscope
- Glass pipette
- Macropipette controller
- Aluminium or stainless steel tray
- Glass coverslips - 22 x 22mm (Global Science: 866101050) [M071]
- Glass screw top jar

Materials

- May and Grunwald stain (Biolab: BDH350254R) [M017]
- Giemsa (Biolab: BDH350864X) [M029]
- Methanol (Biolab: BSPML868.2.5) [M026]
- pH 6.8 Buffer Tablets (Biolab: MER1.11374.0100) [M025]
- Tap water
- Distilled water (Biolab: TCHH20) [M016]
- Incohelp Pads (Oracle Cat no: M12796)
- DPX (Australian Scientific Cat no: 36029 4H)
- Kimwipes (Global Science: 716N4103) [M058]
- Pasteur pipette (Global Science: 642672040) [M086]
- 50mL Falcon Tubes (Global Science: 672548004) [M088]

Set up

- Prepare the pH 6.8 Buffer by dissolving 1 buffer tablet in 1000mL of distilled water (this solution can be used until it has been emptied or there is growth in it).
- The Giemsa solution is made up by adding 5mL of Giemsa to 45mL of pH 6.8 buffer solution (this is made up in a glass beaker).
- The filter paper is folded in half four (4) times.
- Place the funnel in the tripod, and the filter paper in the funnel, place this over a coplin jar.
- Pour the Giemsa solution into the funnel, which is positioned, over the glass coplin jar.
- Pour approximately 50mL of May and Grunwald into a glass coplin jar. This is stable for 3 months (Label with Expiry date).
- Place an incohelp pad into the aluminium tray to catch any solution that may drip, either off the slide or down the side of the coplin jars.
- Place tap water into water jars.
- Place methanol into fixative jars.

Methods

- Place slides in methanol for 10 minutes.
- Using the tweezers move the slides from the methanol to the coplin jar containing the May and Grunwald.
- Stain in May and Grunwald for 10 minutes.
- Again using the tweezers move the slides to a coplin jar containing water and give slides a rinse to remove excess stain.
- Remove from the water and place in the Giemsa solution for 5 minutes.
- Wash in water twice, by placing the coplin jar under running tap water, then emptying the jar and repeating this step.
- Wipe back of slides with Kimwipes (be careful not to wipe the wrong side, as this will remove the cells).
- Allow slides to air dry.
- Coverslip slides:
- Lay slides on bench, cell side up.
- Get out as many coverslips as required, and line them up with the slides.
- Put a drop of DPX on the coverslip using a pasteur pipette that has had about a centimetre cut off the end.
- Slowly lower the slide onto the coverslip ensuring that the mountant covers the cells with no air bubbles.
- Place back up the other way and let the mountant dry.

Appendix 3: Differential Cell Count Protocol for Phenotyping

Purpose: To describe the procedure when performing a differential cell count using cytospin slides stained with May Grunwald Giemsa stain or DIFF-QUIK stain.

Equipment

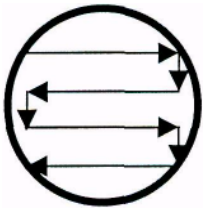
- Microscope (preferably with 40X and 10X objective)
- Totalising Counter
- Calculator

Set Up

- Place slide on microscope, held in place by the clip, with cover slip side facing up.
- Focus on the cells using the 40X objective or similar (first find cell circle using 10X objective then change to 40X and fine focus).We avoid using 100X objective as counts take too long.

Methods

- Once the cells are in focus, scan over the stained area to determine that cells are present as expected. Scan around the stained cytospin circle to find an eosinophil (if they are present).
- Once an eosinophil is located start counting from that position. If no eosinophils are present start counting from anywhere around the top left hand side of the cell circle (as observed through the microscope). I suggest starting in an area where the cells are not too dense, and you can clearly see individual cells - if this is possible.
- Count all cells (including squamous cells) until you reach a total of approximately 400 non-squamous cells.
- Count all cells in one field of view at a time, and then move across to the right to a new field (until you can no longer see any cells from the previous field). When you no longer see cells to the right, move the field down until you no longer see cells from the field above. Then count the cells in that field, and move the field left following the pattern below across the cytospin:



- Identify cells according to the table below (and powerpoint slide previously produced by CPHR). Cell types are counted on separate buttons on the cell counter, or alternatively, by manual tally.

	CELL size/shape	NUCLEUS size/shape/ chromatin	CYTOPLASM colour/granules	COMMENT
Bronchial or columnar Epithelial	10-20µm oval or columnar	8µm single, round, purple, loose chromatin pattern	blue - light purple	Often cilia visible, "treetrunk" like appearance
Squamous Epithelial	40-60µm polygonal	5-10µm single, round, purple, loose	blue – pink, non-complex, bacteria often evident	Very large
Macrophage	20-40µm oval Large to small	12-15µm round 1 or more purple, dense	blue / grey foamy ≅ (white vacuoles)	smokers have black / purple dots
Neutrophil	16µm oval	3-5 lobes purple, tight	pink / blue granules, blue cytoplasm	
Lymphocyte	9-12µm oval	1 round purple fills cell, tight	sky blue thin rim	Large lymphocytes may look like small monocytes
Eosinophil	16µm oval	2 lobes purple, dense	brick red granules, pink cytoplasm	Often have "sunburnt man with sunglasses" appearance
Mast Cell	15-24µm slightly irregular	small, round, purple. Can be obscured by granules	blue / purple with numerous dark granules	Rare, don't count

To calculate the percentage of cell types on the cytospin:

- Divide the number of squamous cells counted by total number of all cells (including squamous) and then multiply that number by 100 to get the percentage of squamous cell contamination.
- For each non-squamous cell type, divide their count by the total number of non-squamous cells (we usually aim for 400, but as stated previously, you could do a quick count of 100 to 200) and multiply by 100 to get the percentage.

Appendix 4: Luminex MAGPIX xMAP technology

xMAP technology

Luminex corporations invented the xMAP technology in late 1990, which created significant progress in the multiplex assay industry. The x in xMAP stands for the biomarker or disease panel that needs to be tested. MAP stands for Multi-Analyte Profiling. According to Luminex, xMAP means multiplex biological testing of up to 500 analytes in a single sample volume. The xMAP technology can be used for either immuno-assays or nucleic acid assays and is executed in a 96-wells plate. Additionally, the detection of enzyme activity can also be determined using xMAP (1). There is a range of possibilities that allow protein, DNA, or enzyme activity detection. For example, the detection of various pathogens like bacteria, viruses, fungi, and parasites in samples (2), Single Nucleotide Polymorphism (SNP) genotyping, genetic disease screening, gene expression profiling, microbial detection (3), and cytokine, chemokine, grow-factor and inflammation marker profiling (4). Luminex has different devices running on xMAP technology. Some devices use the technology slightly differently than others, but all have the same main principle.

xMAP principle

The xMAP technology uses magnetic microspheres internally dyed with two or three different dyes. This internal dye is specific for that bead that the MAGPIX system can detect. The internal dye consists of 2 colors, called red and near-infrared. By using different concentrations of each dye, a wide variety of different dyes can be created, each individually authentic. The outer layer of the polystyrene bead is covered with magnetic particles, which gives the bead magnetic characteristics. The outer layer also consists of very reactive carboxyl groups that can react with antibodies, proteins, DNA, etc. The beads have a diameter of 6.5 micrometers and are able to stay suspended in their solution due to their size. Then, the beads can be conjugated with specific reagents, depending on the executed bioassay. In the case of a cytokine assay, capture antibodies will be attached to the beads by reacting with the carboxyl group on the outer side of the bead. The binding of the capture antibody to the bead happens on a large scale of beads with the same exact color. This makes one color of beads specific to the detection of one target in the sample. The beads with capture antibodies are then exposed to the sample. Any analytes in the sample will be captured by their specific antibodies on the beads. When using a multiplex protocol, multiple antibody-coated beads with different internal dyes and antibodies react with their analytes at the same time in the same well (1, 5).

Any excess material and liquids must be washed away since they might interfere with the rest of the protocol. Using a handheld magnetic washing tray, magnetic beads remain in the assay plate while unwanted fluids can be washed out. The wash solution can then be easily decanted without throwing out the beads. After washing, biotinylated detection antibodies are added. These detection antibodies are able to bind to the analyte that has been bound to the capture antibody, creating a sandwich. Phycoerythrin (P.E.) conjugated streptavidin is added, which binds to the biotin molecule on the detection antibody. After adding PE-conjugated streptavidin, an additional wash step is required to ensure detection is specific to the amount of bound protein and not influenced by leftover P.E. The beads are then resuspended in drive fluid and are ready for sample analysis (1, 5).

The assay is then placed in the MAGPIX, after which it gets injected into the fluid of the system. The beads are transported to the sample chamber that contains a strong magnet, causing the magnetic beads to form a monolayer in the back of the chamber. Light-emitting diodes (LED) light up the monolayered magnetic beads with red light, after which a charging coupled device (CCD) camera takes pictures of the fluorescent beads. The software then processes the images and identifies the beads based on fluorescent patterns. A green LED then illuminates the chamber, and the CCD camera takes additional pictures. The more fluorescent streptavidin P.E. present, the higher the concentration of the detected analyte. The pictures are then transformed into data and shown in the xPONENT

software on the desktop. Fig.3. The data can be processed by xPONENT to generate Excel tables which can be used for statistical analysis (1, 5).

Literature

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Appendix 5: Detectability of mediators per country and phenotype

	Biomarker	United Kingdom (n=50)				New Zealand (n=202)				Brazil (n=91)			
		EA (%)	NA (%)	MGA (%)	PGA (%)	EA (%)	NA (%)	MGA (%)	PGA (%)	EA (%)	NA (%)	MGA (%)	PGA (%)
Inflammation	IL-1β	14/14 (100)	6/6 (100)	2/2 (100)	26/28 (93)	92/93 (99)	14/14 (100)	2/3 (67)	77/87 (89)	28/29 (97)	5/5 (100)	2/2 (100)	53/55 (96)
	IL-6	10/14 (71)	4/6 (67)	2/2 (100)	16/28 (57)	63/93 (68)	12/14 (86)	3/3 (100)	47/87 (54)	17/29 (59)	4/5 (80)	2/2 (100)	37/55 (67)
	IL-8	13/14 (93)	6/6 (100)	2/2(100)	28/28 (100)	91/93 (98)	14/14 (100)	3/3 (100)	85/85 (100)	29/29 (100)	5/5 (100)	2/2 (100)	55/55 (100)
	IL-13	0/14 (0)	1/6 (17)	0/2 (0)	0/28 (0)	9/93 (10)	3/14 (21)	1/3 (33)	6/85 (7)	1/29 (3)	0/5 (0)	0/2 (0)	1/55 (1.8)
	Neutrophil Elastase	14/14 (100)	6/6 (100)	2/2 (100)	28/28 (100)	76/77 (99)	14/14 (100)	2/2 (100)	53/53 (100)	28/28 (100)	5/5 (100)	2/2 (100)	55/55 (100)
	ECP	12/12 (100)	5/5 (100)	2/2 (100)	20/20 (100)	77/77 (100)	14/14 (100)	2/2 (100)	49/49 (100)	24/24 (100)	3/3 (100)	2/2 (100)	50/50 (100)
	PGD-2	12/12 (100)	5/5 (100)	2/2 (100)	19/19 (100)	45/47 (96)	13/13 (100)	2/2 (100)	25/25 (100)	NA	NA	NA	NA
	Histamine	12/12 (100)	5/5 (100)	2/2 (100)	20/20 (100)	68/78 (87)	12/14 (86)	2/2 (100)	39/50 (78)	16/19 (84)	1/3 (33)	1/2 (50)	21/44 (48)
Neural	Neurokinin A	13/13 (100)	4/6 (67)	2/2 (100)	26/27 (96)	46/46 (100)	NA	1/1 (100)	51/51 (100)	18/21 (86)	4/4 (100)	1/2 (50)	42/52 (81)
	Substance P	8/13 (62)	5/6 (83)	2/2 (100)	18/27 (67)	6/36 (17)	1/1 (100)	NA	12/45 (27)	7/17 (41)	3/4 (75)	1/2 (50)	19/52 (37)
	Nociceptin	12/13 (92)	6/6 (100)	2/2 (100)	27/27 (100)	31/31 (100)	1/1 (100)	NA	25/25 (100)	13/21 (62)	1/4 (25)	1/2 (50)	27/50 (54)
	NGF-β	0/14 (0)	0/6 (0)	0/2 (0)	0/28 (0)	18/45 (40)	0/1 (0)	1/1 (100)	25/60 (42)	0/29 (0)	0/5 (0)	0/2 (0)	0/55 (0)
	BDNF	4/14 (29)	1/6 (17)	0/2 (0)	3/28 (11)	0/93 (0)	0/14 (0)	0/9 (0)	4/87 (5)	7/29 (24)	2/5 (0.4)	2/2 (100)	13/55 (24)
Remodelling	MMP-1	11/14 (79)	6/6 (100)	2/2 (100)	25/28 (89)	11/45 (25)	1/1 (100)	1/1 (100)	7/60 (12)	11/29 (40)	3/5 (60)	2/2 (100)	28/55 (51)
	MMP-9	13/14 (93)	6/6 (100)	2/2 (100)	28/28 (100)	92/93 (99)	14/14 (100)	3/3 (100)	85/85 (100)	29/29 (100)	5/5 (100)	2/2 (100)	55/55 (100)
	TIMP-1	13/14 (93)	6/6 (100)	2/2 (100)	28/28 (100)	93/93 (100)	14/14 (100)	3/3 (100)	84/85 (99)	26/29 (90)	5/5 (100)	2/2 (100)	52/55 (95)
	VEGF	13/14 (93)	6/6 (100)	2/2 (100)	26/28 (93)	87/93 (94)	13/14 (93)	2/3 (67)	76/85 (89)	28/29 (97)	5/5 (100)	2/2 (100)	54/55 (98)
	Periostin	14/14 (100)	5/6 (100)	2/2 (100)	27/28 (96)	65/77 (84)	11/14 (79)	2/3 (67)	45/54 (83)	21/29 (72)	2/5 (40)	2/2 (100)	36/55 (65)
	Elastin	14/14 (100)	6/6 (100)	2/2 (100)	28/28 (100)	77/78 (99)	14/14 (100)	2/2 (100)	50/50 (100)	13/18 (72)	3/3 (100)	2/2 (100)	36/47 (77)
	SADAM33	13/14 (93)	6/6 (100)	2/2 (100)	26/28 (93)	47/47 (100)	12/13 (92)	2/2 (100)	20/25 (80)	7/14 (50)	3/3 (100)	2/2 (100)	34/45 (76)

NA=Data not available due to lack of datapoints. Data shown as n/total n (%).

	Biomarker	Ecuador (n=116)				Uganda (n=68)			
		EA (%)	NA (%)	MGA (%)	PGA (%)	EA (%)	NA (%)	MGA (%)	PGA (%)
Inflammation	IL-1β	30/31 (97)	6/7 (86)	5/5 (100)	64/67 (96)	14/15 (93)	26/26 (100)	7/7 (100)	20/20 (100)
	IL-6	19/31 (61)	6/7 (86)	5/5 (100)	48/67/ (72)	5/15 (33)	18/26 (69)	5/7 (71)	8/20 (40)
	IL-8	31/31 (100)	6/7 (86)	5/5 (100)	66/67 (99)	15/15 (100)	26/26 (100)	7/7 (100)	20/20 (100)
	IL-13	1/31 (3)	0/7 (0)	1/5 (20)	1/67 (1)	0/15 (0)	0/26 (0)	0/7 (0)	4/20 (20)
	Neutrophil Elastase	31/31 (100)	6/6 (100)	5/5 (100)	64/64 (100)	15/15 (100)	26/26 (100)	7/7 (100)	20/20 (100)
	ECP	NA	NA	NA	NA	11/11 (100)	23/23 (100)	6/6 (100)	16/16 (100)
	PGD-2	NA	NA	NA	NA	11/11 (100)	22/22 (100)	6/6 (100)	16/16 (100)
	Histamine	NA	NA	NA	NA	11/11 (100)	23/23 (100)	6/6 (100)	16/16 (100)
Neural	Neurokinin A	23/29 (79)	4/6 (67)	5/5 (100)	44/59 (75)	15/15 (100)	24/26 (92)	7/7 (100)	19/20 (95)
	Substance P	8/27 (30)	3/5 (60)	2/5 (40)	19/48 (40)	9/15 (60)	18/24 (75)	5/7 (71)	12/20 (60)
	Nociceptin	18/29 (62)	4/6 (67)	4/5 (80)	47/60 (78)	15/15 (100)	26/26 (100)	7/7 (100)	20/20 (100)
	NGF-β	0/31 (0)	0/7 (0)	0/5 (0)	0/67 (0)	0/15 (0)	0/26 (0)	0/7 (0)	0/20 (0)
	BDNF	6/31 (19)	1/7 (14)	4/5 (80)	8/67 (12)	3/15 (20)	7/26 (27)	4/7 (57)	4/20 (20)
Remodelling	MMP-1	8/31 (26)	0/7 (0)	1/5 (20)	26/67 (39)	13/15 (87)	23/26 (88)	7/7 (100)	20/20 (100)
	MMP-9	31/31 (100)	6/7 (86)	5/5 (100)	66/67 (99)	15/15 (100)	26/26 (100)	7/7 (100)	20/20 (100)
	TIMP-1	31/31 (100)	6/7 (86)	5/5 (100)	66/67 (99)	15/15 (100)	26/26 (100)	7/7 (100)	20/20 (100)
	VEGF	30/31 (97)	6/7 (86)	5/5 (100)	65/67 (97)	13/15 (100)	26/26 (100)	7/7 (100)	19/20 (95)
	Periostin	20/31 (65)	6/6 (100)	5/5 (100)	35/65 (54)	15/15 (100)	20/26 (77)	5/7 (71)	18/20 (90)
	Elastin	13/15 (87)	2/2 (100)	3/3 (100)	26/28 (93)	15/15 (100)	26/26 (100)	7/7 (100)	20/20 (100)
	SADAM33	14/15 (93)	2/2 (100)	3/3 (100)	24/27 (89)	7/15 (47)	25/25 (100)	6/6 (100)	16/20 (80)

NA=Data not available due to lack of datapoints. Data shown as n/total n (%).

Appendix 6: Sputum mediator levels in asthma vs controls overall

	Biomarker	Asthma (n=527)	controls (n=191)
Inflammation	IL-1β	120.2 (48.0 – 368.9)	96.1 (37.8 – 234.6) **
	IL-6	69.1 (41.3 – 199.1)	69.3 (41.3 – 158.5)
	IL-8	954.4 (458.6 – 2033.0)	909.6 (417.6 – 1848.0)
	Histamine	4905.0 (2475.0 – 7686.0)	5297.0 (992.3 – 8678.0)
	Neutrophil elastase (ng/mL	1420.0 (495.0 – 3530.0)	1220.0 (548.0 – 3110.0)
	ECP (ng/mL)	853.9 (156.0 – 2780.0)	189.4 (54.0 – 976.0) ***
	PGD-2	919.0 (496.4 – 1808.0)	692.8 (439.5 – 1101.0) *
	IL-13	24/527 (4.55%)	13/191 (6.81%)
Neural	Neurokinin A	290.0 (69.4 – 775.7)	290.6 (72.3 – 857.7)
	Substance P	43.9 (43.9 – 364.8)	43.92 (43.9 – 271.7)
	Nociceptin (ng/mL)	79.0 (11.6 – 383.4)	31.5 (8.6 – 221.5) #
	NGF-β	29/427 (6.79%)	14/139 (10.01%)
	BDNF	75/518 (14.48%)	6/182 (3.30%) ***
Remodelling	MMP-1	44.37 (44.37 – 224.1)	44.37 (44.37 – 119.6) *
	MMP-9	1526 (600.7 – 4293)	1809.0 (678.4 – 4562.0)
	TIMP-1 (ng/mL)	84.4 (26.4 – 396.6)	78.6 (33.2 – 469.5)
	VEGF	1174.0 (375.1 – 3485.0)	663.6 (209.7 – 2218.0) ***
	Periostin	3420.0 (900.0 – 13590.0)	2025.0 (877.5 – 8640.0) *
	Elastin	15692.0 (6855.0 – 26660.0)	14580.0 (7525.0 – 20033.0)
	SADAM33	15016.0 (3181.0 – 40455.0)	10611.0 (2528.0 – 26562.0)

Data in pg/mL unless indicated otherwise. Data showing median IQR or detectability (%). P-value calculated using Mann-Whitney U test or Chi-square. *****: P≤0.05, ******: P<0.01**, *****: P<0.001, **#:** P<0.1

Appendix 7: Sputum mediator levels in EA, NEA and controls overall

	Analyte	EA (n=208)	NEA (n=319) †	Controls (n=191) ‡	Kruskal-Wallis P- value
Inflammatory	IL-1β	109.2 (47.57 – 284.6)	143.2 (50.04 – 401.9) ‡	96.1 (37.8 – 234.6)	0.0068
	IL-6	70.3 (41.32 – 165.6)	69.1 (41.32 – 237.1)	69.3 (41.3 – 158.5)	0.7611
	IL-8	897.4 (410.3 – 1487) †	1083 (544.8 – 2628)	909.6 (417.6 – 1848)	0.0012
	Neutrophil Elastase (ng/mL)	1100 (359.0 – 3220) †	1590 (624.8 – 3830)	1221 (547.7 – 3112)	0.0199
	ECP (ng/mL)	1300 (340.5 – 3590) ‡	444.8 (100.5 – 1590) ‡	188.9 (53.9 – 976.1)	<0.0001
	PGD-2	1013 (631.6 – 2054)	837.8 (465.5 – 1703)	692.8 (439.5 – 1101)	0.0231
	Histamine	5441 (2862 – 7760)	4356 (1998 – 7632)	5297 (992.3 – 8678)	0.2237
	IL-13	14/201 (6.9%)	15/313 (4.7%)	14/177 (7.9%)	0.3412
Neural	Neurokinin A	292.9 (67.7 – 891.5)	282.2 (69.4 – 734.6)	290.6 (72.3 – 857.7)	0.7829
	Substance P	43.9 (43.9 – 318.1)	43.9 (43.9 – 373.2)	43.9 (43.9 – 271.7)	0.1079
	Nociceptin (ng/mL)	66.4 (11.5 – 418.5)	82.6 (11.2 – 375.3)	31.5 (8.6 – 221.6)	0.2164
	NGF-β	19/151 (12.6%)	25/275 (9.1%)	20/139 (14.3%)	0.2342
	BDNF	31/201 (15.4%) ‡	43/315 (13.7%) ‡	7/182 (3.8%)	0.0011
Remodelling	MMP-1	44.4 (44.4 – 173.8)	44.4 (44.4 – 246.4)	44.4 (44.4 – 119.6)	0.0385
	MMP-9	1452 (576.5 – 3656)	1575 (633.0 – 4653)	1809 (678.4 – 4562)	0.3890
	TIMP-1 (ng/mL)	75.6 (22.1 – 237.2)	88.4 (27.2 – 699.3)	78.6 (33.2 – 469.5)	0.1601
	VEGF	1379 (480.0 – 2772) ‡	1089 (360.6 – 3854) ‡	663.6 (209.7 – 2218)	0.0038
	Periostin	6120 (1170 – 21353) † ‡	2520 (877.5 – 10080)	2025 (877.5 – 8640)	<0.0001
	Elastin	15101 (6051 – 27588)	15884 (8003 – 26325)	14580 (7525 – 20033)	0.3061
	SADAM33	11028 (140.6 – 32692)	17214 (4581 – 46133)	10611 (2528 – 26562)	0.0202

All data presented in pg/mL unless indicated otherwise. Data presented as median IQR or detectability (%). P-value calculated with Kruskal-Wallis or Chi-Square. †: significantly ($p<0.05$) different compared to NEA, ‡: significantly ($p<0.05$) different compared to control.

Appendix 8: Multivariate analysis of mediator levels within countries in asthma, controls, asthma sub-groups and asthma phenotype

	New Zealand	Asthma vs Control	EA vs Control	NEA vs Control	EA vs NEA	EA vs MGA	EA vs NA	EA vs PGA
	Continuous Variables							
Inflammatory	IL-1β	-89.6 (-479.3 – 300.2)	98.3 (-360.5 – 557.1)	-257.5 (-703.4 – 188.4)	355.8 (-101.4 – 813.0)	NA	2423 (1532 – 3313) ***	5.62 (-456.2 – 467.4)
	IL-6	-10.0 (-60.1 – 40.1)	12.2 (-46.2 – 70.6)	-30.8 (-87.9 – 26.3)	43.0 (-14.8 – 100.9)	NA	62.6 (-54.6 – 179.8)	43.3 (-17.8 – 104.4)
	IL-8	217.5 (-68.8 – 503.9)	383.7 (50.2 – 717.1) *	63.7 (-262.6 – 389.9)	320.0 (-10.2 – 650.3) #	NA	858.5 (200.4 – 1516) *	288.7 (-54.3 – 631.7)
	Neutrophil Elastase	-2816 (-9172 – 3540)	-159.4 (-7339 – 7021)	-5767 (-13187 – 1652)	5608 (-1632 – 12849)	NA	30089 (17809 – 4269)***	-885.5 (-8345 – 6574)
	ECP	1513 (-11000 – 14000)	2065 (-11977 – 16107)	1084 (-13681 – 15849)	981.2 (-13219 – 15182)	NA	-4707 (-29658 – 20244)	2241 (-13239 – 17723)
	PGD-2	-2283 (-3907 – -660.2) ***	-1980 (-3781 – -179.1) *	-2619 (-4513 – -726.3) **	639.4 (-1121 – 2400)	NA	1892 (-667.7 – 4452)	260.2 (-1738 – 2259)
	Histamine	-1187 (-3076 – 701.4)	-1738 (-3860 – 383.7)	-491.9 (-2722 – 1738)	-1246 (-3400 – 907.9)	NA	-570.9 (-4380 – 3238)	-1395 (-3741 – 949.8)
Neural	Neurokinin A	475.7 (-79.7 – 1031) #	326.3 (-326.9 – 979.4)	602.5 (-24.8 – 1229) #	-276.2 (-909.5 – 357.0)	NA	NA	-289.1 (-927.1 – 348.9)
	Substance P	-2.88 (-21.56 – 15.78)	6.12 (-15.9 – 28.3)	-8.31 (-28.9 – 12.2)	14.4 (-6.52 – 35.4)	NA	NA	14.9 (-6.20 – 36.1)
	Noiciceptin	-74.7 (-186.9 – 37.3)	-103.1 (-230.4 – 24.0) #	-35.1 (-168.1 – 97.8)	-68.0 (-200.4 – 64.4)	NA	NA	-81.7 (-215.2 – 51.8)
Remodelling	MMP-1	-9.95 (-52.8 – 32.9)	-1.20 (-49.4 – 47.0)	-11.4 (-55.8 – 33.0)	10.2 (-37.1 – 57.6)	NA	NA	17.8 (-29.2 – 64.7)
	MMP-9	26103 (-24404 – 76611)	32710 (-26493 – 91915)	20413 (-37506 – 78333)	12297 (-46335 – 70930)	NA	105175 (-13152 – 223503) #	-3255 (-27106 – 91835)
	TIMP-1	278.7 (-10.6 – 598.1) #	348.9 (-25.1 – 723.1) #	214.4 (-151.6 – 580.4)	134.6 (-235.9 – 505.1)	NA	762.7 (15.6 – 1509) *	21.1 (-368.3 – 410.4)
	VEGF	121.5 (-409.9 – 653.0)	263.9 (-356.7 – 884.6)	-5.79 (-612.9 – 601.4)	269.7 (-344.9 – 884.4)	NA	1852 (623.4 – 3081) **	-14.2 (-375.3 – 860.1)
	Periostin	-18721 (-35355 – -2088) *	-27425 (-46215 – -8635) ***	-8753 (-28160 – 10653)	-18671 (-37616 – 272.7) #	NA	-7467 (-41343 – 26408)	-23307 (-43797 – -2817) *
	Elastin	34961 (-12966 – 82890)	34697 (-19290 – 88686)	35420 (-21309 – 92150)	-722.3 (-55336 – 53892)	NA	19390 (-77185 – 115967)	-6761 (-66229 – 52705)
	SADAM33	8887 (-7746 – 25521)	13868 (-4579 – 32316)	2620 (-16806 – 22047)	11248 (-6959 – 29456)	NA	16608 (-10194 – 43411)*	7740 (-13190 – 28672)
	Dichotomous variables							
	IL-13	0.82 (0.34 – 1.96)	0.75 (0.28 – 2.04)	0.87 (0.32 – 2.38)	0.86 (0.33 – 2.25)	5.41 (0.42 – 68.5)	2.25 (0.50 – 10.1)	0.71 (0.23 – 2.13)
	NGF-β	0.64 (0.32 – 1.28)	0.62 (0.27 – 1.41)	0.62 (0.29 – 1.34)	0.99 (0.45 – 2.16)	NA	NA	1.06 (0.49 – 2.35)
	BDNF	NA	NA	NA	NA	NA	NA	NA

New Zealand: EA: Eosinophilic Asthmatics (EA + MGA), NEA: Non-Eosinophilic Asthmatics (NA + PGA), EA: Eosinophilic asthmatics, NA: Neutrophilic asthmatics, MGA: Mixed Granulocytic asthmatics, PGA: Paucigranulocytic asthmatics. All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. #: p<0.1, *:p<0.05, **: p<0.01, ***:p<0.001.

UK: EA: Eosinophilic Asthmatics (EA + MGA), NEA: Non-Eosinophilic Asthmatics (NA + PGA), EA: Eosinophilic asthmatics, NA: Neutrophilic asthmatics, MGA: Mixed Granulocytic asthmatics, PGA: Paucigranulocytic

	United Kingdom	Asthma vs Control	EA vs Control	NEA vs Control	EA vs NEA	EA vs MGA	EA vs NA	EA vs PGA
	Continuous Variables							
Inflammatory	IL-1β	-167.3 (-403.3 – 68.7)	-56.6 (-357.1 – 243.8)	-220.1 (-471.8 – 31.6) #	163.5 (-112.5 – 439.4)	NA	237.5 (-192.7 – 667.8)	276.9 (-12.3 – 566.3) #
	IL-6	-243.4 (-533.2 – 46.4) #	-8.40 (-369.6 – 352.9)	-355.5 (-658.1 – -52.9) *	347.1 (15.3 – 678.9) *	NA	423.2 (-117.5 – 963.9)	351.6 (-11.9 – 715.2) #
	IL-8	-139.9 (-782.8 – 503.0)	247.6 (-565.5 – 1060)	-324.8 (-1005 – 356.2)	572.4 (-174.3 – 1319)	NA	926.3 (-281.9 – 2134)	631.3 (-499.9 – 1198)
	Neutrophil Elastase	-3511 (-12481 – 5458)	-9119 (-20448 – 2209)	-834.6 (-10323 – 8654)	-8284 (-18689 – 2119)	NA	1319 (-10990 – 13629)	1085 (-7193 – 9363)
	ECP	-10244 (-38315 – 17826)	-26588 (-60712 – 7535)	-86.5 (-30404 – 30230)	-26502 (-58920 – 5916)	NA	-34683 (-87021 – 17654)	-31187 (-67256 – 4881) #
	PGD-2	-212.9 (-474.6 – 48.9)	-361.3 (-676.6 – -46.2) *	-113.2 (-398.2 – 171.8)	-248.1 (-551.3 – 54.9)	NA	-370.7 (-853.4 – 111.9)	-173.6 (-511.6 – 164.5)
	Histamine	1332 (-486.6 – 3151)	1695 (-564.9 – 3955)	1106 (-901.1 – 3115)	588.3 (-1558 – 2735)	NA	993.5 (-2494 – 4481)	721.6 -1682 – 3125)
Neural	Neurokinin A	-3701 (-8189 – 785.7)	-7987 (-13477 – -2497) **	-1673 (-6275 – 2928)	-6313 (-11320 – -1307) *	NA	-5262 (-12632 – 2107)	-3325 (-8400 – 1749)
	Substance P	-255.6 (-540.3 – 29.2) #	-162.5 (-526.1 – 201.1)	-299.6 (-604.4 – 5.15) #	137.2 (-194.4 – 468.7)	NA	354.6 (-171.4 – 880.7)	78.4 (-283.9 – 440.7)
	Noiciceptin	-317.1 (-577.9 – -56.3) *	-535.7 (-858.5 – -212.9) **	-213.6 (-484.2 – 56.9)	-322.1 (-616.4 – -27.8) *	NA	-570.5 (-1035 – -106.0) *	-311.3 (-903.5 – 232.2) #
Remodelling	MMP-1	-196.9 (-489.5 – 95.8)	-186.2 (-562.6 – 190.3)	-201.9 (-517.2 – 113.3)	15.8 (-329.9 – 361.5)	NA	532.2 (16.7 – 1047) *	99.9 (-246.7 – 446.5)
	MMP-9	-6002 (-25450 – 13445)	1621 (-23219 – 26463)	-9640 (-30447 – 11165)	11262 (-11551 – 34076)	NA	4968 (-32165 – 42101)	13782 (-11189 – 38753)
	TIMP-1	123.9 (-664.2 – 912.2)	61.2 (-952.6 – 1074)	153.9 (-695.1 – 1003)	-92.8 (-1023 – 838.2)	NA	-146.9 (-1636 – 1342)	197.1 (-804.4 – 1198)
	VEGF	-732.6 (-1709 – 244.0)	-1139 (-2385 – 106.7) #	-538.4 (-1582 – 505.5)	-601.2 (-1745 – 543.4)	NA	-959.3 (-2823 – 905.0)	-569.5 (-1823 – 684.2)
	Periostin	-23643 (-58006 – 10719)	-53012 (-95694 – -10330) *	-9627 (-45376 – 26122)	-43385 (-82583 – -4186) *	NA	-20992 (-84183 – 42199)	-52875 (-95370 – -10380) *
	Elastin	-8615 (-24514 – 7284)	-14720 (-34927 – 5487)	-5664 (-22675 – 11345)	-14720 (-34927 – 5487)	NA	733.8 (-29252 – 30720)	-9604 (-29772 – 10563)
	SADAM33	-50445 (-206462 – 105571)	21075 (-176599 – 218749)	-85009 (-251411 – 81392)	106084 (-74698 – 286867)	NA	32247 (-261325 – 325821)	131865 (-65585 – 329317)
	Dichotomous Variables							
	IL-13	NA	NA	NA	NA	NA	NA	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA
	BDNF	NA	NA	NA	NA	NA	NA	NA

asthmatics. All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. #: p<0.1, *:p<0.05, **: p<0.01, ***:p<0.001.

	Brazil	Asthma vs Control	EA vs Control	NEA vs Control	EA vs NEA	EA vs MGA	EA vs NA	EA vs PGA
	Continuous Variables							
Inflammatory	IL-1β	68.6 (-574.5 – 711.7)	335.2 (-362.9 – 1033)	-56.8 (-706.8 – 593.1)	392.0 (-32.4 – 816.5) #	NA	322.7 (-617.5 – 1262)	414.7 (-30.8 – 860.1) #
	IL-6	173.9 (-46.5 – 394.2)	224.2 (-17.9 – 466.3) #	150.2 (-75.2 – 375.6)	73.9 (-73.2 – 221.2)	NA	415.2 (98.7 – 731.7) *	55.1 (-94.9 – 205.1)
	IL-8	-2381 (-5315 – 553.1)	-2232 (-5471 – 1006)	-2451 (-5467 – 564.2)	219.5 (-1749 – 2188)	NA	4341 (78.9 – 8603) *	-80.2 (-2099 – 1939)
	Neutrophil Elastase	-632.5 (-2338 – 1073)	-134.3 (-2013 – 1745)	-848.7 (-2584 – 887.5)	714.5 (-431.3 – 1860)	NA	1652 (-844.7 – 4150)	751.5 (-44.6 – 1947)
	ECP	-1627 (-3554 – 300.1) #	-3218.9 (-3444 – -1068) **	-962.6 (-2787 – 862.3)	-2256 (-3444 – -1068) ***	NA	NA	-2352 (-3598 – -1107) ***
	PGD-2	NA	NA	NA	NA	NA	NA	NA
	Histamine	-750.2 (-3466 – 1965)	-864.7 (-3851 – 2122)	-709.2 (-3478 – 2059)	-155.4 (-1783 – 1472)	NA	NA	-227.5 (-1949 – 1494)
Neural	Neurokinin A	-1603 (-8245 – 5037)	-66.6 (-7600 – 7466)	-2128 (-8889 – 4632)	2026 (-2678 – 6802)	NA	NA	2291 (-2723 – 7306)
	Substance P	80.2 (-117.9 – 278.2)	92.6 (-136.7 – 321.8)	75.9 (-127.3 – 279.1)	16.7 (-135.7 – 169.1)	NA	NA	9.80 (-146.6 – 166.2)
	Noiciceptin	-255.7 (-475.3 – 23.8) #	-114.8 (-390.7 – 161.1)	-271.2 (-522.9 – -19.6) *	156.4 (-18.5 – 331.3) #	NA	NA	163.1 (-21.0 – 347.1) #
Remodelling	MMP-1	-185.5 (-499.4 – 128.4)	-255.2 (-600.1 – 89.7)	-152.7 (-473.8 – 168.4)	-102.5 (-312.2 – 107.2)	NA	-154.5 (-605.7 – 296.7)	-39.1 (-252.9 – 174.7)
	MMP-9	-8212 (-22429 – 6004)	-7098 (-22785 – 8588)	-8737 (-23342 – 5868)	1638 (-7899 – 11177)	NA	18858 (-1912 – 39629) #	294.4 (-9546 – 10135)
	TIMP-1	181.5 (-141.5 – 504.6)	279.3 (-74.2 – 632.8)	135.6 (-193.6 – 464.7)	143.7 (-74.2 – 358.7)	NA	307.7 (-167.1 – 782.6)	127.7 (-97.3 – 352.7)
	VEGF	1726 (-1998 – 5451)	2191 (-1914 – 6297)	1507 (-2315 – 5330)	684.3 (-1812 – 3180)	NA	2297 (-3216 – 7811)	711.2 (-1901 – 3323)
	Periostin	-5572 (-13952 – 808)	-8619 (-17813 – 574.7) #	-4249 (-12742 – 4243)	-4369 (-9974 – 1235)	NA	-6143 (-18438 – 6151)	-4237 (-10126 – 1650)
	Elastin	-15594 (-34818 – 3628)	-12044 (-34296 – 10206)	-16580 (-36127 – 2966) #	4535 (-9603 – 18674)	NA	NA	4206 (-10804 – 19217)
	SADAM33	-21636 (-57740 – 14468)	-19702 (-62924 – 23519)	-22096 (-58890 – 14697)	2394 (-26519 – 31308)	NA	NA	753.1 (-30398 – 31904)
	Dichotomous Variables							
	IL-13	NA	NA	NA	NA	NA	NA	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA
	BDNF	NA	NA	NA	0.96 (0.34 – 2.69)	NA	NA	1.26 (0.39 – 4.00)

Brazil: EA: Eosinophilic Asthmatics (EA + MGA), NEA: Non-Eosinophilic Asthmatics (NA + PGA), EA: Eosinophilic asthmatics, NA: Neutrophilic asthmatics, MGA: Mixed Granulocytic asthmatics, PGA: Paucigranulocytic asthmatics. All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. #: p<0.1, *:p<0.05, **: p<0.01, ***:p<0.001.

	Uganda	Asthma vs Control	EA vs Control	NEA vs Control	EA vs NEA	EA vs MgA	EA vs NA	EA vs PGA
	Continuous Variables							
Inflammatory	IL-1β	-328.4 (-1099 – 442.7)	-508.9 (-1422 – 404.5)	-241.3 (-1049 – 566.8)	-267.7 (-988.1 – 452.8)	1079 (-179.8 – 2338) #	55.6 (-838.5 – 949.7)	129.7 (-825.1 – 1084)
	IL-6	-118.7 (-576.8 – 339.4)	-160.4 (-704.7 – 383.9)	-98.6 (-580.2 – 382.9)	-61.7 (-491.0 – 367.6)	345.9 (-409.6 – 1101)	-53.8 (-590.2 – 482.6)	197.4 (-375.4 – 770.3)
	IL-8	-256.2 (-1147 – 634.7)	79.9 (-969.9 – 1129.7)	-418.2 (-1346 – 510.5)	498.0 (-329.9 – 1325)	1336 (-92.7 – 2766) #	1170 (155.8 – 2185) *	615.1 (-614.9 – 1654)
	Neutrophil Elastase	-1277 (-4150 – 1596)	-1890 (-5296 – 1516)	-981.9 (-3995 – 2031)	-908.4 (-3595 – 1778)	8713 (4428 – 12997) ***	2882 (-159.9 – 5923) #	672.4 (-2576 – 3920)
	ECP	-713.2 (-2548 – 1122)	-2291 (-4356 – -226.9) *	-44.0 (-1848 – 1760)	-2247 (-3826 – 669.2) **	2869 (144.6 – 5594) *	-1236 (-3194 – 721.7)	-1141 (-3272 – 989.7)
	PGD-2	-82.9 (-652.4 – 486.7)	-240.5 (-910.3 – 429.3)	-13.8 (-604.7 – 577.2)	-226.8 (-731.4 – 277.9)	643.5 (-241.1 – 1528)	26.9 (-611.8 – 665.6)	-20.0 (-712.1 – 672.1)
	Histamine	-970.6 (-3462 – 1521)	-1617 (-4555 – 1321)	-693.7 (-3278 – 1891)	-923.4 (-3131 – 1284)	2754 (-1063 – 6573)	-626.6 (-3370 – 2117)	1170 (-1816 – 4157)
Neural	Neurokinin A	1926 (-6465 – 10317)	4466 (-5452 – 14385)	702.4 (-8071 – 9476)	3764 (-4058 – 11587)	1498 (-12407 – 15403)	3290 (-6582 – 13162)	5587 (-4955 – 16129)
	Substance P	-312.6 (-739.1 – 113.8)	-333.3 (-840.2 – 173.6)	-302.7 (-751.1 – 145.7)	-30.6 (-430.4 – 369.2)	610.4 (-77.8 – 1298) #	310.8 (-177.8 – 799.4)	-24.4 (-546.2 – 497.4)
	Noiciceptin	13.2 (-295.7 – 321.9)	-126.5 (-488.9 – 235.9)	80.5 (-240.1 – 401.1)	-207.0 (-492.9 – 78.8)	740.3 (259.8 – 1220) **	34.7 (-306.5 – 375.9)	37.9 (-326.4 – 402.3)
Remodelling	MMP-1	-16.9 (-375.7 – 53.8)	-220.2 (-474.3 – 33.8) #	-132.4 (-357.1 – 92.4)	-87.8 (-288.2 – 112.6)	374.2 (28.8 – 719.6) *	-1.31 (-246.5 – 243.9)	85.2 (-176.6 – 347.0)
	MMP-9	24368 (-72204 – 23467)	-1315 (-57354 – 54722)	-35475 (-85045 – 14095)	34159 (-10037 – 78356)	634.8 (-76331 – 77600)	54180 (-463.3 – 108824) #	7078 (-51274 – 65431)
	TIMP-1	154.7 (-994.6 – 1304)	-91.8 (-1454 – 1270)	273.5 (-931.7 – 1478)	-365.3 (-1439 – 709.3)	1931 (74.7 – 3788) *	442.1 (-876.3 – 1760)	30.6 (-1377 – 1438)
	VEGF	-870.9 (-2098 – 356.9)	-1011 (-2469 – 447.5)	-803.4 (-2093 – 486.9)	-207.8 (-1358 – 942.7)	1841 (-162.6 – 3845) #	298.7 (-1124 – 1721)	532.2 (-987.4 – 2051))
	Periostin	-3883 (-14732 – 6965)	-9986 (-22626 – 2653)	-943.0 (-12124 – 10238)	-9043 (-19012 – 925.7) #	-14812 (-32225 – 2601) #	-14888 (-27251 – -2525) *	-12554 (-25757 – 647.6) #
	Elastin	-14308 (-58455 – 29837)	-36156 (-87830 – 15517)	-3782 (-49491 – 41926)	-32373 (-73128 – 8380)	100330 (31529 – 169131) **	5169 (-43677 – 54016)	-5765 (-57928 – 46397)
	SADAM33	-18514 (-58629 – 21599)	-9792 (-57901 – 38316)	-22500 (-64527 – 19526)	12708 (-25644 – 51061)	72690 (5587 – 139793) *	46288 (519.5 – 92057) *	17661 (-30830 – 66153)
	Dichotomous Variables							
	IL-13	NA	NA	NA	NA	NA	NA	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA
	BDNF	0.20 (0.02 – 1.69)	0.43 (0.12 – 1.60)	0.23 (0.03 – 1.97)	0.37 (0.13 – 1.05) #	8.22 (1.01 – 66.7) *	1.82 (0.37 – 8.90)	1.27 (0.22 – 7.40)

Uganda: EA: Eosinophilic Asthmatics (EA + MGA), NEA: Non-Eosinophilic Asthmatics (NA + PGA), EA: Eosinophilic asthmatics, NA: Neutrophilic asthmatics, MGA: Mixed Granulocytic asthmatics, PGA: Paucigranulocytic asthmatics. All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. #: p<0.1, *:p<0.05, **: p<0.01, ***:p<0.001.

	Ecuador	Asthma vs Control	EA vs Control	NEA vs Control	EA vs NEA	EA vs MGA	EA vs NA	EA vs PGA
	Continuous Variables							
Inflammatory	IL-1β	-559.3 (-1682 – 564.3)	-34.3 (-1371 – 1302)	-830.4 (-2011 – 350.9)	796.1 (-312.1 – 1904)	944.2 (-1332 – 3221)	6521 (4585 – 8457) ***	284.4 (-739.4 – 1308)
	IL-6	-43.7 (-491.7 – 404.3)	148.5 (-385.2 – 682.3)	-143.0 (-614.7 – 328.6)	291.6 (-150.9 – 733.9)	264.8 (-704.3 – 1233)	2063 (1239 – 2886) ***	129.4 (-306.4 – 565.1)
	IL-8	197.7 (5431 – 5826)	-630.7 (-7373 – 6112)	625.7 (-5333 – 6584)	-1256 (-6845 – 4333)	27089 (14617 – 39561) ***	1800 (-8802 – 12403)	2270 (-3337 – 7878)
	Neutrophil Elastase	-709.7 (-1759 – 340.4)	-606.1 (-1836 – 623.9)	-767.7 (-1879 – 344.4)	161.6 (-828.1 – 1151)	2266 (147.5 – 4385) *	4390 (2468 – 6312) ***	52.7 (-908.9 – 1014)
	ECP	NA	NA	NA	NA	NA	NA	NA
	PGD-2	NA	NA	NA	NA	NA	NA	NA
	Histamine	NA	NA	NA	NA	NA	NA	NA
Neural	Neurokinin A	-181.1 (-4741 – 478)	-2177 (-7237 – 2881)	980.9 (-3728 – 5690)	-3158 (-6757 – 439.9)	18663 (11210 – 26116) ***	72.6 (-6682 – 6827)	-672.9 (-4161 – 2816)
	Substance P	-69.8 (-249.0 – 109.4)	-85.0 (-282.1 – 112.0)	-60.1 (-247.2 – 127.0)	-24.9 (-155.9 – 106.0)	417.1 (136.7 – 697.5) **	133.7 (-137.9 – 405.3)	25.3 (-109.8 – 160.4)
	Noiciceptin	-149.8 (-491.0 – 191.4)	-115.1 (-500.7 – 270.5)	-169.7 (-526.9 – 187.6)	54.6 (-223.9 – 333.0)	-16.7 (-657.9 – 624.4)	-54.2 (-635.3 – 526.8)	63.6 (-235.2 – 362.4)
Remodelling	MMP-1	104.2 (-16.0 – 224.4) #	114.2 (-29.8 – 258.3)	98.9 (-28.3 – 226.3)	15.2 (-104.2 – 134.7)	-23.4 (-307.0 – 260.2)	-71.8 (-312.9 – 169.3)	21.7 (-105.8 – 149.3)
	MMP-9	731.2 (-5112 – 6575)	-201.8 (-7201 – 6797)	1213 (-4972 – 7398)	-1415 (-7217 – 4387)	24888 (11754 – 38021) ***	3624 (-7540 – 14789)	1572 (-4332 – 7478)
	TIMP-1	283.4 (51.7 – 515.1) *	329.4 (52.0 – 606.8) *	259.6 (14.5 – 504.7) *	69.8 (-160.1 – 299.8)	278.2 (-249.6 – 805.9)	-475.8 (-924.4 – -27.1) *	171.2 (-66.1 – 408.5)
	VEGF	-750.2 (-3848 – 2348)	122.4 (-3581 – 3826)	-1200 (-4474 – 2072)	1323 (-1747 – 4393)	6427 (-797.8 – 13653) #	1374 (-4768 – 7517)	2236 (-1012 – 5485)
	Periostin	-754.6 (-4415 – 2906)	-2728 (-6974 – 1517)	325.3 (-3499 – 4150)	-3054 (-6459 – 350.9) #	-2454 (-10489 – 5580)	-3914 (-11202 – 3373)	-3312 (-6945 – 320.6) #
	Elastin	-2416 (-15899 – 11066)	-1017 (-15979 – 13944)	-3281 (-17414 – 10851)	2263 (-7841 – 12369)	NA	NA	6348 (-3084 – 15781)
	SADAM33	20673 (-35251 – 76599)	14331 (-47517 – 76181)	24819 (-33958 – 83598)	-10488 (-52551 – 31575)	NA	NA	-7666 (-50737 – 35404)
	Dichotomous Variables							
	IL-13	NA	NA	NA	NA	NA	NA	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA
	BDNF	0.75 (0.22 – 2.52)	NA	1.14 (0.31 – 4.25)	NA	11.16 (1.00 – 124.6) *	0.62 (0.06 – 6.12)	0.53 (0.17 – 1.69)

Ecuador: EA: Eosinophilic Asthmatics (EA + MGA), NEA: Non-Eosinophilic Asthmatics (NA + PGA), EA: Eosinophilic asthmatics, NA: Neutrophilic asthmatics, MGA: Mixed Granulocytic asthmatics, PGA: Paucigranulocytic asthmatics. All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. #: p<0.1, *:p<0.05, **: p<0.01, ***:p<0.001.

Appendix 9: Multivariate analysis of mediator levels between countries in EA, NA, MGA, and PGA

	Eosinophilic Asthmatics (EA)										
		NZ vs UK	NZ vs BRA	NZ vs UGA	NZ vs ECU	UK vs BRA	UK vs UGA	UK vs ECU	BRA vs UGA	BRA vs ECU	UGA vs ECU
	Continuous Variables										
Inflammatory	IL-1β	264.7 (-305.4 – 834.8)	167.1 (-135.3 – 469.6)	495.4 (154.9 – 835.8) *	138.6 (-122.1 – 399.3)	-97.5 (-590.9 – 395.8)	230.7 (-382.9 – 844.3)	-126.1 (-773.8 – 521.5)	328.2 (-76.2 – 732.6)	-28.6 (-414.1 – 356.9)	-356.8 (-732.6 – 76.2)
	IL-6	25.8 (-198.9 – 250.4)	45.7 (-73.4 – 164.9)	117.4 (-16.7 – 251.5)	32.3 (-70.4 – 135.0)	19.9 (-174.4 – 214.4)	91.6 (-150.1 – 333.4)	6.56 (-248.6 – 261.7)	71.7 (-87.7 – 230.9)	-13.4 (-165.3 – 138.5)	-85.1 (-241.6 – 71.4)
	IL-8	-2097 (-4619 – 423.6)	1196 (-140.8 – 2534)	-8.34 (-1513 – 1497)	1834 (681.5 – 2987) **	3294 (1112 – 5476) **	2089 (-624.2 – 4803)	3932 (1068 – 6796) **	-1205 (-2993 – 583.4)	637.7 (-1067 – 2342)	1842 (86.0 – 3599) *
	Neutrophil Elastase	2494 (-1372 – 6362)	-400.5 (-2421 – 1620)	219.5 (-1930 – 2369)	-551.6 (-2176 – 1073)	-2895 (-6086 – 295.6)	-2275 (-6239 – 1688)	-3046 (-7288 – 1195)	620.1 (-1898 – 3138)	-151.0 (-2597 – 2295)	-771.1 (-3234 – 1692)
	ECP	47103 (-2005 – 96212)	6675 (-19109 – 32461)	-1580 (-30896 – 27735)	NA	-40427 (-81330 – 474.8)	-48683 (-100534 – 3166)	NA	-8255 (-42509 – 25997)	NA	NA
	PGD-2	7985 (-1652 – 17622)	NA	249.9 (-2941 – 3441)	NA	NA	-7735 (-15455 – -15.4) *	NA	NA	NA	NA
	Histamine	-7507 (-13274 – -1740) *	-5781 (-9024 – -2538) ***	-372.5 (-3834 – 3089)	NA	1726 (-3238 – 6690)	7135 (1019 – 13251) *	NA	5409 (1200 – 9618) *	NA	NA
Neural	Neurokinin A	3241 (-1720 – 8204)	-429.7 (-2612 – 1753)	1329 (-1156 – 3814)	969.5 (-1767 – 3706)	-3671 (-8334 – 991.4)	-1912 (-7781 – 3956)	-2272 (-9082 – 4537)	1759 (-1231 – 4749)	1399 (-2044 – 4843)	-359.9 (-3196 – 2476)
	Substance P	234.2 (-202.9 – 671.4)	115.9 (-77.9 – 309.8)	556.6 (354.4 – 758.9) ***	151.2 (-96.9 – 399.4)	-118.2 (-514.9 – 278.4)	322.4 (-192.5 – 837.3)	-82.9 (-700.2 – 534.5)	440.7 (184.3 – 697.0) ***	35.3 (-286.5 – 356.9)	-405.4 (-640.1 – -170.7) ***
	Noiciceptin	519.5 (-142.9 – 1182)	-103.7 (-392.1 – 184.7)	347.1 (28.9 – 665.3) *	114.1 (-255.5 – 483.8)	-623.2 (-1213 – -33.2)	-172.4 (-946.4 – 601.5)	-405.4 (-1317 – 507.0)	450.8 (70.1 – 831.5) *	217.8 (-245.9 – 681.5)	-232.9 (-585.8 – 119.8)
Remodelling	MMP-1	423.9 (26.5 – 821.2) *	268.9 (99.8 – 437.9) **	230.8 (22.4 – 439.3) *	-27.22 (-250.1 – 195.7)	-154.9 (-513.3 – 203.3)	-193.0 (-664.7 – 278.6)	-451.1 (-995.0 – 92.8)	-38.1 (-280.2 – 204.1)	-296.1 (-572.8 – -19.4)	-258.0 (-491.9 – -24.2) *
	MMP-9	-978.5 (-14464 – 12507)	2345 (-4808 – 9499)	2.45 (-8050 – 8055)	-2627 (-8794 – 3539)	3323 (-8346 – 14994)	980.9 (-13534 – 15496)	-1649 (-13534 – 15496)	-2343 (-11909 – 7223)	-4973 (-14093 – 4146)	-2630 (-12026 – 6766)
	TIMP-1	745.5 (-140.6 – 1631)	157.7 (-312.4 – 627.8)	557.7 (28.6 - 1086) *	213.2 (-191.9 – 618.4)	-587.8 (-1354 – 179.0)	-187.8 (-1141 – 765.9)	-532.3 (-1538 – 474.3)	400.0 (-228.5 – 1028)	55.6 (-543.7 – 654.8)	-344.5 (-961.8 – 272.9)
	VEGF	2842 (-370.1 – 6055)	2929 (1225 – 4634) ***	472.8 (-1445 – 2391)	2703 (1234 – 4173) ***	87.2 (-2693 – 2867)	-2369 (-5828 – 1088)	-138.7 (-3788 – 3511)	-2457 (-4736 –177.9) *	-225.8 (-2398 – 1946)	2231 (-7.36 – 4469)
	Periostin	63434 (-13492 – 140362)	-12139 (-52343 – 28065)	-4155 (-46916 – 38604)	-37705 (-70038 – -5373) *	-75574 (-139052 – -12096) *	-67590 (-146442 – 11260)	-101140 (-185527 – -16754) *	7983 (-42117 – 58083)	-25566 (-74228 – 23095)	-33549 (-82549 – 15449)
	Elastin	-34.7 (-30242 – 30173)	1957 (-15008 – 18923)	33480 (17232 – 49728) ***	2380 (-13782 – 18543)	1992 (-24647 – 28633)	33514 (2673 – 64356) *	2415 (-32451 – 37281)	31522 (11025 – 52019) **	422.6 (-22127 – 22972)	-31099 (-52501 – -9697) **
	SADAM33	15.8 (-82358 – 82390)	9882 (-31900 – 51664)	-5686 (-36676 – 25303)	36396 (11278 – 61514) **	9866 (-45449 – 65182)	-5702 (-73078 – 61673)	36380 (-43824 – 116584)	-15568 (-49777 – 18639)	26513 (-15856 – 68884)	42082 (8391 – 75774) *
	Dichotomous Variables										
	IL-13	NA	0.29 (0.02 – 2.92)	NA	0.33 (0.04 – 2.83)	3.04 (0.35 – 26.2)	NA	NA	NA	1.13 (0.00 – 2.68)	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	BDNF	NA	0.19 (0.00 – 12.50)	0.55 (0.06 – 5.04)	0.78 (0.13 – 4.52)	2.91 (0.22 – 39.5)	4.08 (0.11 – 155.2)	5.25 (0.78 – 344.8)	1.40 (0.21 – 9.54)	1.79 (0.19 – 16.3)	1.28 (0.22 – 7.46)

All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. *:p<0.05, **: p<0.01, ***:p<0.001.

	Neutrophilic Asthma (NA)										
		NZ vs UK	NZ vs BRA	NZ vs UGA	NZ vs ECU	UK vs BRA	UK vs UGA	UK vs ECU	BRA vs UGA	BRA vs ECU	UGA vs ECU
	Continuous Variables										
Inflammatory	IL-1β	-5383 (-19320 – 8552)	-3424 (-10852 – 4004)	-2832 (-7492 – 1826)	4080 (-649.5 – 8810)	1959 (-7871 – 11791)	2551 (-8220 – 13322)	9464 (-4261 – 23190)	591.2 (-4687 – 5870)	7504 (-100.9 – 15109)	6913 (1787 – 12039) **
	IL-6	-525.1 (-4413 – 3362)	27.1 (-2045 – 2099)	-242.5 (-1542 – 1057)	1885 (566.4 – 3205) **	552.1 (-2190 – 3294)	282.6 (-2722 – 3287)	2410 (-1418 – 6240)	-269.5 (-1742 – 1203)	1858 (-262.9 – 3980)	2128 (698.3 – 3558)
	IL-8	6467 (-287.6 – 13222)	7640 (4040 – 11241) ***	1806 (-452.2 – 4064)	2702 (410.0 – 4995) *	1173 (-3591 – 5938)	-4661 (-9881 – 559.7)	-3764 (-10417 – 2888)	-5834 (-8393 – -3276) ***	-4938 (-8624 – -1252) **	896.4 (-1588 – 3380)
	Neutrophil Elastase	-29459 (-151030 – 92111)	-30753 (-95061 – 33554)	-28198 (-68498 – 12102)	-22661 (-65514 – 20191)	-1293 (-86553 – 83965)	1261 (-92602 – 95125)	6798 (-116561 – 130158)	2555 (-42782 – 47892)	8092 (-61221 – 77405)	5536 (-42653 – 53727)
	ECP	816.1 (-3048 – 4680)	1967 (-281.6 – 4217)	-430.0 (-1667 – 807.8)	NA	1151 (-1609 – 3913)	-1246 (-4254 – 1762)	NA	-2397 (-4088 – -707.5) **	NA	NA
	PGD-2	-2801 (-16711 – 11108)	NA	-3703 (-8106 – 698.7)	NA	NA	-902.7 (-11516 – 9711)	NA	NA	NA	NA
	Histamine	-5245 (-15316 – 4825)	-7012 (-12874 – -1149) *	-806.5 (-4032 – 2419)	NA	-1766 (-8963 – 5430)	4439 (-3400 – 12279)	NA	6205 (1800 – 10611) **	NA	NA
Neural	Neurokinin A	NA	7628 (-26618 – 41874)	13985 (-24845 – 52816)	14042 (-35560 – 63645)	7628 (-26618 – 41874)	13985 (-24845 – 52816)	14042 (-35560 – 63645)	6357 (-11325 – 24041)	6414 (-19855 – 32684)	56.6 (-16774 – 16887)
	Substance P	2023 (-1024 – 5071)	761.5 (-1322 – 2845)	863.5 (-980.7 – 2707)	61.1 (-2019 – 2141)	-1262 (-3436 – 911.8)	-1160 (-3647 – 1327)	-1962 (-5051 – 1126)	102.1 (-993.4 – 1197)	-700.3 (-2310 – 910.1)	-802.4 (-1849 – 244.9)
	Noiciceptin	-466.3 (-2196 – 1263)	-391.4 (-1606 – 824.1)	136.5 (-943.6 – 1216)	-46.6 (-1258 – 1165)	74.8 (-1154 – 1304)	602.8 (-791.5 – 1997)	419.6 (-1361 – 2200)	527.9 (-107.0 – 1162)	344.8 (-598.5 – 1288)	-183.1 (-787.5 – 421.2)
Remodelling	MMP-1	757.8 (-951.6 – 2467)	50.0 (-1124 – 1224)	222.5 (-846.2 – 1291)	-6.32 (-1199 – 1186)	-707.7 (-1912 – 497.4)	-535.3 (-1912 – 841.4)	-764.1 (-2519 – 991.7)	172.4 (-403.6 – 748.4)	-56.4 (-940.6 – 827.9)	-228.8 (-809.9 – 352.4)
	MMP-9	-3608 (-551075 – 543859)	-45223 (-337024 – 246577)	-29292 (-212329 – 153744)	-98982 (-284785 – 86820)	-41615 (-427824 – 344593)	-25684 (-448820 – 397452)	-95374 (-634581 – 443832)	15931 (-191435 – 223299)	-53758 (-352520 – 245003)	-69690 (-271061 – 131680)
	TIMP-1	2342 (-1919 – 6604)	768.4 (-1503 – 3040)	771.2 (-653.7 – 2196)	-776.2 (-2222 – 670.3)	-1573 (-4580 – 1432)	-1571 (-4865 – 1722)	-3118 (-7316 – 1079)	2.82 (-1611 – 1617)	-1544 (-3870 – 781.2)	-1547 (-3115 – 20.2)
	VEGF	-5039 (-16781 – 6701)	748.7 (-5509 – 7006)	-2400 (-6326 – 1525)	1840 (-2144 – 5825)	5788 (-2494 – 14071)	2639 (-6435 – 11714)	6880 (-4683 – 18444)	-3149 (-7596 – 1298)	1092 (-5315 – 7499)	4241 (-77.6 – 8559)
	Periostin	-65951 (-173244 – 41341)	-59240 (-115996 – -2485) *	-39440 (-75008 – -3873) *	-28113 (-65933 – 9706)	6710 (-68535 – 81956)	26510 (-56329 – 109351)	37838 (-71033 – 146709)	19799 (-20212 – 59812)	31127 (-30045 – 92299)	11327 (-31203 – 53858)
	Elastin	-11523 (-202558 – 179511)	9573 (-27837 – 96786)	34474 (-27837 – 96786)	18377 (-83822 – 120577)	21096 (-115940 – 158134)	45997 (-101168 – 193163)	29901 (-177265 – 237067)	24900 (-60478 – 110280)	8804 (-132545 – 150154)	-16096 (-123343 – 91149)
	SADAM33	205369 (-84877 – 495617)	86168 (-80548 – 252886)	80587 (-15608 – 176783)	79472 (-60058 – 219003)	-119200 (-316876 – 78474)	-124782 (-341759 – 92194)	-125897 (-430156 – 178361)	-5581 (-122989 – 111826)	-6696 (-204887 – 191494)	-1114 (-150130 – 147900)
	Dichotomous Variables										
	IL-13	0.61 (0.56 – 1.84)	NA	0.29 (0.02 – 4.94)	NA	NA	0.47 (0.00 – 407.2)	NA	NA	NA	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	BDNF	1832.5 (0.79 – 13700000)	71.0 (0.79 – 6353)	11.4 (0.51 – 251.6)	NA	0.04 (0.00 – 11.9)	0.001 (0.00 – 5.82)	0.001 (0.00 – 4.09)	0.16 (0.01 – 2.03)	0.01 (0.0002 – 1.26)	0.09 (0.004 – 1.95)

All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. *:p<0.05, **: p<0.01, ***:p<0.001

	Mixed Granulocytic asthma (MGA)										
		NZ vs UK	NZ vs BRA	NZ vs UGA	NZ vs ECU	UK vs BRA	UK vs UGA	UK vs ECU	BRA vs UGA	BRA vs ECU	UGA vs ECU
	Continuous Variables										
Inflammatory	IL-1β	-4380 (-15201 – 6440)	-2857 (-9591 – 3875)	-460.2 (-4907 – 3987)	375.3 (-3306 – 4057)	1522 (-5436 – 8481)	3920 (-4267 – 12107)	4755 (-4945 – 14456)	2397 (-1951 – 6747)	3233 (-2311 – 8777)	835.5 (-2394 – 4065)
	IL-6	-1176 (-4590 – 2238)	-562.1 (-2686 – 1562)	-20.3 (-1423 – 1383)	144.3 (-1017 – 1306)	614.2 (-1581 – 2809)	1156 (-1427 – 3739)	1320 (-1740 – 4381)	541.8 (-830.6 – 1914)	706.4 (-1043 – 2455)	164.6 (-854.6 – 1183)
	IL-8	-21991 (-180887 – 136904)	-25875 (-124746 – 72995)	-20653 (-85959 – 44652)	14763 (-39306 – 68833)	-3884 (-106065 – 98296)	1337 (-118890 – 121565)	36754 (-105688 – 179198)	5222 (-58645 – 69089)	40639 (-40771 – 122050)	35417 (-12012 – 82846)
	Neutrophil Elastase	84890 (-107294 – 277076)	3190 (-115573 – 121954)	8719 (-73191 – 90630)	1927 (-63597 – 67453)	-81700 (-190925 – 27524)	-76171 (-208991 – 56648)	-82963 (-240872 – 74946)	5529 (-59374 – 70432)	-1262 (-86427 – 83902)	-6791 (-54845 – 41262)
	ECP	18409 (-41972 – 78790)	8506 (-28462 – 45474)	6073 (-19993 – 32141)	NA	-9902 (-41753 – 21947)	-12335 (-53243 – 28572)	NA	-2432 (-20082 – 15217)	NA	NA
	PGD-2	-17890 (-55564 – 19783)	NA	-9424 (-25661 – 6812)	NA	NA	8465 (-17063 – 33994)	NA	NA	NA	NA
	Histamine	-20315 (-57952 – 17321)	-16789 (-39832 – 6253)	-4231 (-20479 – 12017)	NA	3525 (-16326 – 23378)	16084 (-9414 – 41582)	NA	12558 (1556 – 23559) *	NA	NA
Neural	Neurokinin A	-17948 (-140274 – 104377)	-26606 (-104038 – 50824)	-12452 (-72155 – 47251)	15186 (-44523 – 74895)	-8658 (-94033 – 76716)	5496 (-98836 – 109828)	33134 (-90969 – 157237)	14154 (-36154 – 64463)	41792 (-24546 – 108132)	27638 (-9590 – 64867)
	Substance P	NA	-1858 (-4409 – 691.6)	-1629 (-4746 – 1487)	-2549 (-6257 – 1157)	-1858 (-4409 – 691.6)	-1629 (-4746 – 1487)	-2549 (-6257 – 1157)	NA	-690.7 (-2672 – 1291)	-919.9 (-2032 – 192.3)
	Noiciceptin	NA	-1453 (-3588 – 681.6)	-529.5 (-3138 – 2079)	-1611 (-4715 – 1491)	-1453 (-3588 – 681.6)	-529.5 (-3138 – 2079)	-1611 (-4715 – 1491)	924.0 (-334.1 – 2182)	-158.2 (-1817 – 1500)	-1082 (-2013 – -151.2) *
Remodelling	MMP-1	111.5 (-2626 – 2849)	119.0 (-1614 – 1852)	2.28 (-1334 – 1338)	-333.7 (-1670 – 1002)	7.5 (-1903 – 1918)	-109.3 (-2444 – 2225)	-445.2 (-3223 – 2332)	-116.8 (-1242 – 1009)	-452.7 (-1937 – 1032)	-335.9 (-1169 – 497.3)
	MMP-9	-54068 (-182372 – 74235)	-46238 (-126073 – 33597)	-42629 (-95362 – 10104)	-5477 (-49137 – 38182)	7830 (-74678 – 90338)	11439 (-85641 – 108520)	48590 (-66428 – 163610)	3609 (-47962 – 55180)	40760 (-24976 – 106498)	37151 (-1146 – 75449)
	TIMP-1	-1502 (-14615 – 11610)	-2840 (-10999 – 5319)	331.7 (-5057 - 5721)	-510.2 (-4972 – 3951)	-1337 (-9770 – 7094)	1834 (-8087 – 11756)	992.2 (-10763 – 12747)	3171 (-2098 – 8442)	2329 (-4388 – 9048)	-841.9 (-4756 – 3072)
	VEGF	-1855 (-23123 – 19411)	775.5 (-12457 – 14008)	-85.9 (-8826 – 8655)	8600 (1363 – 15837) *	2631 (-11045 – 16307)	1769 (-14322 – 17861)	10455 (-8609 – 29521)	-861.4 (-9409 – 7686)	7824 (-3071 – 18721)	8686 (2337 – 15034) *
	Periostin	21966 (-34507 – 78439)	6964 (-28174 – 42104)	9453 (-13757 – 32663)	-30.9 (-19248 – 19186)	-15001 (-51317 – 21314)	-12513 (-55243 – 30217)	-21997 (-72623 – 28628)	2488 (-20211 – 25187)	-6995 (-35930 – 21938)	-9484 (-26341 – 7372)
	Elastin	-66969 (-539248 – 405308)	-28012 (-314871 – 258845)	120978 (-72963 – 314919)	32992 (-127642 – 193626)	38957 (-210825 – 288739)	187948 (-130273 – 506169)	99962 (-274031 – 473955)	148991 (4918 – 293063) *	61004 (-135983 – 257993)	-87986 (-200839 – 24867)
	SADAM33	-107906 (-675541 – 459728)	-134909 (-474515 – 204695)	-10584 (-226705 – 205535)	70237 (-109071 – 249546)	-27003 (-314511 – 260504)	97321 (-293395 – 488037)	178143 (-268915 – 625202)	124325 (-48204 – 296855)	205147 (-22073 – 432368)	80822 (-37599 – 199243)
	Dichotomous Variables										
	IL-13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	NGF-β	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	BDNF	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. *:p<0.05, **: p<0.01, ***:p<0.001

	Paucigranulocytic Asthma (PGA)									
		NZ vs UK	NZ vs BRA	NZ vs UGA	NZ vs ECU	UK vs BRA	UK vs UGA	UK vs ECU	BRA vs UGA	BRA vs ECU	UGA vs ECU
	Continuous Variables										
Inflammatory	IL-1β	486.7 (-239.2 – 1212)	546.7 (176.6 – 916.9) **	556.6 (80.9 – 1032) *	327.9 (-27.5 – 683.4)	60.1 (-550.6 – 670.7)	69.9 (-738.7 – 787.5)	-158.7 (-1043 – 726.5)	9.84 (-670.7 – 550.6)	-218.8 (-710.6 – 273.0)	-228.6 (-749.6 – 292.3)
	IL-6	299.1 (-134.2 – 732.4)	58.9 (-162.5 – 280.4)	274.2 (-9.5 – 557.8)	96.8 (-115.1 – 308.7)	-240.2 (-603.7 – 123.3)	-24.9 (-506.3 – 456.4)	-202.3 (-729.4 – 324.8)	215.2 (-95.1 – 525.5)	37.8 (-254.9 – 330.6)	-177.3 (-487.4 – 132.7)
	IL-8	-290.3 (-4217 – 3637)	1494 (-513.1 – 3501)	269.7 (-2301 – 2840)	4473 (2552 – 6394) ***	1784 (-1510 – 5079)	560.0 (-3803 – 4923)	4763 (-14.3 – 9541)	-1224 (-4036 – 1588)	2979 (4036 – 1588) *	4203 (1393 – 7013) **
	Neutrophil Elastase	2826 (519.3 – 5132) *	870.0 (-343.5 – 2083)	1201 (-193.4 – 2595)	833.6 (-196.5 – 1863)	-1956 (-3705 – 206.7) *	-1625 (-3949 – 698.9)	-1992 (-4604 – 619.2)	331.0 (-1103 – 1766)	-36.4 (-1456 – 1384)	-367.4 (-1816 – 1082)
	ECP	13015 (-18822 – 44854)	1664 (-14698 – 18027)	-3250 (-22132 – 15632)	NA	-11351 (-35017 – 12314)	-16265 (-48009 – 15477)	NA	-4914 (-24309 – 14480)	NA	NA
	PGD-2	-1405 (-8735 – 5924)	NA	-2067 (-4752 – 617.0)	NA	NA	-662.4 (-6066 – 4742)	NA	NA	NA	NA
	Histamine	8301 (1973 – 14630) *	-363.7 (-3668 – 2941)	3868 (135.9 – 7601) *	NA	-8665 (-13437 – -3893) ***	-4433 (-10792 – 1925)	NA	4232 (323.2 – 8141) *	NA	NA
Neural	Neurokinin A	6365 (-1729 – 14460)	2864 (-946.4 – 6676)	6304 (1277 – 11330) *	-1668 (-6702 – 3365)	-3500 (-10669 – 3668)	-61.3 (-9599 – 9477)	-8033 (-19276 – 3208)	3439 (-1880 – 8759)	-4533 (-10410 – 1343)	-7972 (-13435 – 2509) **
	Substance P	241.9 (-88.2 – 572.0)	87.6 (-67.2 – 242.4)	493.3 (295.9 – 690.7) ***	229.5 (17.9 – 441.0) *	-154.3 (-446.7 – 138.1)	251.4 (-137.8 – 640.6)	-12.4 (-478.7 – 453.8)	405.7 (194.4 – 617.0) ***	141.8 (-103.5 – 387.2)	-263.9 (-482.6 – -45.2) *
	Noiciceptin	202.1 (-335.6 – 739.7)	95.9 (-183.3 – 375.2)	381.6 (42.9 – 720.3)	322.9 (-19.9 – 665.8)	-106.1 (-558.3 – 345.9)	179.5 (-415.9 – 774.8)	120.9 (-591.7 – 833.5)	285.6 (-32.5 – 603.7)	227.0 (-135.9 – 589.9)	-58.6 (-388.7 – 271.5)
Remodelling	MMP-1	289.3 (2.32 – 576.3) *	154.2 (22.0 – 286.3) *	283.1 (106.1 – 460.1) **	92.5 (-77.0 – 262.0)	-135.2 (-387.3 – 117.0)	-6.23 (-343.7 – 331.2)	-196.8 (-590.9 – 197.2)	128.9 (-61.4 – 319.3)	-61.7 (-266.6 – 143.3)	-190.6 (-384.1 – 2.83)
	MMP-9	17492 (-1645 – 36629)	3938 (-5843 – 13719)	460.1 (-12067 – 12987)	-2452 (-11813 – 6908)	-13554 (-29609 – 2501)	-17032 (-38294 – 4230)	-19944 (-43226 – 3337)	-3477 (-17182 – 10226)	-6390 (-19323 – 6542)	-2912 (-16607 – 10781)
	TIMP-1	682.4 (126.9 – 1237) *	209.7 (74.2 – 493.6)	256.8 (-106.8 – 620.4)	463.2 (191.5 – 734.9) ***	-472.7 (-938.7 – -6.68) *	-425.6 (-1042 – 191.6)	-219.2 (-894.9 – 456.6)	47.1 (-350.6 – 444.8)	253.5 (-121.8 – 628.8)	206.4 (-191.1 – 603.8)
	VEGF	272.6 (-3619 – 4164)	3132 (1143 – 5122) **	1450 (-1097 – 3998)	5297 (3393 – 7200) ***	2860 (-405.4 – 6125)	1178 (-3146 – 5502)	5024.4 (289.2 – 9759) *	-1681 (-4469 – 1105)	2164 (-465.9 – 4794)	3846 (1061 – 6631) **
	Periostin	10209 (-3419 – 23838)	-3374 (-10533 – 3785)	2032 (-6227 – 10292)	-11909 (-17993 – -5825) ***	-13583 (-23955 – -3211) *	-8176 (-21951 – 5597)	-22118 (-37592 – -6645) **	5406 (-3109 – 13922)	-8535 (-16950 – -120.6) *	-13941 (-22539 – -5344) **
	Elastin	-6890 (-34660 – 20879)	9063 (-5800 – 23928)	30902 (14850 – 46954) ***	11257 (-3269 – 25783)	15954 (-4713 – 36622)	37792 (10221 – 65363) **	18147 (-14467 – 50762)	21838 (4993 – 38683) *	2193 (-17102 – 21488)	-19645 (-38283 – -1007) *
	SADAM33	200390 (-125640 – 526420)	46551 (-137011 – 230114)	15459 (-139654 – 170573)	26386 (-92512 – 145286)	-153838 (-340097 – 32420)	-184930 (-439876 – 70016)	-174003 (-492255 – 144249)	-31091 (-163793 – 101610)	-20164 (-195569 – 155240)	10927 (-136677 – 158531)
	Dichotomous Variables										
	IL-13	NA	0.23 (0.02 – 2.28)	3.17 (0.76 – 13.3)	0.21 (0.02 – 2.07)	1.12 (0.04 – 28.3)	15.3 (1.34 – 175.6) *	NA	13.8 (1.25 – 151.7) *	0.89 (0.04 – 22.8)	0.06 (0.006 – 0.75) *
	NGF-β	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	BDNF	0.73 (0.06 – 9.20)	5.66 (1.53 – 20.9)	7.05 (1.51 – 32.9)	4.59 (1.09 – 19.4)	7.76 (1.06 – 56.7) *	9.65 (0.71 – 132.3)	6.29 (0.30 – 131.9)	1.25 (0.30 – 5.01)	0.81 (0.17 – 3.94)	0.65 (0.14 – 2.99)

All data adjusted for age and sex. Data presented as Regression Coefficient (95% CI) for continuous variables, and Odds Ratio (95% CI) for dichotomous variables. NA: Data not shown if n<5 or data not available. *:p<0.05, **: p<0.01, ***:p<0.001

Appendix 10: Spearman correlation analysis between mediators and leukocytes

		Macrophages			Eosinophils			Neutrophils			Lymphocytes			Epithelial Cells		
		Asthma	EA	NEA	Asthma	EA	NEA	Asthma	EA	NEA	Asthma	EA	NEA	Asthma	EA	NEA
Inflammatory	IL-1β	-0.22 ***	-0.24 ***	-0.24 ***	-0.07	-0.1	0.01	0.27 ***	0.31 ***	0.25 ***	-0.06	-0.09	-0.04	-0.10 *	-0.06	-0.12 *
	IL-6	-0.19 ***	-0.24 ***	-0.18 **	-0.00	-0.14	0.09	0.25 ***	0.37 ***	0.19 ***	-0.13 **	-0.14 *	-0.11 *	-0.04	0.02	-0.05
	IL-8	-0.05	-0.12	-0.07	-0.16 ***	-0.14	-0.05	0.15 ***	0.09	0.25 ***	-0.06	-0.00	-0.1	-0.14 **	-0.11	-0.17
	Neutrophil Elastase	-0.37 ***	-0.30 ***	-0.46 ***	-0.07	-0.13	0.12 *	0.43 ***	0.37 ***	0.47 ***	-0.13 **	-0.15 *	-0.12 *	-0.01	-0.02	-0.02
	ECP	-0.19 ***	-0.22 *	-0.17 *	0.31 ***	0.04	0.26 ***	0.11 *	0.20 *	0.17 *	0.16 **	0.17 *	0.18 *	-0.20 ***	-0.12	-0.26 ***
	PGD-2	-0.04	-0.10	-0.01	0.19 *	0.04	0.21 *	0.01	0.12	-0.00	-0.11	-0.16	-0.10	0.11	0.13	0.12
	Histamine	-0.27 ***	-0.12	-0.35 ***	0.09	0.05	0.05	0.28 ***	0.16	0.36 ***	-0.19 ***	-0.10	-0.27 ***	0.13 *	0.14	0.13
Neural	Neurokinin A	-0.20 ***	-0.21 *	-0.20 ***	0.02	-0.14	0.03	0.24 ***	0.31 ***	0.20 ***	-0.01	-0.04	0.01	0.02	-0.02	0.05
	Substance P	-0.32 ***	-0.22 *	-0.38 ***	-0.1	-0.09	-0.04	0.35 ***	0.27 **	0.39 ***	-0.00	0.07	-0.03	-0.02	-0.04	-0.01
	Nociceptin	-0.23 ***	-0.26 **	-0.25 ***	0.02	-0.00	0.08	0.27 ***	0.31 ***	0.24 ***	-0.08	-0.04	-0.11	0.14 **	0.08	0.18
	NGF-β	0.04	0.10	0.02	0.05	0.07	-0.02	-0.04	-0.10	-0.01	-0.09	-0.17 *	-0.04	0.02	-0.02	0.06
	BDNF	-0.12 **	-0.15 *	-0.10	0.00	-0.04	-0.02	0.13 **	0.19 **	0.10	0.03	0.07	0.00	-0.11 **	-0.13	-0.10
Remodelling	MMP-1	-0.31 ***	-0.38 ***	-0.30 ***	-0.00	0.01	0.08	0.34 ***	0.39 ***	0.30 ***	-0.06	0.07	-0.13 *	0.04	0.01	0.06
	MMP-9	-0.24 ***	-0.21 **	-0.28 ***	-0.03	-0.11	-0.07	0.29 ***	0.29 ***	0.29 ***	-0.14 **	-0.11	-0.16 **	-0.04	-0.01	-0.06
	TIMP-1	-0.13 **	-0.18 **	-0.13	-0.04	-0.03	0.02	0.17 ***	0.24 ***	0.13 *	-0.11 **	-0.05	-0.14 **	-0.05	-0.08	-0.04
	VEGF	-0.06	-0.18 *	-0.01	0.00	-0.11	0.01	0.08	0.25 ***	0.01	-0.03	-0.04	0.00	-0.05	-0.06	-0.08
	Periostin	-0.22 ***	-0.14	-0.23 ***	0.23 ***	0.14	0.14 *	0.17 ***	0.12	0.22 ***	-0.05	-0.07	-0.04	0.17 ***	0.10	0.22 ***
	Elastin	-0.21 ***	-0.19 *	-0.24 ***	-0.04	-0.23 **	0.01	0.28 ***	0.34 ***	0.27 ***	-0.01	-0.04	0.02	-0.07	-0.16	-0.01
	SADAM33	-0.17 **	-0.20 *	-0.18 **	-0.08	-0.27 **	0.12	0.27 ***	0.43 ***	0.19 **	-0.13 *	-0.13	-0.14 *	-0.03	-0.03	-0.02

Includes data from asthmatics, EA, and NEA from all countries. Data presented as Spearman's correlation coefficient (r). *=p<0.05, **=P<0.01, ***=P<0.001

Appendix 11: Spearman correlation analysis between mediators

	Analytes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Inflammatory	1. IL-1β	x																			
	2. IL-6	0.64 ***	x																		
	3. IL-8	0.70 ***	0.62 ***	x																	
	4. Neutrophil Elastase	0.59 ***	0.43 ***	0.53 ***	x																
	5. ECP	0.43 ***	0.47 ***	0.42 ***	0.46 ***	x															
	6. PGD-2	0.31 ***	0.47 ***	0.21 **	0.20 **	0.50 ***	x														
	7. Histamine	0.15 *	0.08	0.04	0.26 ***	0.06	0.13	x													
	8. IL-13	0.09 *	0.16 ***	-0.02	0.01	-0.01	-0.01	0.03	x												
Neural	9. Neurokinin A	0.16 **	0.19 ***	0.15 **	0.29 ***	0.17 **	0.48 ***	0.21 **	-0.01	x											
	10. Substance P	0.29 ***	0.17 **	0.21 ***	0.34 ***	0.13	0.28 **	0.28 ***	0.01	0.32 ***	x										
	11. Noiciceptin	0.06	0.1 *	0.06	0.29 ***	0.12	0.50 ***	0.37 ***	0.06	0.63 ***	0.35 ***	x									
	12. NGF-β	-0.16 ***	0.08	-0.11 *	-0.11	-0.21 **	NA	-0.08	0.27 ***	-0.19 ***	-0.17 ***	-0.07	x								
	13. BDNF	0.39 ***	0.39 ***	0.39 ***	0.30 ***	0.32 ***	0.08	-0.06	0.06	0.11 *	0.18 ***	0.01	-0.07	x							
Remodelling	14. MMP-1	0.39 ***	0.36 ***	0.42 ***	0.44 ***	0.30 ***	0.51 ***	0.23 ***	-0.01	0.39 ***	0.42 ***	0.46 ***	-0.18 ***	0.32 ***	x						
	15. MMP-9	0.69 ***	0.53 ***	0.65 ***	0.54 ***	0.23 ***	0.16 *	0.09	-0.02	0.12 *	0.23 ***	0.03	-0.08	0.35 ***	0.40 ***	x					
	16. TIMP-1	0.37 ***	0.43 ***	0.60 ***	0.35 ***	0.23 ***	0.23 **	0.04	0.05	0.25 ***	0.25 ***	0.33 ***	-0.01	0.21 ***	0.57 ***	0.42 ***	x				
	17. VEGF	0.59 ***	0.63 ***	0.63 ***	0.33 ***	0.50 ***	0.52 ***	0.06	0.05	0.20 ***	0.23 ***	0.14 **	-0.18 ***	0.35 ***	0.39 ***	0.50 ***	0.63 ***	x			
	18. Periostin	-0.03	0.17 ***	-0.06	0.18 ***	0.33 ***	0.33 ***	0.19 ***	0.05	0.45 ***	0.18 **	0.49 ***	0.06	-0.01	0.48 ***	0.07	0.15 ***	0.15 **	x		
	19. Elastin	0.29 ***	0.42 ***	0.44 ***	0.48 ***	0.43 ***	0.19 *	0.18 **	0.01	0.50 ***	0.40 ***	0.60 ***	-0.05	0.25 ***	0.46 ***	0.18 ***	0.47 ***	0.52 ***	0.41 ***	x	
	20. SADAM33	0.49 ***	0.54 ***	0.66 ***	0.63 ***	0.48 ***	0.27 ***	0.11	0.02	0.33 ***	0.26 ***	0.39 ***	NA	0.26 ***	0.41 ***	0.33 ***	0.60 ***	0.53 ***	0.14 *	0.70 ***	x

Includes data from asthmatics from all countries. Data presented as Spearman's correlation coefficient (r). *=p<0.05, **=P<0.01, ***=P<0.001

Appendix 12: Relationship between asthma medication and sputum mediators

	Analyte	Preventer (n=311)	Reliever (n=317) †	No medication (n=252) ‡
Inflammatory	IL-1β	93.96 (39.96-248) ‡	88.92 (34.39-241.2) ‡	231.6 (65.3-621.9)
	IL-6	61.47 (41.32-172)	68.67 (41.32-177.7)	97.47 (41.32-251.1)
	IL-8	776.1 (385.9-1489) ‡	757 (371.2-1410) ‡	1349 (697.3-2949)
	IL-13	15/192 (7.8%)	22/309 (7.1%)	14/245 (5.7%)
	Neutrophil Elastase (ng/mL)	1394 (478.1-3499)	1409 (340.7-3900)	1669 (637.1-4278)
	ECP (ng/mL)	969.8 (143.1-2757)	705.7 (101.5-2543)	753.1 (192.5-3600)
	PGD-2	968.2 (538.4-2024)	928.7 (591.8-2121)	543.2 (456.9-1330)
	Histamine	4968 (2709-7434)	5175 (2520-7380)	4734 (2376-8586)
Neural	Neurokinin A	259.1 (65.75-736)	186.9 (65.75-786.1)	379.4 (130.6-826.9)
	Substance P	43.92 (43.92-253.6)	43.92 (43.92-308.1) ‡	98.37 (43.92-596.7)
	Noiciceptin (ng/mL)	74.2 (15.7-383.2)	78.1 (21.2-383.9)	100.6 (10.8-452.9)
	NGF-β	24/130 (18.5%)	38/227 (13.7%)	22/208 (10.6%)
	BDNF	15/196 (7.6%)	34/311 (10.9%)	14/245 (5.7%)
Remodeling	MMP-1	44.37 (44.37-170.9)	44.37 (44.37-194.2)	71.91 (44.37-266.7)
	MMP-9	1412 (602.3-4142)	1519 (605.2-4049)	2148 (690.5-4711)
	TIMP-1 (ng/mL)	77.2 (28.1-218.9)	78.4 (29.8-206.4)	95.7 (21.1-1201)
	VEGF	984.8 (337.5-2421) ‡	786.8 (266.2-2020) ‡	1955 (397.4-4510)
	Periostin	5850 (1170-18720) ‡	6120 (1440-18720) ‡	2070 (877.5-9360)
	Elastin	14540 (6062-24935)	12933 (6034-24717)	17279 (8759-30588)
	SADAM33	12376 (2855-33434)	15506 (2266-33161)	19047 (3184-43828)

†: p<0.01 compared to Reliever, ‡: p<0.01 compared to None (no medication usage). Concentrations provided in pg/mL unless indicated otherwise. Data shown as Median (IQR) or detectability (%). P-values obtained through Dunn's post-comparison test or Chi-Square test.