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ASPECTS OF INFLAMMATION IN ASTHMA

Ismail Sikander Kalla

A Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

2017

DECLARATION

I, Ismail Sikander Kalla declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

_____

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DEDICATION

I dedicate this work:

To my beloved mother, the late Tahera and my dearest uncle, Haroon Kalla whose unconditional love, support and encouragement I will always cherish.

To my parents, the Late Sikander and Mumtaz Kalla whose constant prayers, love and support remain the cornerstone of my success.

To my wonderful uncle, Asgar Ali Kalla who has been the beacon of light throughout my career. I will always treasure his unconditional love, unwavering support and valuable input.

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- **Sub-Saharan and Oceanic Pulmonology Society Conference**

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- **Respiratory Expert Symposium 2011**

2011: Assessing Airway Inflammation

- **South African Thoracic Society: National Combined Congress meetings**

2012: COPICON Congress: Critical4Africa:Combined meeting of the Critical Care Society of South Africa (CCSSA), South African Thoracic Society (SATS), Southern Society for Parenteral and Enteral Nutrition (SASPEN) and Trauma Society of South Africa (TSSA): Yes to NO: An update on Inhaled Nitric Oxide.

2013: South African Thoracic Society Annual Conference: Is assessing Airway Inflammation important?

2014: Airwaves Congress: Combined meeting of the South African Thoracic Society (SATS), South African Society of Sleep Medicine (SASSM) and National Asthma Education Program (NAEP): Aspects of Airway Inflammation.

2015: South African Thoracic Society Annual Conference: Biologicals in asthma.

- **University of the Witwatersrand: Annual Physician Conference**

2011:Asthma Update

2015:GINA Guidelines 2015

- **South African Critical Care Society Congress**

2015: The Critical Care Congress 2015**:** Management of Severe Acute Asthma.

ABSTRACT

Asthma is the most prevalent chronic respiratory disease worldwide, with South Africa having the fourth highest asthma mortality rate in the world (1.5 per 100,000 people) and the fifth highest asthma mortality among five to thirty-five year old asthmatics (18.5 per 100,000 asthmatics). Current guidelines recommend that the achievement of 'asthma control' may still be plagued with persistent airway inflammation despite normalisation of spirometric parameters and control of asthmatic symptoms. This may result in structural damage with airway remodelling, fibrosis and progressive loss of lung capacity over time. Assessment of inflammation,'Inflammometry' is now increasingly used to titrate therapeutic interventions and achieve better asthma control. The current study aims to identify simple, objective, non-invasive and reproducible biomarkers for airway inflammation; that would allow documentation of the presence or absence of airway inflammation in individual asthmatic patients. The availability of such tests may allow titration of therapeutic interventions to an outcome parameter that is a true reflection of the state of inflammation of the airways.

This was a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic at the Charlotte Maxeke Johannesburg Academic Hospital Asthma Clinic. Patients attending the clinic were identified from patient records and those who met the inclusion and exclusion criteria, were selected for the study.

The level of asthma control of each patient was determined using the Asthma Control Questionnaire (ACQ). Patients undertook the Asthma Control Test (ACT), had an independent assessment of their asthma control as well as performed lung function tests in a dedicated lung function laboratory. Blood and exhaled breath samples from patients were analysed for inflammatory biomarkers. Optimisation experiments were performed to establish a protocol for the measurement of serum leukotrienes in the asthmatic patient. For the statistical analyses, patients were characterised into asthma control groups as defined by the ACQ.

The current study found that the ACQ and the ACT, as well as the ACQ and an independent physician's assessment of the level of asthma control were in synchrony. Baseline FEF 25-75 levels, expressed as percentage of predicted, were low in the totally controlled, well-controlled and uncontrolled groups of asthmatic patients with medians of 35.7%, 27.3% and 17.3% respectively. The FEF 25-75 values in the three groups demonstrated an absolute post-bronchodilator reversibility of 32%, 27% and 31% respectively. These findings confirmed the

presence of bronchial airway hyper-reactivity (BAH), and suggest probable remodelling in the small airways. Impulse oscillometry (IOS) had a greater value in differentiating small airway resistance in the controlled when compared to the uncontrolled adult asthmatic patient. Biomarker assessments found that CRP, IL-2 and RANTES were significantly higher in the uncontrolled group when compared with the controlled group of asthmatic patients ($p = 0.03$, $p = 0.02$ and $p = 0.03$ respectively). Of the three serum leukotrienes measured (i.e. LTB4, LTC4 and LTE4), LTE4 levels were significantly higher in the totally controlled group compared to the uncontrolled and well-controlled groups ($p = 0.007$ and $p = 0.006$ respectively). Therefore, an elevated LTE4 coupled to a suppressed level of RANTES in the same patient may identify a different asthma phenotype. This could provide opportunities for identifying asthma phenotypes and using biomarkers in assessing asthma control.

The study also found that TGFβ1 and TGFβ2 levels were significantly higher in patients using high-dose inhaled corticosteroids (ICS) (dose category as defined by the Global Initiative for Asthma (GINA)), compared to those patients not using high-dose ICS ($p = 0.01$ and $p = 0.001$ respectively). By comparison, TGFβ1 and TGFβ2 levels were significantly lower in the patients using moderate-dose ICS (dose category as defined by GINA), compared to those not using moderate-doses of ICS ($p = 0.02$ and $p = 0.001$ respectively). These findings suggest that airway inflammation is modulated by the upregulation of Treg cells as TGFβ is produced by Treg cells. It is also possible that Treg cells under the influence of elevated IL-2 levels inhibited the expression of the other Th1 as well as Th2 cytokines measured. Therefore, an elevated TGFβ level coupled with an elevated IL-2 level in the same patient may also identify a different asthma phenotype. Collectively, biomarker assessments may prove useful in assessing asthma control. Such assessments may require individualisation for different asthma endotypes and phenotypes, possibly even using biomarker profiling using multiple biomarkers for future patient care considering the heterogeneity of inflammation in asthma.

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GLOSSARY

Abd Circ	Abdominal circumference
AChE	Acetylcholinesterase
ACOS	Asthma-COPD overlap syndrome
ACQ	Asthma Control Questionnaire
ACT	Asthma Control Test
ANOVA	One-Way Analysis of Variance
AP-1	Activator protein 1
APC	Antigen presenting cell
AST	Physicians Asthma Rating
ATS	American Thoracic Society
AX	Reactance area
B or BLK	Blank
B_0	Maximum binding between tracer and antibody without any analyte
BAL	Bronchoalveolar lavage
B cell	B-lymphocytes
BAH	Bronchial airway hyper-reactivity
BLT	Leukotriene B4 receptors
BMI	Body mass index
C	Capacitance
CA	California
Ca^{2+}	Calcium
°C	Celsius
cAMP	Cyclic adenosine monophosphate
CC	Controlled
CCL5	Chemokine (C-C motif) ligand 5
CD^{+}	Cluster of differentiation
CD23	Low-affinity IgE receptor
cGMP	Cyclic guanosine monophosphate
Δ ch	Change
CI	Confidence interval
95% CI	95% confidence intervals
cm	Centimetre
cNOS	Cellular nitric oxide synthase

CPAP	Continuous positive airway pressure
COPD	Chronic obstructive pulmonary disease
Conc	Concentration
CRP	C-reactive protein
CXC	α-Chemokines
CXCR3	Chemokine receptor R3
CXC4	Chemokine receptor 4
Cys	Cysteinyl
cys-LT	Cysteinyl leukotriene receptor
DALY	Disability-adjusted life years
dH_2O	De-ionized water
ECCS	European Community for Coal and Steel
ECP	Eosinophilic cationic protein
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example (exempli gratia)
EGFR	Epithelial growth factor receptor
EIA	Enzyme immuno-assay
ELISA	Enzyme-linked immunosorbent assay
eNO	Exhaled nitric oxide
eNOS	Endothelial nitric oxide synthase
Ers	Elastance of the lung and thorax
F	Frequency
Fcε	Fragment, crystallisable epsilon receptor
FcεRI	High-affinity IgE receptor
FcεRII	Low-affinity IgE receptor
fdR	Frequency dependence of resistance = R5-R20
FEFx	Forced expiratory flow related to some specific portion of the FVC, denoted as subscript x, referring to the volume of FVC already exhaled at the time of measurement
FEF25-75	Average expired flow over the middle half of FVC
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in first second
FEV1/FVC	Percentage of the FVC expired in the first second of forced expiration
Foxp3	Forkhead box P3

FVC	Forced vital capacity
FLAP	5-Lipoxygenase-activating protein
FOT	Forced oscillation technique
Fres	Resonant frequency
FRC	Functional residual capacity
g	Gravitational force or Centrifugal force
GATA 3	Nuclear transcription factor belonging to the GATA family
GINA	Global Initiative for Asthma
Gi	G protein
GM-CSF	Granulocyte monocyte colony stimulating factor
GMP	Guanosine monophosphate
G-TSF	Glioblastoma-derived T-cell suppressor factor
Gq	G protein
H1 RA	Histamine 1 receptor antagonist
H2 RA	Histamine 2 receptor antagonist
hd	High-dose
HETE	12-(*R*)-Hydroxyeicosatetraenoic acid
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HRCT	High-resolution computed tomography
HREC	Human Research Ethics Committee (Medical)
hs-CRP	Highly sensitive C-reactive protein
HTS	High Throughput Screening system
Hz	Hertz
I	Inertance
ICAM-1	Intercellular adhesion molecule 1
ICF	Informed consent form
ICS	Inhaled corticosteroid
IFNγ	Interferon gamma
Ig	Immunoglobulin
INS	Intranasal corticosteroid
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
ILC2	Group 2 innate lymphoid cells

iNOS	Inducible nitric oxide synthase
IOS	Impulse oscillometry
IP3	Inositol triphosphate
IU/ml	International units per millilitre
JAK	Janus protein kinase
kg/m^2	Kilogram per square meter
kPa	Kilopascal
$kPa/l.s^{-1}$	Kilopascal per litre per second
LA	Los Angeles
LABA	Long acting β2 agonist
LAMA	Long acting anti-muscarinic antagonist
ld	Low-dose
LLN	Lower limit of normal
LPS	Lipopolysaccharide
l/s	Litre per second
5-LO	5-Lipoxygenase
LT	Leukotriene
LRTA	Leukotriene receptor antagonist
LTA4H	Leukotriene A4 hydrolase
LTC4S	Leukotriene C4 Synthase
MAP	Mitogen-activated protein
MBP	Major basic protein
MCP	Macrophage chemoattractant protein
MCT	Mast cell tryptases
md	Moderate-dose
MHC	Major histocompatibility complex
mins	Minutes
MIP-1α	Macrophage inflammatory proteins - alpha
µl	Microlitre
ml	Millilitre
ml/s	Millilitre per second
mm	Millimetre
MPO	Myeloperoxidase
MRC	Medical Research Council

mRNA	Messenger RNA
n	Number
NAEPP	National Asthma Education and Prevention Program
NF-κB	Nuclear factor kappa B
NHLS	National Health Laboratory Services
ng	Nanograms
NKT	Natural killer T cells
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NSB	Non-specific binding
NU	Not using
OCS	Oral corticosteroid
OR	Odds ratio
P	Pressure
PAI 2	Plasminogen activator inhibitor 2
PGD2	Prostaglandin D2
pg	Picogram
PEF	Peak expiratory flow
PI	Phosphatidylinositol
PIP-3	Phosphatidylinositol-3-trisphosphate
PLA2	Phospholipase A2
Post	Post - bronchodilator
PPI	Proton pump inhibitor
Pre	Pre - bronchodilator
QC	Quality control
r	Rho
r^2	Coefficient of determination
R	Resistance
R5	Resistance at lowest oscillation frequency 5 Hz, Total airway resistance
R20	Resistance at high oscillation frequency 20 Hz, Resistance of the large airways
R5-R20	Resistance of the total airways minus resistance of large airways = resistance of small airways
RANTES	Regulated on Activation, Normal T Expressed and Secreted

Rc	Central resistance
RBM	Reticular basement membrane
ROC	Receiver operating characteristic
RORγ	Retinoic acid receptor-related orphan nuclear receptor gamma
ROS	Reactive oxidant species
Rp	Peripheral resistance
rpm	Revolutions per minute
Rrs	Total respiratory resistance
RT	Room temperature
S	Standard
SAAP	Serum amyloid associated protein
SABA	Short acting β2 agonist
SAMA	Shorting acting anti-muscarinic antagonist
sd	Standard-dose
SI	Standard international unit
SMA	Smooth muscle airway
SST	Serum separator tubes
STAT	Signal transducer and activator of transcription
Std	Standard concentration
Std. Err.	Standard error
TA	Total activity
TB	Tuberculosis
TBB	Transbronchial biopsy
T-bet	T-box nuclear transcription protein
TC	Totally controlled
T cell	T lymphocyte
Tc	Cytotoxic T cell
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th	Helper T cell
Th0	Naive T cell
TLR	Toll-like receptors
TNFα	Tumour necrosis factor alpha
TRAF	TNF receptor associated adaptor factor proteins

Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
U	Mouthpiece (as per figure legend)
UC	Uncontrolled
USA	United States of America
V	Flow
v	Version
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
Vol	Volume
vs	Versus
WC	Well-controlled
X	Reactance
X5	Reactance at lowest oscillation frequency of 5 Hz, Peripheral capacitive reactance
Xrs	Total respiratory reactance
Z	Impedance
Z5	Impedance at lowest oscillation frequency of 5 Hz

CHAPTER 1: INTRODUCTION

1.1 Definition and epidemiology of asthma

Asthma is defined as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role (1). In the genetically susceptible individual, this inflammation causes recurrent episodes of wheezing, breathlessness and coughing particularly at night and in the early morning. Asthma is the most prevalent chronic respiratory disease worldwide and its prevalence has increased by 12.6% from 1990 to 2015 (2, 3). Approximately 360 million people worldwide currently have asthma and the rate of asthma is increasing as communities adopt western lifestyles and become urbanised (4). With the projected increase in the proportion of the world's population that is urban from 45% to 59% in 2025, there is likely to be a marked increase in the number of asthmatics worldwide over the next two decades. It is estimated that there may be an additional 100 million persons with asthma by 2025 (5, 6). Unfortunately, despite the great strides we have made in diagnosing and treating asthmatic patients, asthma continues to be under-diagnosed and under-treated (7). The number of disability-adjusted life years (DALYs) lost due to asthma worldwide is approximately 15 million per year, with the age-standardised asthma DALY rates approximately 650 per 100 000 people in South Africa (2). Universally, asthma accounts for around 1.1% of all DALYs lost, which reflects the high prevalence and severity of asthma (8). The number of DALYs lost due to asthma is similar to that for diabetes mellitus and cirrhosis of the liver, both conditions with less therapeutic options available to the treating physician.

Asthma is the eighth leading contributor to the burden of disease in South Africa and is the second most important chronic disease after human immune deficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (9). The prevalence of doctor diagnosed asthma within the age group 18-45 years, in South Africa, is estimated to be about 5.9% (10).This prevalence increases to 12.4%, within the same age band, if a less stringent definition, based on symptoms of wheezing or usage of asthma medication, to identify asthmatic patients is used (10). In South Africa, asthma is the third most common cause of hospital admission in children, after pneumonia and gastroenteritis. A greater concern is that South Africa has the fourth highest asthma mortality rate in the world among the general population (1.5 deaths per 100,000 people) and the fifth highest asthma mortality among five to thirty-five year old asthmatics (18.5 deaths per 100,000 asthmatics) (5). Although progressive reductions in mortality have been made over

the last few decades, asthma mortality remains unacceptably high within the southern African region (5, 8). These statistics beg the question: “Are we monitoring and treating our asthmatic patients appropriately?”

1.2 Pathophysiology of asthma

Asthma is a complex disease with several key cells and numerous proteins involved in mediating the airway inflammatory response (11). The following paragraphs will highlight the most important of these elements, with specific reference to their role in asthma.

1.2.1 Interaction between the cellular and cytokine responses in asthma

There is no single mechanism that serves to explain the development of asthma in all individuals (1). The initiation of the inflammatory process is probably a result of the introduction of specific allergens into the airways of susceptible individuals (12, 13). There are, however, common events that characterise the asthma phenotype (14).

Epithelial cell disruption is a key feature in asthma (1). The denuding of the epithelium and the subsequent recruitment of eosinophils and other inflammatory cells to the airway is largely orchestrated through the activation of chemotactic proteins (15). These chemotactic proteins are commonly referred to as cytokines and chemokines. They attract a variety of leucocytes including lymphocytes, granulocytes and monocytes to the inflamed airways by increasing the transcription of cell adhesion molecules, of the selectin and immunoglobulin supergene family, on the endothelial surface of the lungs (1, 15-18). These cell adhesion molecules include intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin and they are upregulated byinterleukin 1 (IL-1), tumour necrosis factor alpha (TNFα) and lipopolysaccharide (LPS) (19). The leukocytes then attach to the vascular endothelial cells expressing these cell adhesion molecules, through receptor-ligand interactions between the endothelial cell and the leukocyte (15). This attraction and attachment of the leukocytes, and in particular the T helper lymphocytes (Th cells) and eosinophils, to the airway endothelial cells forms the hallmark pathophysiological feature of asthma (17). These leukocytes, once attached to the endothelial cells, become activated and further perpetuate the airway inflammatory response. This leads to a state of sustained airway hyper-responsiveness, disruption of the airway epithelium and release of further pro-inflammatory mediators such as histamine, leukotrienes, bradykinin and eosinophil cationic protein (17).

The resultant airway inflammation causes airway smooth muscle hyper-reactivity and hypertrophy with airway narrowing and a significant increase in airway resistance throughout the bronchial tree (16, 20, 21). This change in airway calibre causes the characteristic asthma symptoms of cough, shortness of breath and wheezing (8). Under normal physiological circumstances, the small-calibre peripheral airways, defined as those with an internal diameter of less than two mm, do not contribute significantly to airflow resistance. In patients with asthma, these inflamed small airways become the most important site of increased airway resistance. The reason for this physiological phenomenon is determined by the *Hagen–Poiseuille law* in which minor changes of small airway wall thickness, with a resultant decrease in airway radius, has an exponential effect in the increase in small airway flow resistance (22). Currently, the most validated tool used to assess airway calibre and airflow dynamics i.e. spirometry, is not very sensitive in detecting these changes in the small airways. As a result small airway inflammation can progress undetected (23). Thus, the small airways are often referred to as the "SILENT ZONE" of the bronchial tree (21).

1.2.2 The Th1 – Th2 cellular response

Helper T lymphocytes (Th cells) are the principal orchestrators of the immune response in asthma (11). They are several different Th cells subclasses and are they needed for activation of the cytotoxic T (Tc) cells as well as antibody-secreting plasma cells (24). Th cell activation occurs early in an immune response and once activated, the Th cell begins secreting a cytokine known as interleukin 2 (IL-2). Simultaneously, the Th cell also begins to express a specific high affinity IL-2 receptor on its surface. IL-2 thus acts in a predominantly autocrine way to enhance Th cell activation (25). In addition to IL-2, activated Th cells secrete other cytokines that promote the growth, differentiation, and functions of B-lymphocytes (B cells), macrophages and other cell types. Th cells also provide the signals that augment cell-mediated immune responses that are necessary for B cells to differentiate into antibody producing cells. In asthma, the predominant antibody that is produced is immunoglobulin E (IgE) and its production mediated by the cytokines IL-4 and IL-5 (11, 19, 26).

Most of the mature Th cells belong to one of two distinct subclasses; i.e. Th1 or Th2, identified by the cytokines they produce (27, 28). The divergent pattern of cytokine expression allows each of these Th subsets to promote distinct types of immune reactions that are best suited to orchestrating a specific immunological response. Th1 cells produce IL-2, interferon gamma

(IFNγ) and TNFα (18). These cytokines promote defensive reactions that promote intracellular killing of pathogens. Macrophages and other phagocytic cells mediate this intracellular killing. IFNγ also activates macrophages by inducing nitric oxide synthase (NOS) and other metabolic enzymes that increase cellular microbicidal activity (29).

Th2 cells, in contrast, do not produce IL-2, IFNγ or TNFα but instead secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (13). The Th2-derived cytokines act together to chemoattract B cells, mast cells, basophils and eosinophils (30). These cytokines are also responsible for promoting the growth and differentiation of mast cells, basophils and eosinophils. In addition, IL-4 promotes a switch in the B cell class from producing the IgG to producing the IgE immunoglobulin isotope (13). This class switching enables the mast cells and eosinophils to recognise and respond rapidly to specific antigens, and forms the basis of the underlying pathogenesis of asthma (31).

With better understanding of the immunopathogenesis of asthma, the cellular-cytokine interactions perpetuating the inflammatory milieu are becoming more complex than just the Th1- Th2 axis (32, 33). Several newer T cell subpopulations have been identified by their cytokine thumbprint as well as their role in the pathogenesis of asthma. They will be discussed briefly in the following chapter (34). Furthermore, the innate immune system, as well as the disruption of the airway epithelial lining, are increasingly being recognised as playing vital roles in driving the airway inflammation in asthma (35).

Innate immune responses are triggered by the recognition of conserved molecules of both endogenous and exogenous origin, such as pathogen-associated molecular patterns (PAMPs) and damage/danger-associated molecular patterns (DAMPs) (36). These molecules are recognised by a highly conserved family of cell surface receptors known as a pattern recognition receptors (PRRs) (37, 38). Toll-like receptors (TLRs) belong to this family of PRRs and they are expressed on many inflammatory cell types implicated in the asthma pathogenesis (Chapter 2), as well as on airways epithelial cells and airway smooth muscle cells (39, 40). TLRs recognise PAMPs on viral particles and bacterial tissue, and DAMPs on aeroallergens. Through a process of intracellular signalling, TLRs activate epithelial cells, as well as antigen presenting cells, to release several cytokines (41, 42). These cytokines include IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) and were previously not thought to play a role in the

pathogenesis of asthma (43, 44). However, they are responsible for activating group 2 innate lymphoid cells (ILC2), which are able to produce a Th2 cytokine response and mediate IgE class switching. The importance of this finding is that the Th2 cytokine response (IL-4, IL-5 & IL-13) can be activated by the innate immune system, and is not purely a function of the adaptive immune response (45, 46).

Airway epithelial lining disruption and/or injury by viral particles or aeroallergens also results in the production of an abnormal amount of cytokines directly by the activated epithelial cells (47). The most important of these cytokines are transforming growth factor beta (TGFβ) and epithelial growth factor receptor (EGFR) (48). Release of these cytokines has the effect of promoting an exaggerated and persistent repair process in the airways of asthmatic patients, ultimately leading to airway fibrosis and remodelling (49).

1.3 Asthma control

Asthma control is defined as the extent to which the various manifestations of asthma have been reduced or removed by treatment (8). This includes two components, firstly, the level of clinical control (i.e. day-to-day control) determined from features such as symptoms and the performance of activities of daily living. Secondly, it is the minimizing of the risk of future adverse events including loss of control, exacerbations and accelerated decline in lung function (8, 50, 51). This definition has been emphasised in the latest GINA guidelines published in 2017 (8).

“Asthma control” in clinical practice refers to the achievement of an acceptable clinical state, often based on goals of asthma management. It differs from the assessment of disease severity, which quantifies departure from 'normal' (52). Asthma is a heterogeneous condition that is characterised by frequent acute episodic exacerbations. These exacerbations occur against a background of chronic persistent inflammation and structural changes, which lead to recurrent symptoms and reduced lung function (50). This has led to a change in the goal of asthma therapy. The current GINA guidelines now recommend titrating asthma therapy to achieve “asthma control” (7, 8, 52, 53).

Initially, practice guidelines for asthma treatment aimed to minimise patient symptoms, optimise lung function and prevent exacerbations (54, 55). While referring to this aim, normal

lung function was frequently the primary endpoint. With the subsequent recognition of the importance of the patient perspective, and the poor correlation between lung function, airway inflammation and symptoms, therapeutic interventions have increasingly focused on the assessment of ‘‘asthma control’’(8, 56). Asthma control is thus a summary term implying a global assessment of symptoms, use of reliever medication, lung function and the frequency of exacerbations (50).

The most validated assessment of asthma control currently available to us, is a questionnaire developed by Juniper *et al*. which incorporates all the required aspects of asthma control i.e. symptoms, quality of life, frequency of exacerbations and lung functions (57, 58). It is called the Asthma Control Questionnaire (ACQ) (Appendix 1) and has seven domains. The first five domains are questions that assess the patient’s level of control based on their specific asthma symptoms over the last week. The sixth domain is a score based on the patient’s need for reliever therapy and the seventh domain uses the spirometric assessment of the patient’s airways. The lower the average score of all seven domains the better the asthma control, with a composite score of less than 1.5 out of a maximum score of 7 defining controlled asthma, and a score of less than 0.75 defining total asthma control (59).

Another internationally validated asthma scoring scale is the Asthma Control Test (ACT) (Appendix 2) (60, 61). The ACT assesses patient symptom control over the last month with a score of more than 19 indicative of control (62). The most significant difference between the ACQ and the ACT is that the ACT does not use spirometry as part of the assessment of control. Hence, the ACT can be used in a facility without access to spirometry i.e. a primary health care facility (60). There are several studies confirming the excellent inverse correlation between the ACQ and ACT, thus confirming the ACQ as a valuable alternative tool in assessing asthma control (63). An abridged version of the ACQ (i.e. ACQ 6), where the spirometric assessment domain has been excluded is as robust an assessment of asthma control as the full version of the ACQ and the ACT (63-65).

The fallacy in our current assessment of patients with asthma, with the increasing focus on asthma control, has been the assumption that by titrating therapeutic interventions to normalise symptoms and improve airflow dynamics (i.e. targeting asthma control), we will have suppressed the inflammation present in the airways (7). Unfortunately, this is not true as the

airway inflammation may persist despite normalisation of spirometric parameters and control of asthmatic symptoms (66). The result of this persistent airway inflammation is structural damage within the airways, with airway remodelling, fibrosis and progressive loss of lung capacity over time (67, 68).

1.4 Inflammometry

Our understanding of airway inflammation in asthma was predominantly derived from studies of the large airways. These studies included methodologies such as induced sputum analysis, proximal airway endobronchial biopsy and bronchial washings. They have led us to the perception that asthma is predominantly a disease of the larger airways (69). Recently, the paradigm of airway inflammation in asthma has changed. It is becoming increasingly evident that asthma is an inflammatory lung disease, not only affecting the central airways, but also involving the small airways as well as the lung parenchyma. (70). Thus, therapeutic interventions directed at symptom control, reversal of airflow obstruction and resolution of airway hyper-reactivity have yielded disappointing results in some patients (7, 71, 72). Assessment of inflammation, 'Inflammometry' is now increasingly recommended to titrate therapeutic interventions and achieve better asthma control (73-76).

The nature of asthma as an illness is that one of its fundamental characteristics is the presence of significant symptom variability. Individual patients can have periods of their illness where they are symptom-free or periods where they have increasing symptoms that may require the need for short acting bronchodilators, and even an increase in anti-inflammatory medication. This variability and fluctuation in an individual patient's symptoms can cycle within hours, days or even weeks where the patient is completely well and then moments later, becomes symptomatic or even suffers an acute exacerbation. There are several triggers for this variability in symptoms. They include stress, anxiety, allergen exposure, cold air, humidity and infections (8, 50). This variability in symptoms might not always be associated with airway inflammation. However, when these acute episodic exacerbations occur against a background of chronic persistent airway inflammation, the patient is at risk for airway remodelling and severe bronchospasm, sometimes requiring hospital admission or even supportive mechanical ventilation (7, 8, 50, 56).

With a greater understanding of the molecular mechanisms driving the inflammation in asthma, newer therapeutic targets for treating asthma have been developed (77). With this greater understanding of the molecular mechanisms driving the disease process we are now able to redefine subgroups of asthma into phenotype-specific and endotype-specific (78). A major step forward in the attempt to understand this inflammation and the heterogeneity of asthma, with its protean clinical manifestations, is the concept of asthma phenotypes and asthma endotypes (79, 80). The asthma phenotype describes the clinically observable characteristics of the disease (asthma) without a direct relationship to an underlying pathophysiological process. It refers specifically to clinical and morphologic characteristics as well as unique responses to treatment. Phenotypes are clinically relevant but do not necessarily relate to, or give insights into the underlying molecular and pathological mechanism driving the disease (79). Asthma endotypes refer to subtypes of the disease defined by intrinsically distinct pathogenic or pathophysiological mechanisms. Endotyping describes the disease-specific subtypes based on cellular and molecular mechanisms that drive the clinical manifestations of the illness (80).

Currently, the latest GINA guidelines recommend titrating asthma therapies to a composite score that includes the patient's symptoms, the need for reliever therapy and spirometric lung function tests (i.e. Asthma Control), but direct measures of airway and systemic inflammation are not part of the assessment and treatment algorithm (7, 8). What is lacking is a simple, objective, reproducible and non-invasive point-of-care test that will allow a treating physician to identify those patients who have ongoing airway inflammation. Hence, the clinician can be more scientific with therapeutic interventions. 'Inflammometry' will allow better titration of asthma therapeutics, not just to address patient's symptoms but also to treat airway inflammation (7, 73).

With the concept of 'Inflammometry' being the major theme of this thesis, some of the more important inflammatory molecules that perpetuate the inflammatory milieu in asthmatic patients will be discussed in more detail in later sections. Specific emphasis on their role in airway inflammation in asthma will be highlighted. Furthermore, novel methods to better assess and characterise the airways and in particular, the small airways, as well as assessment of inflammatory biomarkers in patients with asthma will be explored further in this study and form the basis of this thesis.

CHAPTER 2: LITERATURE REVIEW

As indicated above, asthma is an inflammatory disease of the airways in which many cells and various cellular mediators may play an important role.

2.1 Cell mediated regulation of airway inflammation in asthma

2.1.1 Mast Cells

Mast cells are part of the granulocytic cell lineage. They are increased in numbers in the airway epithelium in patients with asthma. This has been confirmed by cell counts in supernatant obtained from bronchoalveolar lavage (BAL), as well as on histology from tissues obtained via transbronchial biopsy (TBB) (81). Mast cells play a critical role in mediating the acute asthmatic response. Their activation occurs following the binding of antigens to the membrane bound antigen-specific IgE Fcε receptor. Mast cells release powerful mediators (histamine, prostaglandins, leukotrienes and tryptases). These mediators act on bronchial smooth muscle and small blood vessels thereby perpetuating the inflammatory response (17). Mast cells are also responsible for the production of vascular endothelial derived growth factor (VEGF) as well as fibroblast growth factor (FGF) which most likely contribute to the airway remodelling in patients with chronic asthma (82).

2.1.2 Eosinophils

These cells are concentrated in large numbers in the bronchial walls and secretions of asthmatic patients. The number of eosinophils are increased in both peripheral blood and the airways of atopic and non-atopic asthmatic patients. Furthermore, their absolute numbers on BAL correlate with persistent eosinophilic airway inflammation (83). Eosinophils are attracted by various cytokines including IL-5, IL-13 and chemokines including eotaxin. Eosinophils also release several mediators including eosinophilic cationic protein (ECP) and major basic protein (MBP) which are toxic to the bronchial epithelium (84, 85).

Asthma is a heterogeneous disease and its clinical manifestations vary with respect to disease severity, frequency of exacerbations and overall response to treatment (8). This has led to the description of several different asthma phenotypes (86). The most important of the various phenotypes described, is the eosinophil predominant subtype. The distinction has been made on the basis of the presence or absence of tissue eosinophils (87). Patients with asthma who have airway eosinophilia have greater airway remodelling and more exacerbations, whereas

those without eosinophilia have more airway obstruction (88). Subsequently, studies have supported the concept that although many patients with asthma do not have any eosinophilic inflammation, the presence of such inflammation identifies a more exacerbation-prone phenotype (86-88).

2.1.3 CD4 T-Helper Lymphocytes (Th Cell)

T-helper lymphocytes (Th cells) are responsible for orchestrating the inflammatory response in the airways of patients with asthma. As indicated above, they produce different cytokines and either subtype of T-helper cells may dominate in the airways of patients with asthma at various stages of the disease process (negative feedback) (18). The cytokines produced by Th1 cells are immunosuppressive to Th2 cells and vice versa (89). The cytokines produced by the Th1 cells include IL-1, IL-12, IFNγ and TNFα. The transcription of IFNγ, the hallmark Th1 cytokine, is regulated by the nuclear transcription factor T-bet, which belongs to the T-box transcription factor family of regulatory genes (90).

In contrast, the Th2 cells produce the cytokines IL-4, IL-5 and IL-13. These cytokines are regulated by the nuclear transcription factor GATA-3, which belongs to the GATA family of transcription factors (91).The Th2 cytokines are responsible for chemotaxis, migration and activation of mast cells and eosinophils, as well as maintaining the allergic inflammatory phenotype (92). They also promote the switching of antibody production by B lymphocytes (B cells) to the IgE class (17).

Table 2.1 is a summary of the Th1 cytokines, and Table 2.2 is a summary of the Th2 cytokines, outlining the basic functions of these molecules as well as highlighting their specific roles in asthma.

Table 2.1: Summary of Th1 cytokines and their role in asthma. Adapted from Barnes *et al*. (1)

Cytokines Secreted	Major Immunological Effects	Effects on Eosinophils	Effects on IgE Secretion	Effects on Airways
IL-1	B cell growth factor. Neutrophil chemoattractant. T cell and epithelial cell activation.	↑ adhesion to vascular endothelium	↔ IgE production	↑ BHR
IL-2	Promotion & activation of antigen-specific Th cells.	Promotes eosinophilia	↔ Ig E production	↑↑ BHR
IL-6	Promotes B cell growth and immunoglobulin production. T cell growth factor.	No effects	↑ Ig E production	↔ BHR
IL-12	Promotes natural killer cell recruitment.	↓eosinophil influx into airways	↓ IgE production	↓ BHR
IFNγ	Activates macrophages. Promotes B cell proliferation and class switching to IgG.	↓eosinophil influx into airways	↓ Ig E production	↓ BHR
TNFα	Activates macrophages and neutrophils. Promotes B cell growth and immunoglobulin production.	↑ adhesion of eosinophils to vascular endothelium	↔ Ig E production	↔ BHR

Th: T Helper Cell, IL: Interleukin, Ig: Immunoglobulin, BHR: Bronchial Hyper Responsiveness, IFNγ: Interferon γ, TNFα: Tumour necrosis factor α, ↑: Increase, ↓: Decrease, ↔: No change.

Table 2.2: Summary of Th2 cytokines and their role in asthma. Adapted from Barnes *et al*. (1)

Cytokines Secreted	Major Immunological Effects	Effects on Eosinophils	Effects on IgE Secretion	Effects on Airways
IL-4	Chemoattractant for lymphocytes, mast cells & basophils. Enhances growth of mast cells and eosinophils. Promotes B cell proliferation and class switching to IgE. Inhibits Th1 cell differentiation.	↑ eosinophil growth	↑ Ig E production	↔BHR
IL-5	Enhances growth and development of eosinophils. Promotes Th2 cell differentiation.	↑eosinophil maturation, ↓apoptosis	↑ Ig E production	↑ BHR
IL-8	Chemoattractant for migration of neutrophils, monocytes and eosinophils.	↑eosinophil influx into airways	↔ Ig E production	↔BHR
IL-10	Inhibits production of cytokines, Th1 cells, macrophages and APC. Inhibits Th1 cell differentiation. Promotes B cell growth and immunoglobulin production.	↑ eosinophil survival	↔ Ig E production	↓ BHR
IL-13	Chemoattracts lymphocytes, mast cells and basophils. Enhances growth of mast cells and eosinophils. Promotes B cell proliferation and class switching to IgE. Inhibits Th1 cell differentiation.	Activates eosinophils, ↓ apoptosis	↑ Ig E production	↔BHR

Th: T Helper Cell, IL: Interleukin, Ig: Immunoglobulin, BHR: Bronchial Hyper Responsiveness, ↑: Increase, ↓: Decrease, ↔: No change.

The importance of the cytokines released by the Th2 cell is becoming increasingly more evident as our understanding of the pathogenesis of asthma improves. Gene expression analyses have identified two distinct Th2 inflammatory asthmatic phenotypes i.e. “Th2-high” and “Th2-low” (93). The Th2-high phenotype has a greater expression of IL-5 and IL-13 and a more significant presence of sub-epithelial fibrosis in bronchial biopsies. The Th2-high phenotype is also associated with more airway hyper-responsiveness, higher serum IgE levels, as well as blood

and airway eosinophilia (93). This characterisation of the various asthma phenotypes is vitally important to the future of asthma therapeutics as the advent of cytokine blocking therapies (i.e. biological anti-cytokine monoclonal antibodies) have allowed scientists to target the molecular mechanisms that drive the inflammation in asthma (94).

With better molecular identification techniques, several newer Th cell populations have been identified (95). They are recognised by the specific cytokine profiles they produce; a situation not too dissimilar to the previously known Th1 versus Th2 cell populations that have been well documented previously (29). While the exact role of these new Th cell subpopulations in asthma have not been clearly defined, a brief description of their postulated roles is worthy of mention (33). Th9 cells produce the cytokine IL-9 which binds to an IL-9 receptor on mast cells, promoting mast cell degranulation and perpetuation of the inflammatory response characteristic of asthma (96). Th17 cells are another of the newer Th cell subpopulations identified by its ability to produce IL-17, regulated by the nuclear transcription factor RAR-related orphan receptor gamma (RORγ), a member of the retinoic acid receptor family of nuclear transcription factors (97). IL-17 has been found to be increasingly expressed in patients with severe asthma and the Th17 cells appear to play a significant role in patients with a neutrophil-predominant asthma phenotype (98). IL-17 produced by Th17 cells, has also been shown to promote airway smooth muscle cell proliferation and airway remodelling (99).

Regulatory T (Treg) cells have also been identified to play a significant role in patients with asthma by limiting airway inflammation via the production of the cytokines IL-10 and TGFβ (100). Treg cell function is regulated by the intracellular transcription factor forkhead box P3 (Foxp3) (101), which is the main control gene in the development and function of Treg cells (102). TGFβ plays an important role in that it increases the expression of Foxp3 which promotes the transition of naïve T cells toward a Treg phenotype (103). This Treg phenotype has a significant immunosuppressive potential within the asthmatic airways by modulating and regulating other T cell activation, including Th1 and Th2 cells, and by inhibiting the expression of other effector cytokines including IL-4, TNFα, IFNγ, IL-17 and IL-21 (104).

Hence, the modulation and upregulation of Treg cell function is becoming more important as they play a major role in counteracting the allergic inflammation in asthma. Treg cells are able to suppress the activation of inflammatory dendritic cells that promote the allergic

inflammation, reduce the survival of mast cells, eosinophils, neutrophil and basophils. They inhibit the degranulation of mast cells and basophils and most importantly, they can reduce B cells isotype switching to IgE production (100).

2.1.4 Neutrophils

Neutrophils were initially thought not to play a significant role in the pathogenesis of the inflammation of asthma (16). With a greater understanding of the cellular mechanisms underlying the inflammatory nature of the asthmatic airways, the neutrophil is being recognised as a vital cell in the immunopathogenesis of acute asthma (14, 46). Increasingly, we are sub-classifying the inflammation at a cellular level based on cell counts in BAL fluid. As alluded to in the preceding paragraphs, two distinct asthma phenotypes have been identified - the eosinophil-predominant phenotype and the neutrophil-predominant phenotype (17, 87, 105). These phenotypes are not driven by the presence of any identifiable infective component and are integral to the underlying pathogenesis of asthma.

This was confirmed in an antigen challenge asthma model developed by Nocker *et al*. (106) wherein it was demonstrated that neutrophils were the first cells recruited to the airways in the initial phase of the asthma inflammatory response. Neutrophils contribute to the inflammation by producing a variety of inflammatory mediators of which reactive oxidant species (ROS), peroxidases, proteases and lipid mediators (especially leukotriene B4 (LTB4)) are the most important. Furthermore, the molecular characterisation of the Th2-low asthma phenotype that manifests with clinical features of asthma, despite the paucity of Th2 driven inflammation, has clearly highlighted the neutrophil as an important cellular mediator in the pathogenesis of asthma (93, 107).

2.1.5 Group 2 Innate Lymphoid Cells (ILC2)

These cells belong to the innate immune mediated immunological response. Within the lung, ILC2 cells are activated either by viral pathogens via toll-like receptors (TLRs) or allergen-induced tissue damage of airway epithelial cells (45). The cytokines responsible for activating ILC2 cells include IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (108). After activation, lung ILC2 cells have the ability to produce IL-5 and IL-13, but do not produce IL-4 (109). Through the production of IL-5 and IL-13 by the activated ILC2 cells after allergen exposure, eosinophils are recruited into the inflamed airways and initiate mucous hyper-

production (45). Unlike Th2 cells of the adaptive immune system, ILC2 cells do not express antigen-specific receptors and their function is regulated by the cytokine environment and possibly through cell-cell interactions (110). However, ILC2 cells do express class II major histocompatibility complex (MHC) antigens which allow the ILC2 cell to function as an antigen presenting cell (APC) and perpetuate a Th2 phenotype through antigen presentation to Th2 cells (45).

2.2 Cytokine mediators of airway inflammation

There is a vast array of cytokines and chemokines involved in the regulation and perpetuation of inflammation in asthmatic airways. These proteins are secreted by a variety of cells either native to the airways or recruited to the airways by the inflammatory response. These secreted proteins allow cells in the airways to communicate with one another and orchestrate inflammation in the asthmatic airways. The result is a complex network of interactions involving cellular recruitment, proliferation, differentiation and effector functions that include antibody switching and release of active cellular mediators (1, 111, 112). Figure 2.1 is a summary diagram depicting the cellular and cytokine interactions in the pathogenesis of asthma.

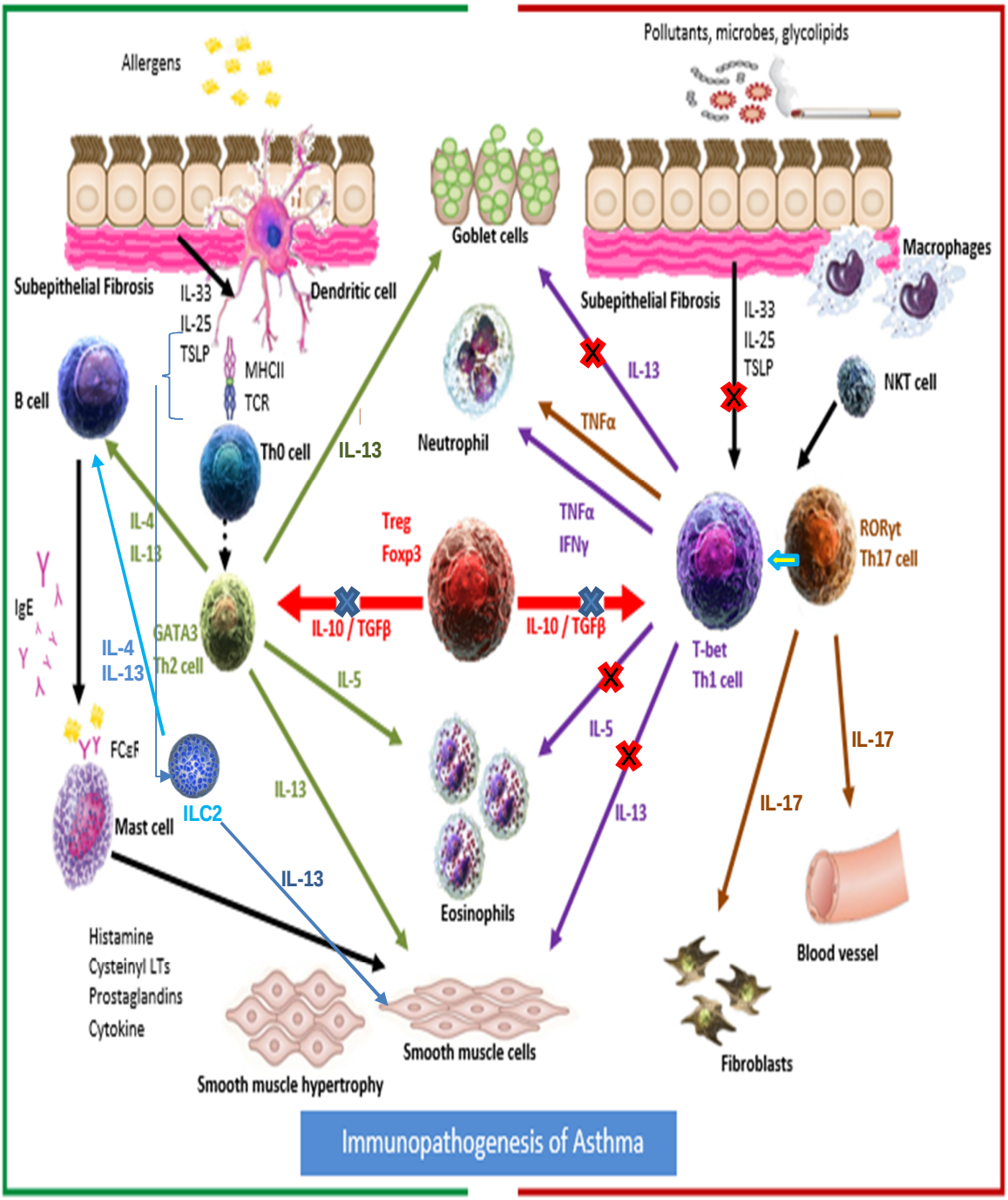

Figure 2.1: The cellular and cytokine interactions in the immunopathogenesis of asthma. Adapted from Lambrecht *et al*. (113), Anderson *et al*. (114) and Seroogy *et al*. (100)

B cell: B lymphocytes, FCεRI: High-affinity IgE receptor, Foxp3: Forkhead box P3, GATA 3: Nuclear transcription factor belonging to the GATA family, IFNγ: Interferon gamma, IgE: Immunoglobulin E, IL: Interleukin, ILC2: Group 2 Innate Lymphoid cells, LT: Leukotrienes, MHC II: Major histocompatibility complex class II, NKT: Natural killer, RORγt: Retinoic acid receptor-related orphan nuclear receptor gamma, T-bet: T-box nuclear transcription protein, TCR: T-cell receptor, Th0: Naive T cell, Th: T helper cells, Treg: Regulatory T cell, TGFβ:Transforming growth factor beta, TNFα: Tumour necrosis factor alpha, TSLP: Thymic stromal lymphopoietin,T cells: T lymphocytes.

However, before the complex interactions of these cytokine and chemokine mediators can be understood, it is important to understand how these molecules act independently before understanding their collective role in patients with asthma. The literature review below will be restricted to the most important cytokines and chemokines pertinent to this study and their individual roles in perpetuating inflammation in asthma.

2.2.1 Interleukin-1 (IL-1)

Thus far, two isoforms of the IL-1 molecule, namely IL-1α and IL-1β have been identified (111). While they share 20% homology of their nucleic acid sequencing, they are produced from two different genes (111). Importantly, they bind to the same receptor and have nearly identical properties. IL-1β is the most active form and is released into the extracellular space and into the circulation. In contrast, IL-1α is usually retained intracellularly. The major source of IL-1 in the airways is from the monocyte/macrophage cell lineage (115). While IL-1α is clearly distinct from IL-1β, their effects will be discussed as one molecule – IL-1.

There are currently two IL-1 receptors described, type 1 and type 2. Both are transmembrane glycoproteins that bind IL-1α, IL-1β as well as interleukin 1 receptor antagonist (IL-1RA). Both receptors are found on most hematopoietic cells, as well as vascular endothelial and bronchial epithelial cells (116). Interestingly, it is only the type 1 receptor that transduces a signal in response to IL-1 binding (117). It has thus been postulated that the type 2 receptor may act as a decoy receptor (118). Intracellular signal transduction occurs via TNF receptor associated factor adaptor proteins (TRAF) as well as via the cyclic adenosine monophosphate (cAMP) pathways (119).

IL-1 is an important growth factor for mature and immature T and B cells, as well as inducing the transcription of several other cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, Regulated on Activation, Normal T Expressed and Secreted (RANTES), granulocyte monocyte colony stimulating factor (GM–CSF), interferon gamma (IFNγ) and TNFα (111, 120, 121). Importantly, IL-1α induces the transcription of IL-1β, which subsequently increases the synthesis of fibronectin and collagen, thus promoting airway remodelling (111, 122). IL-1, together with TNFα and IFNγ can induce and up-regulate the expression of ICAM-1 and VCAM-1 on endothelial cells and respiratory epithelial cells leading to increased adhesion of eosinophils (111, 123). In BAL studies of patients with asthma, levels of IL-1β are elevated and

furthermore, IL-1β has been shown to promote airway neutrophil chemotaxis and increase bronchial airway hyper-reactivity (124).

2.2.2 Interleukin-2 (IL-2)

IL-2 is synthesised predominantly by activated T lymphocytes, especially Th1 cells. It acts in an autocrine fashion causing, initially, the up-regulation of IL-2 receptors and then binds to the IL-2 receptor (25). This process stimulates T cell proliferation, cytokine secretion and expression of growth factor receptors (125). Eosinophils and airway epithelial cells also produce IL-2 (126, 127). IL-2 levels are increased in BAL fluid in asthmatic patients with steroid resistant asthma and this response is not abolished by pre-treatment with oral corticosteroids (128). In asthma, IL-2 functions synergistically with IL-12 in promoting a predominant Th1 cellular response (129).

2.2.3 Interleukin-4 (IL-4)

IL-4 is produced by Th2 helper cells, eosinophils, basophils and mast cells. IL-4 synthesis is regulated by CD40 ligand expressed on CD4 T cells as well as by IgE Fcε receptor cross-linking in mast cells and basophils (130, 131). The IL-4 receptor is also found on activated B cells and T cells (132). In addition, the IL-4 receptor is expressed on mast cells, macrophages, endothelial as well as epithelial cells (132, 133). IL-4 induces phosphorylation of the IL-4-induced phosphotyrosine substrate, which is associated with the p85 subunit of phosphatidylinositol-3-triphosphate (PIP-3), as well as with Signal Transducer and Activator of Transcription (STAT) 6 and Janus protein kinase (JAK) (134). The transcription factor STAT6 is essential for mediation of the cellular effects of IL-4 (134). IL-4 also stimulates phosphatidylinositol (PI) hydrolysis yielding inositol triphosphate (IP3). IP3 promotes cellular calcium influx followed by increased intracellular cAMP levels and subsequent cellular activation (135).

The main function of IL-4 is in B cell activation, resulting in an increased expression of class II MHC molecules (136). It also promotes immunoglobulin (Ig) synthesis by B cells and plays a central role in Ig class switching of activated B cells to the synthesis of IgG4 and IgE subtypes (137). IL-4 promotes the development of Th2 CD4 cells and inhibits the development of Th1 CD4 cells (137). IL-4 also exerts effects on monocytes and macrophages by enhancing the surface expression of MHC class II molecules and the antigen-presenting capacity of macrophages, but inhibits the release of TNFα, IL-1, IL-12, IFNγ, IL-8 and macrophage

inflammatory protein (MIP)1α (138). Together with other cytokines such as granulocyte monocyte colony stimulating factor (GM-CSF) and IL-6, IL-4 can promote the growth of mast cells and myeloid cells. IL-4 also has inhibitory effects, such as suppression of metalloproteinase biosynthesis in human alveolar macrophages (139), inhibition of the expression of inducible nitric oxide synthase (iNOS) in human epithelial cells (140), and reduction of RANTES and IL-8 expression in human airway smooth muscle cells (141).

In asthma, IL-4 has pleotropic effects but the most notable include induction of airway goblet cell metaplasia and pulmonary eosinophilia (142). Furthermore, IL-4 is vitally important in the early stages of Th2 cell development, which can influence the maintenance of an allergic phenotype (143-145).

2.2.4 Interleukin-5 (IL-5)

IL-5 is produced by T lymphocytes and its transcription is upregulated in patients with asthma (146). It is a potent growth and differentiation factor for eosinophils and prolongs the survival of eosinophils (147). IL-5 also serves as a potent chemoattractant for recruiting eosinophils to areas of inflammation, especially the airways of patients with asthma (148, 149). The expression of the IL-5 receptor is restricted to eosinophils and their immediate precursors. There is an increase in the number of IL-5 receptors in bronchial biopsies from asthmatic patients with the transcription of IL-5 receptor messenger ribonucleic acid (mRNA) proteins occurring predominantly within eosinophils (150). IL-5 levels in serum and BAL fluid correspond with severity of asthma and decrease after treatment with corticosteroids (151, 152). In patients with asthma, IL-5 serves to promote an eosinophilic asthma phenotype.

2.2.5 Interleukin-6 (IL-6)

IL-6 is secreted by monocytes, macrophages, T cells, B cells, fibroblasts, bone marrow stromal cells, keratinocytes and endothelial cells (153). Human airway smooth muscle cells, upon activation by IL-1β or TGFβ, can also release IL-6 (154). IL-6 has growth-regulatory effects on many cells and is involved in T cell activation, growth and differentiation (112). It is a terminal differentiation factor for B cells and induces Ig (IgG, IgA and IgM) secretion (155). In asthmatic patients, IL-6 is an important cofactor for IL-4 dependent IgE synthesis (156). IL-6 is a pleiotropic cytokine with both pro-inflammatory as well as anti-inflammatory effects. Besides being a cofactor in IgE synthesis it can also inhibit the expression and release of IL-1 and TNFα

from macrophages in vitro and can inhibit endotoxin-induced TNF production and neutrophil influx into the airways in vivo (157, 158). More importantly, IL-6 has been shown to promote Th2 differentiation of CD4 T cells while suppressing Th1 differentiation, as well as modulating the intensity of the immune response by inhibiting T regulatory (Treg) cell development (159). IL-6 may also be a key factor in determining the balance of CD4 T cells in becoming Treg or inflammatory Th17 cells (160). Hence, IL-6, besides being a marker for inflammation, may have a more functional role in driving the immunopathogenesis of asthma (161).

2.2.6 Interleukin-8 (IL-8)

IL-8 is a member of the CXC chemokine subfamily of cytokines. It acts as a chemoattractant for the migration of neutrophils, monocytes and eosinophils to sites of inflammation, injury or infection (162). In neutrophils, IL-8 induces shape changes, exocytosis of stored proteins and the respiratory burst, resulting in the release of superoxide anion and hydrogen peroxide. IL-8 concentration levels are elevated in sputum, BAL fluid and bronchial tissues of patients with pulmonary diseases such as severe asthma, occupational asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD) (163). The potential sources of IL-8 in the airways include neutrophils, airway epithelial cells, fibroblasts, mast cells and macrophages (164). IL-8 promotes a neutrophilic inflammatory phenotype in some asthmatic patients, and these patients have a more severe illness course (165).

2.2.7 Interleukin-10 (IL-10)

Various cell types including cytotoxic T cells, activated monocytes, alveolar macrophages, peripheral blood T cells and mast cells produce IL-10 (111, 166). Constitutive IL-10 secretion occurs in healthy lungs, with the major source being alveolar macrophages, but circulating monocytes appear to be able to secrete more IL-10 than alveolar macrophages (167). The IL-10 receptor is a member of the class II subgroup of cytokine receptors (the IFNγ receptor family). The IL-10 receptor is highly effective in recruiting the signalling pathways of IL-6-type cytokine receptors, including STAT factor 1 and 3 (168). IL-10 is a pleiotropic cytokine that can exert either an immunosuppressive or an immunostimulatory effect on a variety of cell types.

In asthma, IL-10, via its production from Treg cells, functions as a potent inhibitor of monocyte/macrophage function, suppressing the production of several pro-inflammatory

cytokines, including TNF-α, IL-1β, IL-6, MIP-1α and IL-8 (169, 170). IL-10 inhibits the stimulated release of RANTES and IL-8 from human airway smooth muscle cells in culture (141). IL-10 also inhibits IFNγ and IL-2 production by Th1 lymphocytes (171), as well as IL-4 and IL-5 production by Th2 cells (111) by interfering with B7/CD28-dependent signals (172). IL-10 also inhibits eosinophil survival and IL-4 induced IgE synthesis (111).

2.2.8 Interleukin-12 (IL-12)

Antigen-presenting cells (APC), B cells and macrophages secrete IL-12 (173, 174). IL-12 acts synergistically with IL-2 to increase cytotoxic T cell responses as well as promote induction of IFNγ synthesis by resting human peripheral blood mononuclear cells (174). IL-12 stimulates natural killer cells and T cells to produce IFNγ (175). Furthermore, IL-12 promotes in vitro differentiation of mouse and human T cells into IFNγ and TNF-α secreting cells (176). More importantly, IL-12 inhibits the differentiation of T cells into IL-4 secreting cells (176, 177). IL-12 also indirectly inhibits IL-4 induced human IgE responses by IFNγ-dependent and independent mechanisms (178). Thus, IL-12 can primarily regulate Th1 cell differentiation, while suppressing the expansion of Th2 cell clones by early priming of undifferentiated T-helper cells for IFNγ transcription and secretion (177, 179).

In asthma, IL-12 plays an important role in promoting the development of Th1 cell responses, while inhibiting the development of Th2 cell responses and IgE synthesis (180, 181). This dichotomy whereby IL-12 functions synergistically with IL-2 in promoting the development of a Th1 cell response directed against infections and exposures to microorganisms, and/or their components particularly early in life, has formed the basis of the “Hygiene Hypothesis” (182). Epidemiological evidence collected over the last 50 years from observations of living conditions within larger families and farm workers suggests that children raised in a more sanitary, modern environment have an increased susceptibility to develop allergic conditions and asthma (183). This is the premise for the hygiene hypothesis, and it proposes that children who are exposed to childhood infections as a result of unhygienic contact with siblings and the environment, have a lower prevalence of atopy, eczema, hay fever, and asthma (184, 185).

2.2.9 Interleukin-13 (IL-13)

IL-13 is synthesised by activated CD4 and CD8 T lymphocytes, as well as by Th1, Th2, and Th0-like CD4 T cell (186). IL-13 is a potent modulator of human monocyte and B cell function

(186). IL-13 suppresses the synthesis of pro-inflammatory cytokines, chemokines and colony-stimulating factors including IL-1β, IL-6, IL-8, IL-10, IL-12, IFNγ and GM-CSF from blood monocyte; MIP-1α, IL-1 and TNFα from human alveolar macrophages; and RANTES and IL-8 from airway smooth muscle cells (141, 187-190). These actions of IL-13 are similar to those of IL-4 and IL-10 (111). In contrast to IL-4 and IL-10, IL-13 stimulates the synthesis of IL-1RA from blood monocytes (191).

In patients with asthma, increased expression of IL-13 mRNA has been reported in the airway mucosa, and levels of IL-13 together with IL-4 are increased after a segmental airway allergen challenge (192-194). IL-13, synergistically with IL-4, exerts a pro-remodelling effect on airway smooth muscle cells (195). IL-13 exerts this effect by inducing the expression of several genes in airway epithelial cells. This promotes epithelial goblet cell metaplasia, and by increasing epithelial–mesenchymal signalling, leads to increased sub-epithelial collagen deposition and hyperplasia of airway smooth muscle cells (93). The major genes induced by IL-13 include POSTN, CLCA1, and SERPINB2 and their over-expression leads to airway remodelling (196). Thus, in asthma, IL-13 promotes airway epithelial cell metaplasia, drives smooth muscle hyperplasia and sub-epithelial fibrosis eventually leading to airway remodelling.

2.2.10 Tumour Necrosis Factors (TNF)

TNF is synthesised in the body in two major isoforms i.e. TNFα and TNFβ. TNFα is released from cells by the proteolytic cleavage of the membrane-bound form by a metalloproteinase called TNF-converting enzyme, thereby releasing the soluble form of TNFα into plasma (197). TNFα is the most active isoform involved in the pathogenesis of asthma and will be discussed further. Several cells, including macrophages, T lymphocytes, mast cells and epithelial cells produce TNFα, but the principal source is macrophages (198). Its secretion is regulated by other cytokines such as IL-1, GM-CSF and IFNγ, all promoting the transcription of TNFα. Human eosinophils, together with airway epithelial cells, are also capable of releasing TNFα (199).

TNF receptors are expressed on most cell types, except red blood cells and resting T-lymphocytes. After TNFα binds to a receptor several signalling pathways are activated, leading to the transcription of different molecules including nuclear factor kappa B(NF-κB), activator protein 1 (AP-1) and TNF receptor associated adaptor factor proteins (TRAF). The TRAF family of adaptor proteins, particularly TRAF-2, are involved in signalling from TNF receptors

via the regulation of nuclear transcription of various proteins (200). TRAF-2 also plays a role in the signal transduction from the TNF receptors to activation of the mitogen-activated protein (MAP) kinase cascade (200).

Most of the actions of TNFα occur in combination with other cytokines as part of the cytokine network. Of note, the effects of TNFα are very similar to those of IL-1β, because there are close interactions between the signal transduction pathways of these two cytokines (201). In asthmatic patients, TNFα is a potent stimulant for airway epithelial cells to produce RANTES and IL-8 (140, 202). TNFα increases the expression of ICAM-1 and together with IL-4 and IFNγ, increases VCAM-1 expression on the surface membrane of endothelial cells. In patients with asthma, this results in the adhesion of pro-inflammatory leukocytes, such as neutrophils and eosinophils to the airway endothelial surface (111). TNFα enhances the expression of class II MHC molecules on antigen-presenting cells as well as the release of IL-1 by these cells. It acts as a co-stimulatory factor for activated T lymphocytes, enhancing proliferation and expression of IL-2 receptors. TNFα also induces proliferation of fibroblasts and this may contribute to airway remodelling in patients with uncontrolled asthma (203). Thus, in asthma, TNFα amplifies airway inflammation by increasing transcription of pro-inflammatory cytokines and promoting inflammatory cell chemotaxis (204).

2.2.11 Interferon γ (IFNγ)

IFNγ is a type II IFN produced by CD4, CD8 and NKT cells IFNγ (1), and its receptor is expressed on lymphocytes, granulocytes and epithelial cells (205). IFN-γ transcription is induced by IL-12 and IL-18 and it acts as an autocrine cytokine priming macrophages and epithelial cells for antimicrobial actions, such as inducing nitric oxide (NO) production (4, 13, 38, 39, 40). IFNγ is structurally different from the type I and III IFNs, which promote innate immune activation through the TLR pathways by activating STAT transcription factors in response to viral pathogens (39). IFNγ is a potent inhibitor of IL-4-induced IgE synthesis and amplifies the release of TNFα from alveolar macrophages induced by IgE triggering (111). IFNγ also acts synergistically with the effects of TNFα by increasing the production of RANTES from airway smooth muscle cells (111, 141, 206). Paradoxically, IFNγ inhibits IL-10 production from monocytes, which leads to an up-regulation of TNFα transcription (207, 208). Thus, in asthma, IFNγ promotes cell-mediated cytotoxic responses (i.e. Th1 responses) while inhibiting allergic inflammation and IgE synthesis (i.e. Th2 responses) (111).

2.3 Chemokine mediators of airway inflammation

Chemokines are chemotactic cytokines that are involved in attracting leukocytes into tissue and regulating leucocyte trafficking (1). They are divided into families based on their structure (209). The major groups are C-C chemokines (β-chemokines), in which two cysteine residues are adjacent to each other, and the CXC chemokines (α-chemokines), in which the cysteine residues are separated by another amino acid. The two major sub-families of chemokines that perpetuate the inflammation in asthma differ in the cells they primarily attract (210). The C-C chemokines, including RANTES and eotaxin, selectively recruit eosinophils into the airways making them extremely relevant in the pathogenesis of eosinophilic asthma (211-213). The CXC chemokines, on the other hand, attract neutrophils and thus play a role in non-eosinophilic asthma (214).

2.3.1 Regulated on Activation, Normal T Expressed and Secreted (RANTES/CCL5)

RANTES is a member of the C-C chemokine family and is a product of activated T cells (215). Epithelial cells stimulated by IL-1β or TNFα produce RANTES (212, 216). RANTES is a powerful eosinophil chemoattractant and induces exocytosis of eosinophil cationic protein (ECP) from eosinophils (211, 213). Co-operation between IL-5 and C-C chemokines (i.e. RANTES) is essential for mobilisation of eosinophils from the bone marrow during allergic reactions. This local release of chemokines induces homing and migration of eosinophils into bronchial airway tissues (111, 217). In patients with asthma, including mild asthmatics, RANTES expression is upregulated as demonstrated on bronchial biopsies from these patients (218).

2.3.2 Eotaxin / CCL11

Eotaxin is unique in that it is a potent chemoattractant for eosinophils, but not chemotactic for mononuclear cells or neutrophils (111). In patients with asthma, its synthesis is upregulated by IL-4 and TNFα. It is released by a variety of cells in the airways including alveolar macrophages and airway epithelial cells (111). In asthma, eotaxin not only functions as the most important chemoattractant for eosinophils, but it is also responsible for eosinophil activation and degranulation (212). Furthermore, eotaxin is also responsible for promoting a positive feedback loop that produces a continued Th2 cytokine-driven airway inflammation in patients with asthma (219, 220).

2.4 Lipid-derived (eicosanoid) mediators of airway inflammation - Leukotrienes (LTs)

LTs are potent lipid mediators produced by arachidonic acid metabolism in the cell membrane or nuclear membrane of granulocytic cells (i.e. mast cells, basophils and eosinophils) (221, 222). They are derived from arachidonic acid, which is released from membrane phospholipids via the activation of phospholipase A2 (221). The free 5-lipoxygenase (5-LO) enzyme found in the cytoplasm of cells cannot metabolise arachidonic acid, but after it has been activated, it is translocated to the cells nuclear membrane. Here a membrane-bound protein, termed 5-Lipoxygenase-activating protein (FLAP), stabilises the translocated 5-LO, thus allowing it to transform arachidonic acid into LTA4 (223, 224). LTA4 is further metabolised to either LTC4 via the activation of LTC4 synthase, or to LTB4 by LTB4 hydrolase. After release into the extracellular environment, LTC4 can be further metabolised to LTD4 and LTE4 by cleavage of the peptide side chain of LTC4. In patients with asthma, eosinophils are a rich source of LTC4 (225). LTC4, LTD4 and LTE4 are the active constituents of what was once termed “slow-reacting substance of anaphylaxis”(111, 226).

The biological effects of LT occur via their ability to bind and stimulate specific receptors characterised as:

- Leukotriene B4 (BLT) receptors activated by LTB4 and 12-(*R*)-hydroxyeicosatetraenoic acid (HETE) (227)
- Cysteinyl leukotriene (cys-LT) receptors activated by the cys-LTs (i.e. LTC4, LTD4 and LTE4) (226).

BLT receptors are expressed on lymphocytes as well as granulocytes. There are currently 2 distinct BLT receptors identified (228). BLT1 is a high affinity receptor, and BLT2, a low-affinity receptor (227). The BLT receptor is a G-protein-coupled receptor and is responsible for maturation and differentiation of hematopoietic stem cells as well as recruitment of neutrophils and eosinophils into inflamed airways (229).

Cys-LT1 receptors mediate all of the known airway effects of cys-LT in humans (230). In patients with asthma cys-LT are potent bronchospastic agents and increase vascular permeability in the airways leading to airway oedema as well as increased mucous secretion (231). The latter effects are mediated via the cys-LT effects on goblet cells and submucosal

glandular cells (231-233). Elevated levels of cys-LT have been detected in plasma, BAL fluid and sputum samples obtained from asthmatic patients during spontaneous exacerbations of their asthma or after allergen exposure (234) (Figure 2.2).

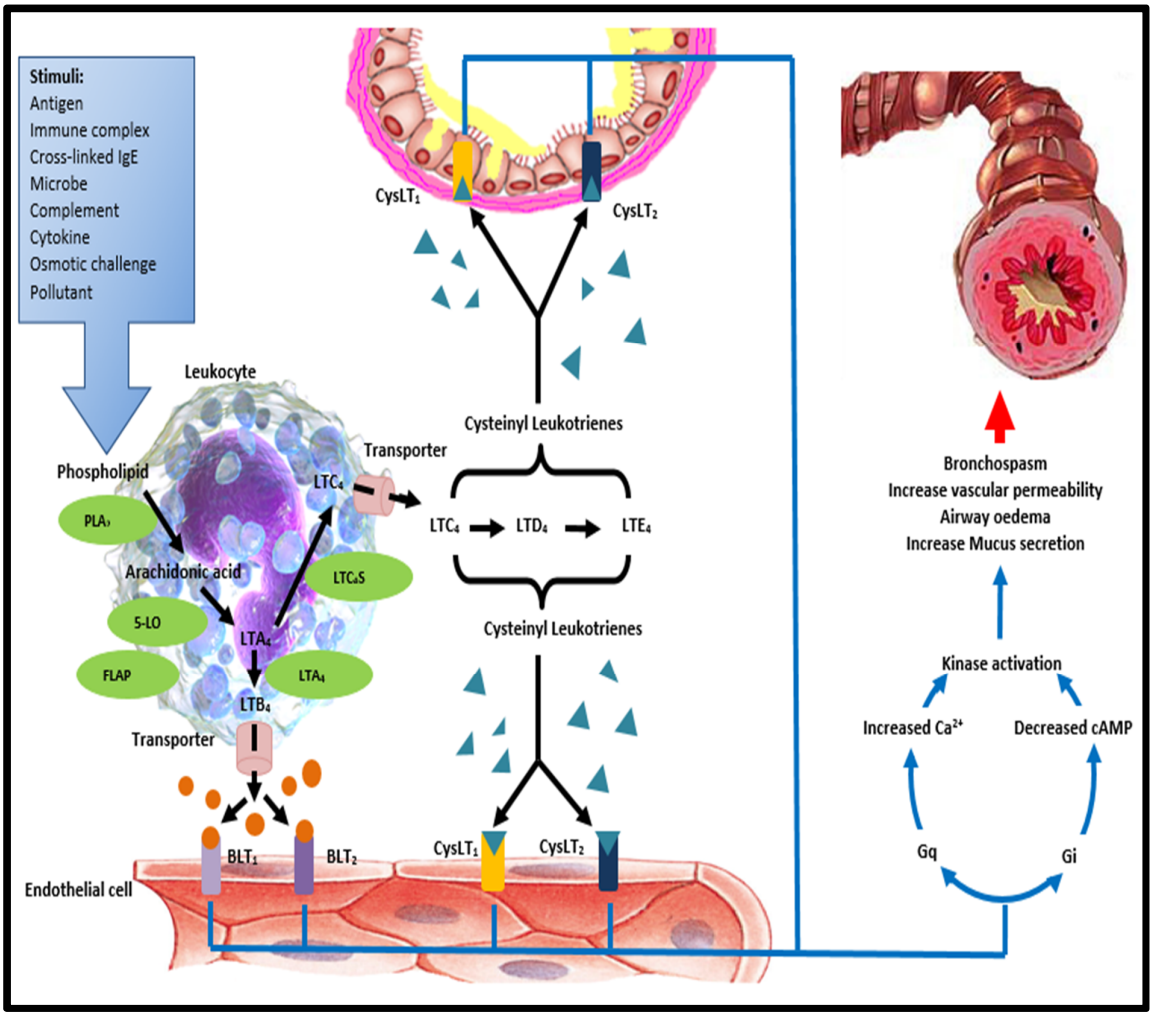

Figure 2.2: Leukotriene interactions in the pathogenesis of asthma. Adapted from Peter-Golden *et al.* (235)

PLA2: Phospholipase A2; FLAP: 5-Lipoxygenase-activating protein; 5-LO: 5-Lipoxygenase; LTA4H: Leukotriene A4 hydrolase; LTC4S: Leukotriene C4 Synthase; LTB4: Leukotriene B4; LTC4: Leukotriene C4; LTD4: Leukotriene D4; LTE4: Leukotriene E4; BLT1: B Leukotriene receptor 1; BLT2: B Leukotriene receptor 2; CysLT1: Cysteinyl leukotriene receptor 1; CysLT2: Cysteinyl leukotriene receptor 2; Gq: G protein; Gi: G protein, Ca^{2+}: Calcium; cAMP: Cyclic adenosine monophosphate.

2.5 Regulators of airway remodelling – growth factors

Persistent airway inflammation leads to airway remodelling. This process is mediated by several cytokines and growth factors, of which vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) are the most important. They will be discussed in more detail below.

2.5.1 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells (236). The expression of VEGF is increased in response to hypoxia by activated oncogenes and by a variety of cytokines including transforming growth factor β (TGFβ), IL-1β and IL-6 (217, 237-239). VEGF expression is also upregulated by nitric oxide and hypoxia inducible factor (HIF) (240). Nitric oxide contributes to the blood vessel permeability effects of VEGF and to VEGF-stimulated vasodilatation (241, 242). The production of nitric oxide is in turn upregulated by VEGF, indicating a positive feedback loop between these two factors (236, 243).VEGF plays a prominent role in promoting and maintaining airway remodelling in asthmatic patients (48).

2.5.2 Transforming Growth Factor β (TGFβ)

Transforming growth factor beta (TGFβ) is part of a super family of cytokines that have several regulatory functions in angiogenesis, wound healing and embryonic development (244). TGFβ is constitutively produced by activated monocytes (34), as well as pulmonary macrophages (245). Fibroblasts, granulocytes, airway smooth muscle cells and airway epithelial cells also secrete TGFβ (246-248). TGFβ is a growth modulating cytokine that regulates the turnover of cellular matrix proteins (249). TGFβ promotes airway epithelium repair by inducing differentiation of normal airway myoepithelial cells into fibroblasts and stimulating airway fibroblasts to produce collagen and other extra-cellular matrix proteins (250, 251). TGFβ signalling promotes sub-epithelial fibrosis by increasing the deposition of extracellular matrix, airway remodelling by increasing airway smooth muscle mass and airway microvascular congestion through enhancing the production and secretion of several proangiogenic factors, including VEGF (48, 244, 252).

The secreted TGFβ cytokine exists in three isoforms i.e. TGFβ1, TGFβ2 and TGFβ3. TGFβ1 plays a critical role in controlling immunity as it is secreted by most immune cells and acts on

various cells promoting cellular proliferation, differentiation, chemoattraction and production cytokines such as TNFα and IL-1 (253). In patients with asthma, TGFβ1 through signalling via the Smad pathway, promotes transformation of airway epithelium into the epithelial-mesenchymal trophic with terminal differentiation into fibroblasts which form collagen and secrete growth factors, perpetuating the remodelling process (49). TGFβ2, also known as glioblastoma-derived T-cell suppressor factor (G-TSF), plays a role in embryonic development and suppresses the effects of interleukin-dependent T-cell tumours (254). It affects the development of the cardiac, lung, and the urogenital systems. TGFβ3 regulates cellular adhesion molecules, extracellular matrix formation and lung and palate development (252).

In asthma there is greater expression of TGFβ1 mRNA and its expression correlates with the severity of asthma and the degree of sub-epithelial fibrosis (252, 255). Asthmatic airway inflammation is modulated by the upregulation of Treg cells by TGFβ (100), and this has been confirmed by the documented release of TGFβ1 into BAL fluid after a segmental airway allergen challenge (256). Treg cells have a significant immunosuppressive potential within the airways in patients with asthma (103). Their function is regulated by the intracellular transcription factor forkhead box P3 (Foxp3) (101), and via a positive feedback loop, TGFβ increases the expression of Foxp3, which promotes the transition of naïve T cells toward a Treg endotype. This has the potential to modulate other T cell activation, including Th1 and Th2 cells, by inhibiting the expression of effector cytokines including IL-4, TNFα, IFNγ, IL-17 and IL-21. Thus, TGFβ ultimately functions as a predominantly immunosuppressive cytokine in the asthmatic airway, while still promoting airway remodelling (104).

2.6 Systemic mediators of airway inflammation – serum proteins

Concentrations of non-specific acute-phase reactants and serum immunoglobulin levels correlate with the amount of airway inflammation. Thus, their measurement may be of value in the diagnosis and treatment of asthmatic airway inflammation. The most important of these molecules include C–reactive protein (CRP), serum amyloid associated protein (SAAP) and immunoglobulin E (IgE) and they will be discussed in more detail below.

2.6.1 C–Reactive Protein (CRP)

CRP is a highly conserved molecule belonging to the family of pentraxins. It was discovered in humans in 1930 as a serum component that bound the C polysaccharide of *Streptococcus*

pneumoniae (257). CRP is an integral member of the acute-phase protein response, which includes structurally unrelated mannose-binding protein and fibrinogen (258). Levels of these acute phase proteins rise rapidly during infection, inflammation and after injury. The transcription of CRP is triggered by IL-6 and amplified by IL-1β (257). It is predominantly produced in the liver, but monocytes, lymphocytes and inflamed lung also have the ability to produce CRP (257). CRP functions as an opsonin to agglutinate bacteria and promote phagocytosis and is directly bactericidal (259). CRP activates the classical complement cascade, which can be intensely pro-inflammatory, by binding directly to the complement fragment C1q (260). CRP also activates NF-κB in endothelial cells and mononuclear cells to induce proteases and pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and IL-18 (261). In patients with asthma, CRP is measured as a non-specific marker of airway and systemic inflammation (262, 263).

2.6.2 Serum Amyloid Associated Protein (SAAP)

SAAP is another acute-phase reactant induced by several inflammatory mediators including IL-6, IL-1β and TNFα.SAAP is secreted from the liver as the predominant apolipoprotein associated with plasma high density lipoprotein cholesterol (264). The major physiological function of SAAP has not yet been clearly established but prolonged elevation of plasma SAAP levels, as in chronic inflammation, results in a pathological condition called secondary amyloidosis (265). SAAP induces mast cell adhesion, migration and adhesion of T cells and migration, adhesion and tissue infiltration of monocytes and polymorphonuclear leukocytes to the extracellular matrix (266). SAAP also enhances eicosanoid synthesis by human monocytes (267). Measurement of SAAP is of value in the assessment of ongoing airway inflammation and response to therapy with anti-inflammatory medications (268).

2.6.3 Immunoglobulin E (IgE)

IgE is a protein that belongs to the repertoire of antibodies produced in the human body. Antigen specific IgE production by the body is promoted when specific antigens are recognised by toll like receptors (TLRs) on antigen-presenting cells (APCs) lining the airways (269). These aeroallergens are processed intracellularly by the APCs, and then presented to naive T cells. This process is unique in that the T helper cells, under the influence of the cytokines IL-4 or IL-13, acquire a Th2 phenotype (11, 19, 26). This process forms the basis of the immunopathogenesis of asthma (1).

Th2 cells then engage B cells through the B cell class II MHC and co-stimulatory molecules, and in the presence of IL-4 and IL-13 promote the switching of antibody production by B-lymphocytes from the IgG subclass to the IgE subclass (17). This Th2 - B lymphocyte co-stimulation includes a second signal that is delivered when CD40 on the B lymphocyte binds to its ligand on the Th2 lymphocyte (270, 271). IgE antibody class switching can also be induced by IL-4 and/or IL-13 derived from cells other than the Th2 lymphocyte, which include mast cells and basophils (272). These antigen specific IgE antibodies produced by the primed B cell (post class switching) circulate in the blood before binding to either the high-affinity IgE receptors (FcεRI) found on the surface of mast cells in tissue or basophils in blood, or to the low-affinity IgE receptors (FcεRII / CD23) found on the surface of lymphocytes, eosinophils, platelets and macrophages (273).

Once an individual has developed IgE antibodies to certain antigen epitopes, several secondary priming mechanisms lead to a more robust IgE response. IgE priming is mediated by the binding of antigen to the low-affinity IgE receptors (FcεRII / CD23) (269, 274). In a patient with asthma, whose tissue mast cells already have antigen specific IgE bound to the high affinity FcεRI, re-exposure to the original antigen results in the crosslinking of adjacent FcεRI-bound IgE (273) (Figure 2.3). This process triggers a complex subcellular signalling event in mast cells and basophils that results in the secretion of biologically active proteins stored within cytoplasmic granules within these cells, a process known as mast cell degranulation (275). The proteins released include histamine, serotonin, and proteases such as tryptases. Furthermore, newly formed lipid-derived mediators, for example, prostaglandin D2 (PGD2), LTC4, LTD4 and LTE4 are synthesised and released as well.

These proteins, once released, induce a response termed an immediate hypersensitivity (or early phase) reaction within minutes of their release. If localised to the airways, this response is characterised by increased vascular permeability, contraction of the airway smooth muscle and enhanced secretion of mucus, resulting in acutely reduced airflow and wheezing. These changes are responsible for the characteristic clinical features seen in a patient with asthma (276). This early-phase reaction usually resolves within an hour. However, in some individuals, a second phase of inflammation, called the late phase reaction, develops at the site of antigen challenge, typically beginning a few hours after antigen exposure. This late-phase reaction with obstruction of airflow develops as a result of cytokines and chemokines generated by resident

activated inflammatory cells (e.g., mast cells, macrophages, and epithelial cells) and recruited inflammatory cells (lymphocytes and eosinophils) (273, 276). This late phase reaction is responsible for the morbidity and sometimes mortality associated with acute asthmatic attacks (Figure 2.3).

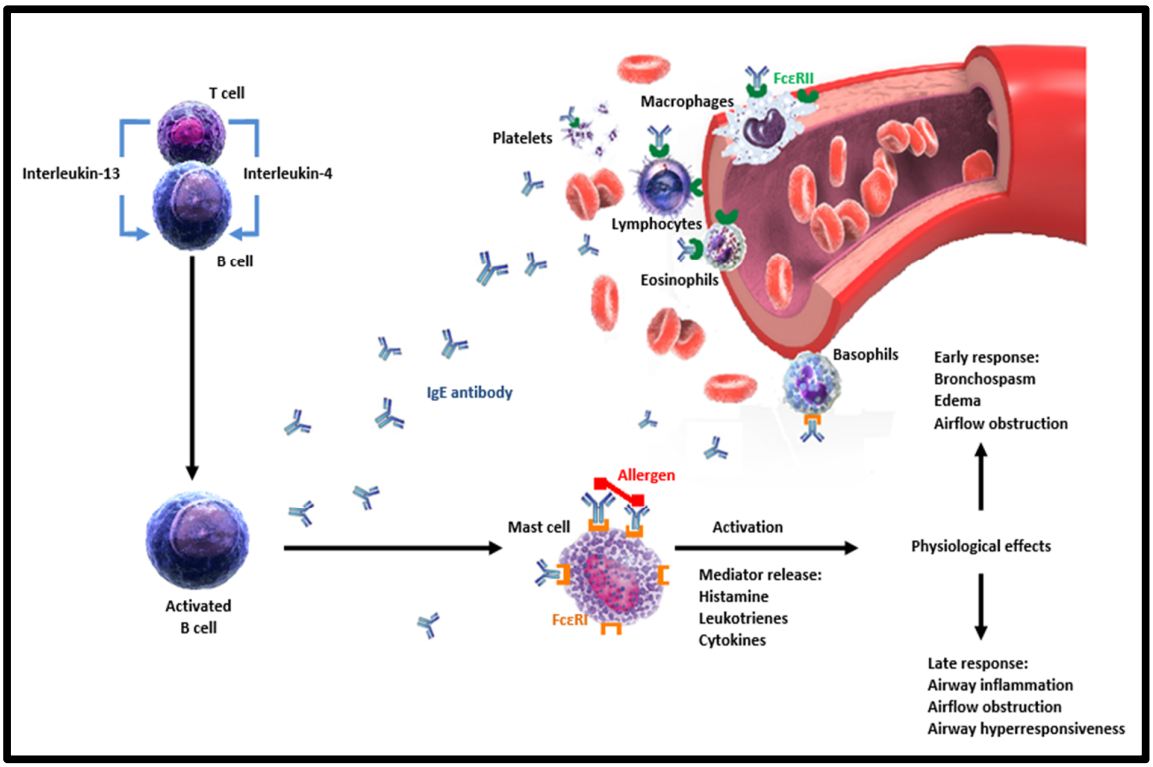

Figure 2.3: IgE interactions in the pathogenesis of asthma. Adapted from Busse *et al.* (273)
FcεRI: High-affinity IgE receptors, FcεRII: Low-affinity IgE receptors.

2.7 Exhaled breath condensates – Nitric Oxide (NO)

There is an overwhelming body of evidence suggesting that endogenous NO plays a key role in physiological regulation of airway smooth muscle functions and hence, NO has been implicated in the pathogenesis of several airway diseases, including asthma (277). NO is a gas that is derived from the amino acid L-arginine by the enzyme nitric oxide synthetase (NOS). There are three important isoforms of the enzyme NOS. Neuronal tissue NOS (nNOS) together with endothelial and epithelial tissue NOS (eNOS) are constitutively expressed on neuronal tissue and endothelial and epithelial tissue respectively. The third isoform, inducible nitric oxide synthase (iNOS), is not normally expressed, but is induced by inflammatory cytokines and endotoxins (278). This isoform once induced, produces much larger amounts of NO than do the constitutively expressed nNOS and eNOS. NO produced by nNOS and eNOS are

involved in physiological regulation of airway function. NO produced by iNOS is synthesised predominantly in inflammatory diseases of the airways including host defence responses to infection (278-280). In asthmatic airways, there is increased immunocytochemical staining for iNOS, which is localised predominantly in airway epithelial cells (281). There is also localisation to inflammatory cells, including macrophages and eosinophils (281, 282). NF-κB induced by pro-inflammatory cytokines also plays a critically important role in increasing the transcription of the iNOS gene (283).

Corticosteroids inhibit the induction of iNOS in epithelial cells, most likely by a direct inhibitory interaction between the activated glucocorticoid receptor and NF-κB (284). NO does not have conventional receptors but diffuses into cells and activates soluble guanylcyclase, resulting in an increase in the formation of cyclic guanosine monophosphate (cGMP). While NO has many different effects on airway function, the effects of endogenous NO depends on the site of its production and the quantity produced (1).

In the context of asthma, the increased production of NO leads to the formation of hydroxyl radicals by the generation of peroxynitrite, which results in increased plasma exudation, increased mucous secretion and epithelial shedding (285). NO inhibits Th1 lymphocytes and favours the development of a Th2 response (286, 287). NO also promotes eosinophilia and eosinophil chemotaxis (288). Airway inflammation resulting from the activation of mast cells and antigen-specific Th2 lymphocytes leads to the increased production of IL-4, IL-5 and IL-13 (32). IL-4 and IL-13 subsequently cause up-regulation of epithelial iNOS via STAT-6, a process that is corticosteroid sensitive (289). NO synthesised via the upregulated iNOS is thus a direct biomarker of the Th2-mediated, pro-inflammatory cytokine milieu that is characteristic of the pathophysiology of an allergic airway inflammatory process (290).

While NO plays a significant pathophysiological role in promoting airway inflammation in the asthmatic airways, its major role today is that it can be measured non-invasively as a marker of airway inflammation (288). In asthmatic patients, portable handheld devices can measure fractional exhaled nitric oxide (FeNO) levels. The FeNO values obtained from these devices have been shown to correlate with sputum eosinophils, blood eosinophils, as well as serum eosinophilic cationic protein (ECP) and serum IgE levels (291). Furthermore, not only is the FeNO level measured in exhaled breath condensates a biomarker of allergic airway

inflammation, but its measured level is also being used to titrate to a response to corticosteroid therapy in patients with asthma (290, 292, 293).

2.8 Assessment of small airway calibre

The pathological end result of airway inflammation is airway narrowing (17). Hence, assessment of airway calibre has been the cornerstone of therapeutic interventions in patients with asthma (7). Until recently the only tool available to assess airway calibre was spirometry (294, 295), but today, several heterogeneous methods for the assessment of the small airways are available. These can be either functional or static. Functional measurements include measuring airflow by spirometry and plethysmography (295), or measuring airway resistance with impulse oscillometry (IOS) (296). Static measurements of airway calibre are derived from imaging tools, in particular, high resolution computed tomography HRCT (297).

Measurement of static pulmonary volumes can provide indirect evidence of the state of the most distal airways, revealing two effects of their alteration, air trapping and dynamic hyperinflation, but it does not differentiate adequately between the larger and small airways (21). Assessment of small airway calibre using HRCT also has several potential limitations. These include technical issues with standardisation of radiological protocols, inaccuracy of assessment of small airway calibre when the airways have not been imaged in the correct plane and the safety issue of cumulative radiation exposure (298). Radiation exposure from CT imaging poses a significant risk factor for secondary malignancies and thus limits the potential repeated HRCT imaging as a viable tool in the assessment of the small airways (299).

In view of the limitations of using static techniques, in particular HRCT imaging, newer techniques of assessing the small airways have to be explored. Functional measurements of airways calibre are reproducible, safe and provide valuable real-time information of the state of the airways (300). Of the functional measurements of airway calibre available, impulse oscillometry has been the tool least investigated in the evaluation of adult patients with asthma, and hence will be explored in this thesis as a tool to assess the small airways in adults patients with asthma (294).

In this discussion, the topics have been limited to spirometry and impulse oscillometry as these techniques bear the most relevance to this thesis.

2.8.1 Spirometry

Spirometry is a measure of lung function using a device called a spirometer that measures specifically the amount (volume) and speed (flow) of air that can be inhaled and exhaled using a hot-wire anemometer. It is helpful in assessing breathing patterns that identifies conditions such as asthma, emphysema and pulmonary fibrosis (295). Spirometry generates pneumotachographs, which are charts that plot the volume and flow of air coming in and out of the lungs from one inhalation and one exhalation. The test is highly dependent on patient co-operation and effort, and thus the validity of the procedure has been standardised by the American Thoracic Society. The most common parameters measured in spirometry are vital capacity (VC), forced vital capacity (FVC), forced expiratory volume (FEV) at timed intervals of 0.5, 1.0 (FEV1), 2.0, and 3.0 seconds and forced expiratory flow 25–75% (FEF 25–75). Results are expressed as a percentage of predicted, using validated reference values, for patients of similar characteristics (height, age, sex, and sometimes race and weight). The interpretation of the results can vary but usually, results over 80% of predicted are often considered normal. This is in keeping with the ATS/ERS consensus document on "interpretative strategies for lung function tests" published by Pellegrino *et al.* (301). This consensus document defining lung function parameters below the fifth percentile of the predicted value (i.e. below the lower limit of normal (LLN)) or values below 80% of predicted as being abnormal. The document also defines a post-bronchodilator increase in the FEV1 $\geq$ 12% and $\geq$ 200 ml as indicative of a positive bronchodilator response indicating airway reversibility (i.e. bronchial airway hyper-reactivity (BAH)) (301).

The main shortcoming of conventional spirometry is that it is predominantly a measurement of airflow within larger airways (295). The small airways are those with an internal diameter of less than 2mm (22). The contribution of these airways to total airflow resistance is small in healthy individuals, but can represent up to 80% of total airflow resistance in asthmatic patients (302). Unfortunately, conventional spirometry does not provide a robust assessment of peripheral airway obstruction, despite the general information contained within the shape of the flow–volume curve and values of mid-flow rates (FEF 25–75 - forced expiratory flow between 25% and 75% of the forced vital capacity). Suspicion of small airways disease was often inferred from the spirometric flow-volume loop and by observing a reduction of the FEF 25–75. However, there is a wide variability in these values and the absence of a sufficiently validated cut-off point has limited its use in clinical applications (294).

Furthermore, while inflammation in the small airways contributes to the total respiratory resistance, the assessment of the small airways is so variable that extensive damage of the small airways may occur before the appearance of any symptoms, and more importantly, before any spirometric changes are detected (303). This is the reason why the small airways are characterised as the "silent zone" of airways (22, 294, 304). The result of the persistent airway inflammation is structural damage with airway remodelling and progressive loss of lung capacity (67, 68). The peripheral localisation of the small airways and their small diameter also makes direct assessment more difficult (72). Small airway inflammation can thus only reliably be assessed by invasive techniques such as transbronchial or open lung biopsy (305), hence, necessitating the need for reproducible, validated and non-invasive methods for assessing the small airways in patients with asthma.

2.8.2 Impulse Oscillometry - Forced Oscillation Technique (FOT)

The forced oscillation technique (FOT) is a novel measurement tool that can be used to differentiate obstruction of the small airways from that of larger airways using non-invasive techniques that are validated, reproducible and easy to use (302). It requires minimal subject co-operation and is thus suitable even for a patient who cannot cooperate or manage spirometry. There is currently only one commercially available FOT system in South Africa, called the impulse oscillometry system (IOS) by Jaeger Master-Screen (Hochberg, Germany) (296, 306). Impulse oscillometry (IOS) is an updated application of FOT, which was initially described by Dubois *et al*. 50 years ago (307, 308). The main difference is that in FOT, the sound waves of different frequencies are transmitted sequentially, whereas in IOS, sound waves with different frequencies are composed into a single impulse and transmitted simultaneously. This helps in reducing the time of test.

IOS is a modified application of echo and sonar technology whereby sound waves are transmitted into the lungs during normal tidal breathing. The purpose of IOS is to obtain information about lung mechanics passively, without the use of any forced inhalation or exhalation techniques by the patient. The electrically controlled deflection of a membrane within the loud speaker generates sound waves of different pressure and energy. The waves are directed into the lungs and a pneumotachograph measures the return waves of the sound impulse after it has bounced back from the lungs. The pneumotachograph is connected to two

transducers, each measuring the flow (V) and the pressure (P) of the return sound wave (Figure 2.4).

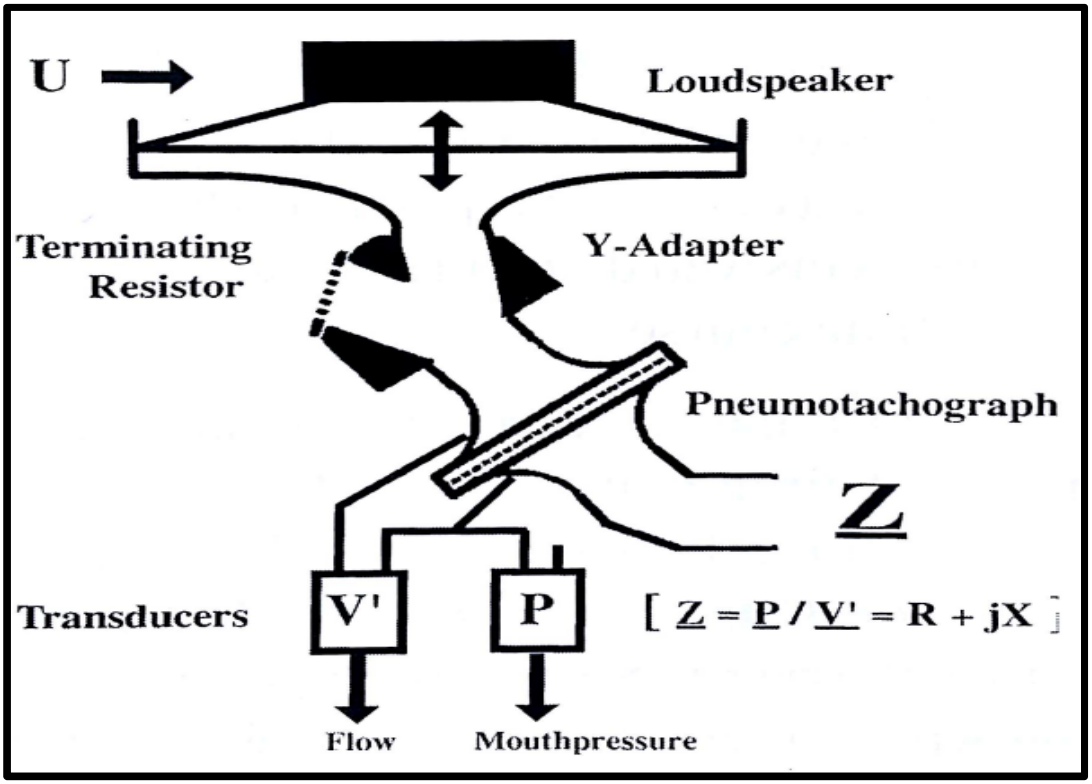

Figure 2.4: A diagrammatic representation of the basic principles of IOS (Courtesy of Jaeger Master-Screen)
Z: Impedance, R: Resistance, jX: Reactance, P: Pressure, F: Frequency, Fres: Resonant frequency, V': Flow, U: Mouthpiece.

Changes in the energy and frequency of the return wave are converted mathematically into information about the nature of the lungs. IOS uses the principle whereby disturbances in flow and pressure caused by the externally applied sound waves are recorded and used to calculate two main parameters. The first parameter calculated is the resistance to airflow, which is surrogate marker of airway calibre. The other important parameter calculated is reactance to the external sound wave applied, and relates to the measurement of storage and return of energy by the lungs in the form of a return sound, which is surrogate markers of lung compliance (309).

Resistance (R) is calculated as a ratio of pressure to flow (i.e. pressure (P) divided by flow (V); R = P/V). Sound waves at 5 Hz have longer wavelengths and travel to the distal airways. Therefore, the P/V measured by the IOS machine during a sound-wave pulse at 5 Hz estimates the resistance at the distal airways. Resistance to sound waves at 20 Hz (shorter wavelength) is

more likely to reflect the mechanics of the upper airway because the waves do not travel as far into the lungs as compared to the longer wavelength sound waves. Resistance (R) values measured include the resistance due to central airways (R20), total airways (R5) and the peripheral/small airways (R5-R20). Frequency-dependence of resistance (fdR or R5-R20) is simply the subtraction of the measured resistance at 20 Hz from the resistance at 5 Hz. Frequency-dependence of resistance is a characteristic of peripheral/small airway dysfunction (310).

Reactance (X) is a measurement of the lung capacitance, a property that reflects elasticity of the lung. This is a surrogate measurement of lung elastance, which is the inverse of lung compliance. Anything that reduces lung compliance (e.g., obesity, interstitial lung disease, small-airways disease) should produce a more negative value for X.

Impedance (Z) is a measurement of the sum of all forces that oppose the generated impulse, and it is derived from the ratio of the difference in pressure and changes in the flow of the sound wave at any specific frequency. Impedance includes the in-phase (real) component resistance (R), and an out-of-phase (imaginary) component reactance (X). Simply put, R can be viewed as the energy dissipation whereas X is viewed as energy storage (309). Z is the combination of R and X. The following paragraphs will elucidate the mathematical aspects of the IOS/FOT technique.

2.8.3 Impulse oscillometry - Technical aspects of application

IOS involves applying a forced perturbation of flow (i.e. a continuous sound wave applied at various frequencies) at the mouth down to the lungs and measuring the resulting pressure generated (306). This occurs while the subject continues to breathe quietly. IOS is thus a non-invasive measurement of pulmonary function that can examine the resistance of total (R5), large (R20), and small airways (R5–R20) separately. IOS also measures the low frequency reactance area (AX), considered a measurement of small airways dysfunction (311, 312). The rationale behind this modality of assessing airway calibre is that sound waves with frequencies greater than 15Hz are mostly damped out before reaching peripheral airways, while those with frequencies less than 10–15Hz can penetrate much further to the lung periphery and hence give vital information on the state of the smaller airways (313).

IOS derives its assessment of airway calibre by measuring *impedance* of the respiratory system to 'forced' pressure oscillations produced by a loudspeaker. Respiratory impedance is a complex quantity that includes both *resistance* and *reactance* (312). Resistance is the more familiar parameter, comprising the pressure-flow relationship of that portion of the pressure oscillation which is 'in phase' with airflow. "In phase" implies that the oscillation wave travels in parallel with the movement of air (i.e. in the same direction), thereby minimizing any potential energy that maybe lost by the sound wave as it traverses the airways. This in phase resistance is designated *Rrs* and essentially, it is a measure of the energy dissipated with the loss of resistance as the airways branch towards the periphery in patients with normal lungs. However, in patients with airway narrowing, as in patients with asthma, this resistance experienced by the sound wave may increase, depending on the calibre of the airways and the amount of bronchospasm present. Reactance, designated *Xrs,* is a complex quantity related to those portions of pressure oscillations that are not 'in phase' with airflow (i.e. moving in the opposite direction to the column of air during normal tidal breathing). Reactance is thus an imaginary component that measures the lung elastance during the lag phase of the sound impulse and the lung inertance during the leading phase of the sound wave. The most striking characteristic of IOS in relation to spirometry is that there is a relatively greater sensitivity of IOS to assess the peripheral airways more reliably (296, 312, 314).

2.8.3.1 Respiratory Resistance

The resistive component of respiratory impedance, *Rrs,* includes measurement of the proximal and distal airways (central and peripheral), lung tissue and chest wall resistance. Normally, the central airway resistance dominates this measurement, depending on airway calibre and the surface structure of the airway walls, while lung tissue and chest wall resistance usually contribute a negligible value in patients with airway diseases only (315). However, because inflammation is a cellular process, it has mechanical consequences as manifested by an increased tone of airway smooth muscles in patients with bronchoconstriction (19). When airway smooth muscle tone increases, *Rrs* increases because of decreased airway lumen size. Scarring or oedema in the walls of the airways also decreases the airway lumen, with an resultant increase in Rrs (313). Peripheral airways have a much smaller lumen than central airways, and inflammation and oedema in the walls of peripheral airways have a proportionately larger effect on lumen size than in larger airways. In asthmatic patients, both high-frequency and low-frequency *Rrs* decrease, but there is a relatively greater decrease in low-frequency *Rrs*

because of the proportionately larger effect of inflammation on the smaller airways (314). The lower frequency waves are designated R5 and higher frequency waves are designated R20.

2.8.3.2 Respiratory Reactance

The reactive component of respiratory impedance (i.e. respiratory reactance), *Xrs*, incorporates in its calculation the mass inertia force of the moving air column in the conducting airways. Low frequency capacitive *Xrs* (i.e. Xrs5) is essentially an expression of the ability of the respiratory tract to store capacitive energy. This adsorptive capacity is primarily resident in the lung periphery and is a surrogate marker for lung compliance (312). Hence, distal or low capacitive reactance at 5Hz (*Xrs*5) manifests increasingly negative values either with restricted or with hyperinflated lungs (i.e. implying stiff or over inflated lungs). Thus, *Xrs*5 characterises the lung periphery, but is nonspecific as to the type of limitation that is occurring within the peripheral airways (296). Interpretation of *Xrs*5 is clearly different from that of the conventional lung function test parameters, and in particular, lung compliance as its value is negative (Figure 2.5). The minus sign (-) differentiates elastic properties from moments of inertia, the latter of which are always positive. Therefore, the minus sign simply confirms a relationship with elastic properties of the lung or lack thereof i.e. the more negative the *Xrs*5 is, the less compliant - or alternatively, the more stiff the lungs are (312). Peripheral airway obstruction may result in relatively small increments to airways resistance because of the large cumulative cross-sectional diameter of all airways in the lower generations of airways. Conversely, if peripheral airway inflammation is decreased by administration of inhaled corticosteroids, peripheral airway lumen increases and the patency of small airways expand in the direction of the lung periphery (316). As a result, a larger surface area is presented to the low-frequency oscillations with a subsequent improvement in lung elasticity (i.e. increase lung compliance) and this acts to decrease the magnitude of low frequency *Xrs* (296).

AX is another parameter that can be obtained from IOS. It is a value derived from the area under the curve between the measurement of X5 and *Fres*, also sometimes called Goldman's Triangle (76, 296, 312) (Figure 2.6). It is a qualitative index of the total respiratory reactance *Xrs* at all frequencies between 5Hz and *Fres.* AX is a more sensitive measurement of the degree of peripheral airway obstruction (317, 318). There is a close correlation between the frequency dependence of resistance (fdR or R5-R20), which is simply the subtraction of the measured resistance at 20 Hz from the resistance at 5 Hz, and AX (Goldman's triangle) (310). The

demonstration of a change in the measured values of the above parameters (i.e. R5-R20 and AX) in patients with asthma is consistent with both parameters reflecting small airway dysfunction and hence the state of airway inflammation in patients with asthma (296, 313).
Figure 2.5 represents an IOS report of a patient with heathy lungs. By comparison, an example of an IOS report of a patient with peripheral / distal airway obstruction as would occur in a patient with asthma is depicted in Figure 2.7. It clearly shows the elevated R5 value in relation to the R20 value measured. Notably, the diagram clearly demonstrates that X5 is markedly more negative when compared with the normal reactance curve depicted in Figure 2.5.

Figure 2.8 represents an IOS report of a patient with central/proximal airway obstruction as would occur in a patient with tracheal stenosis. By comparison, in Figure 2.5 and 2.7, it can clearly be seen that the R5 value in relation to the R20 value measured are virtually similar. X5 remains negative when compared with the report of the patient with healthy lungs (Figure 2.5) but less negative when compared with the reactance curve in a patient with peripheral airway obstruction (Figure 2.7).

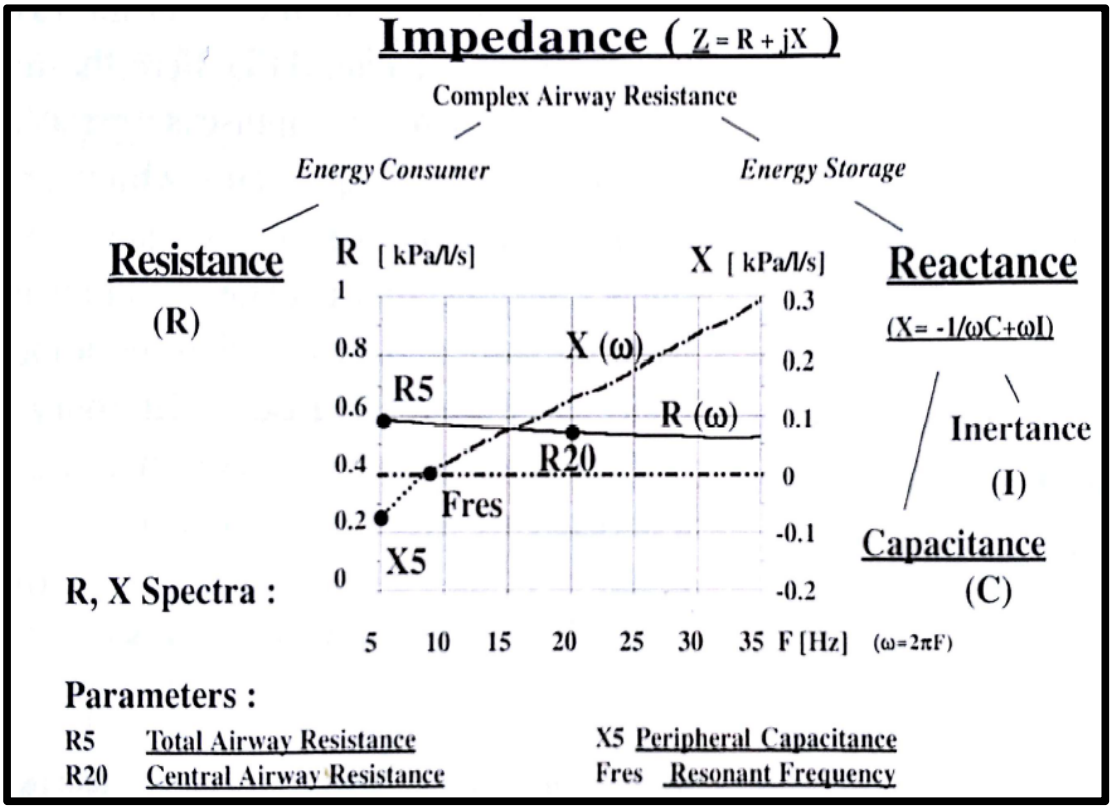

Figure 2.5: The IOS report of a patient with a healthy lung (Courtesy of Jaeger Master-Screen)
Z: Impedance, R: Resistance, X: Reactance, R5: Total airway resistance, X5: Reactance at lowest oscillation frequency of 5Hz = Peripheral capacitive reactance, Hz: Hertz, kPa/l.s^{-1}: Kilopascal per litre per second, F: Frequency, Fres: Resonant frequency, C: Capacitance, I: Inertance.

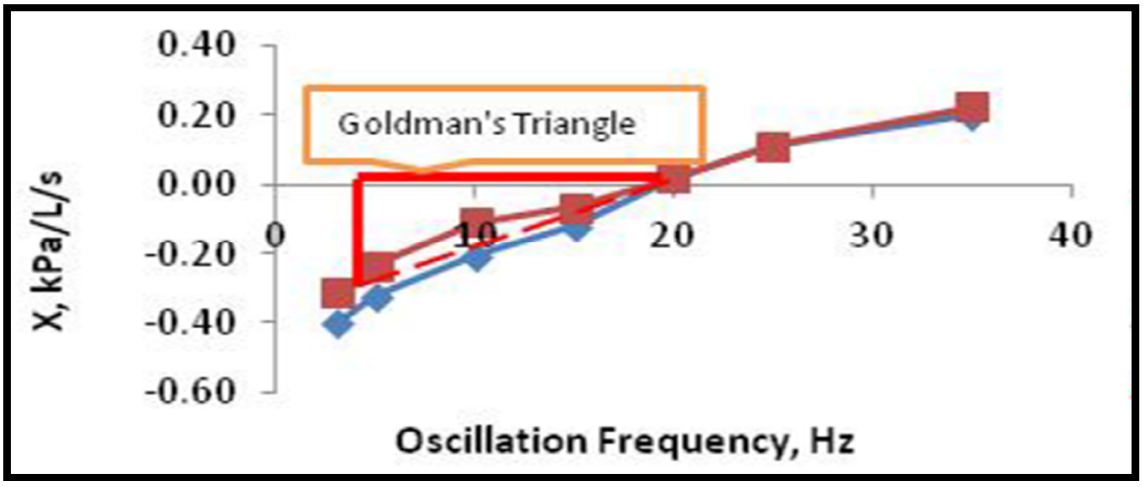

Figure 2.6: AX (Goldman' triangle) value on the IOS reactance curve - a sensitive measurement of the degree of peripheral airway obstruction (Courtesy of Jaeger Master-Screen).With permission from Meraz *et al.* (314)
X: Reactance, kPa/l.s^{-1}: Kilopascal per litre per second, Hz: Hertz.

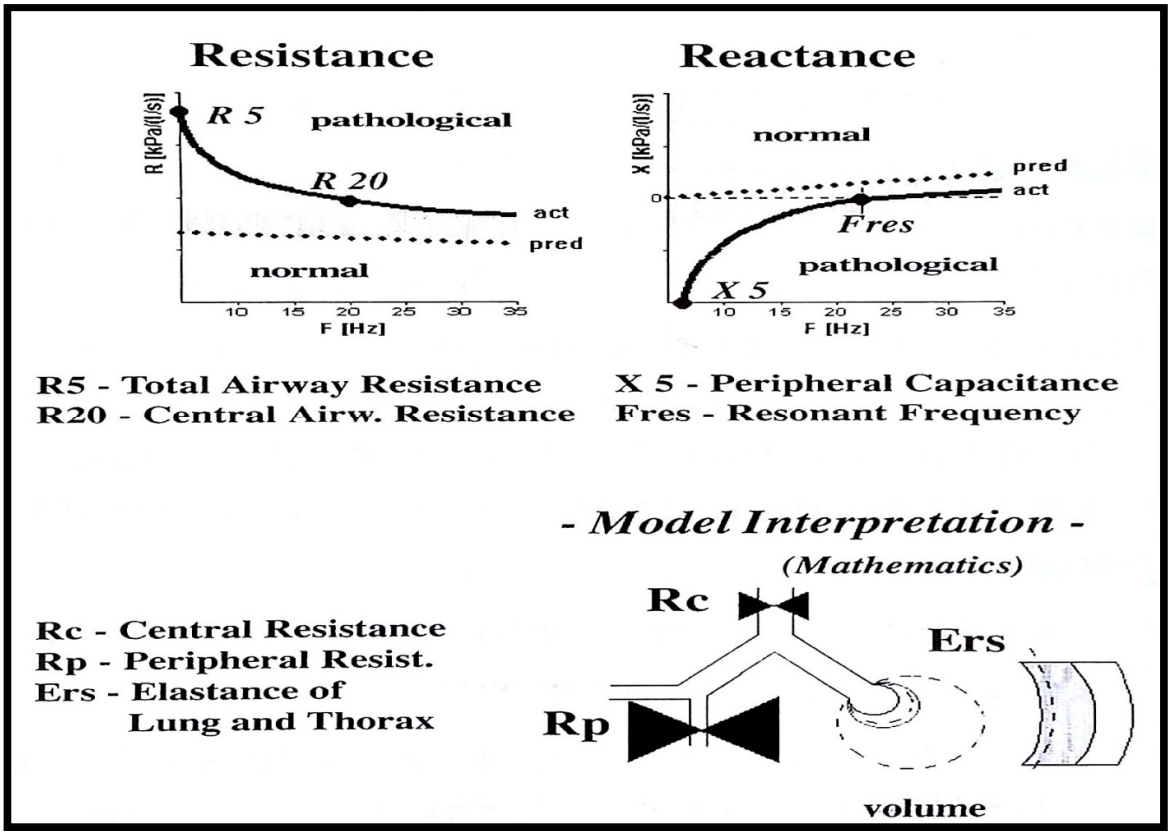

Figure 2.7: The IOS report of a patient with peripheral airway obstruction (i.e. Asthma) (Courtesy of Jaeger Master-Screen)
R: Resistance, X: Reactance, F: Frequency, kPa/l.s^{-1}: Kilopascal per litre per second, Hz: Hertz, R5: Total airway resistance, R20: Resistance of the large airways, X5: Reactance at lowest oscillation frequency of 5 Hz = Peripheral capacitive reactance, Fres: Resonant frequency.

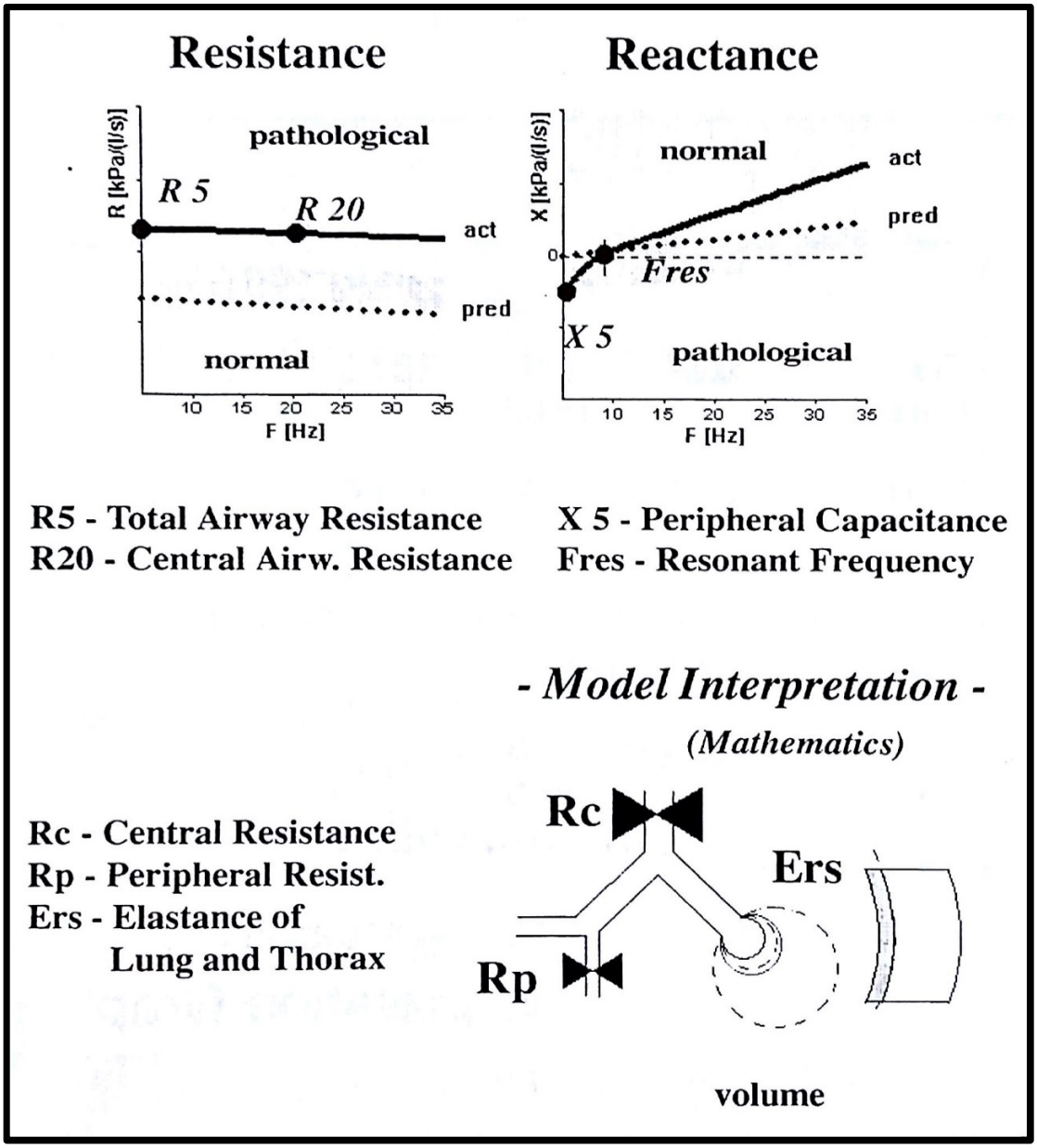

Figure 2.8: The IOS report of a patient with central airway obstruction (i.e. Tracheal stenosis) (Courtesy of Jaeger Master-Screen)
R: Resistance, X: Reactance, F: Frequency, kPa/l.s^{-1}: Kilopascal per litre per second, Hz: Hertz, R5: Total airway resistance, R20: Resistance of the large airways, X5: Reactance at lowest oscillation frequency of 5 Hz = Peripheral capacitive reactance, Fres: Resonant frequency.

CHAPTER 3: RATIONALE, AIMS AND OBJECTIVES OF STUDY

3.1 Rationale of Study

There has been a paradigm shift in our understanding of asthma. It is becoming increasingly evident that asthma is an inflammatory lung disease, not only affecting the central airways, but also involving the small airways as well as the lung parenchyma (22, 72, 319, 320). There are several studies demonstrating that if persistent inflammation in these smaller airways is left untreated, there are likely to be future asthma exacerbations and airway remodelling (47, 66, 74, 321-323). The most recent GINA guidelines recommend titrating asthma therapy to a goal of achieving "asthma control" (8). Asthma control is defined as the extent to which the various manifestations of asthma have been reduced or removed by treatment. This includes two components. Firstly, it is the level of clinical control (i.e. day-to-day control), determined from features such as symptoms and the performance of activities of daily living. Secondly, it is the minimizing of the risk of future adverse events including loss of control, exacerbations and accelerated decline in lung function (8, 50, 51).

The fallacy in our current assessment of patients with asthma, with increasing focus on asthma control, has been the assumption that by titrating therapeutic interventions to normalise symptoms and improve airflow dynamics (i.e. targeting asthma control), we will have suppressed the inflammation present in the airways (7). Unfortunately, this is not always true as the airway inflammation may persist despite normalisation of spirometric parameters and control of asthmatic symptoms (66). Presently, guideline recommendations for titrating asthma therapy to achieve asthma control have significant limitations in the assessment of airway inflammation. The result of this persistent airway inflammation may be the occurrence of structural damage within the airways, with airway remodelling, fibrosis and progressive loss of lung capacity over time (67, 68).

Furthermore, asthma as a chronic illness is unique in that the guidelines, including the latest GINA guidelines, recommend a "step-down" in therapy once asthma control has been maintained for three months (8). This is counterintuitive, as we know that asthma is an inflammatory airway disease, yet none of the current therapeutic titration protocols recommended to achieve asthma control, or objectively assess airway inflammation. The medication we use to achieve asthma control are locally acting anti-inflammatory medications

(i.e. inhaled corticosteroids - ICS), yet we do not monitor airway inflammation to assess their efficacy. In patients who have achieved asthma control but who have persistent subclinical inflammation, “step down” therapy as recommended by GINA guidelines, may predispose these patients to increasing exacerbations risks, worsening of spirometric parameters and eosinophilic airway inflammation (324, 325). This is supported in a study by Jatakanon *et al.* (324) wherein doses of anti-inflammatory (ICS) medication were reduced in stable asthmatic patients, without monitoring for changes in markers of airway inflammation, resulting in increases in the exacerbation events in those patients. Hence, a strategy where ‘inflammometry’ identifies the patient with persistent airway inflammation would potentially prevent over-treatment, but more, importantly under-treatment (326, 327).

Therefore, studies are needed to identify simple, objective, non-invasive and reproducible methods of testing for airway inflammation in patients with asthma. These tests may allow the clinician to document the presence, or absence of airway inflammation in individual asthmatic patients, whether or not these patients appear to be controlled, as assessed by current parameters. Availability of such tests may assist the clinician to treat asthma better, allowing titration of therapeutic interventions to an outcome parameter that is a true reflection of the state of inflammation of the airways. This will possibly allow the clinician to safely reduce therapy in those patients whose airway inflammation appears to be controlled (328-330).

3.2 Aims of study

The aims of this study were firstly, to identify relatively simple, non-invasive, and reproducible biomarkers which might aid in the documentation of the likely presence or absence of airway inflammation in patients with asthma. The second aim was to determine whether there was any association between these biomarkers of inflammation and asthma control or non-control as defined by the Asthma Control Questionnaire (ACQ). Finally, if these biomarkers appear to be able to identify the likely presence of airway inflammation, then to further document whether such airway inflammation is present in cases having adequately controlled asthma as defined by the ACQ (331-333).

3.3 Objectives of study

3.3.1 Primary objectives

The primary objective of the present study was to identify and describe a group of asthmatic patients attending a tertiary referral asthma clinic at a University Hospital in Johannesburg, South Africa, and to characterise their level of asthma control as defined primarily by the ACQ, with a comparison of the agreement between the ACQ, the ACT and the Physicians Asthma Rating (AST) in assessing asthma control.

3.3.2 Secondary objectives

3.3.2.1 To measure small airway calibre via impulse oscillometry (IOS) and to compare and correlate IOS with conventional spirometry, as well as to levels of asthma control as defined by the ACQ

3.3.2.2 To measure non-specific systemic inflammatory biomarkers (CRP and SAAP) and to evaluate the comparison and correlation of these non-specific serum biomarkers to asthma control as defined by the ACQ

3.3.2.3 To measure specific systemic inflammatory cytokine biomarkers (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IFNγ, and TNFα i.e. Th1 vs Th2 cytokines) and to evaluate the comparison and correlation of these serum biomarkers to asthma control as defined by the ACQ

3.3.2.4 To measure the airway inflammatory biomarker FeNO, and to evaluate the comparisonand correlation of FeNO to asthma control as defined by the ACQ

3.3.2.5 To measure total serum IgE levels and to evaluate the comparison and correlation of IgE to asthma control as defined by the ACQ

3.3.2.6 To measure systemic chemokine biomarkers (Eotaxin and RANTES) and to evaluate the comparison and correlation of these serum chemokine biomarkers to asthma control as defined by the ACQ

3.3.2.7 To develop and optimise a protocol for the measurement of the serum leukotrienes LTB4, LTC4 and LTE4

3.3.2.8 To measure serum leukotriene biomarkers (LTB4, LTC4 and LTE4) levels and to evaluate the comparison and correlation of these serum leukotriene biomarkers to asthma control as defined by the ACQ

3.3.2.9 To measure serum biomarkers of remodelling and angiogenesis (TGFβ and VEGF) and to evaluate the comparison and correlation of these serum biomarkers to asthma control as defined by the ACQ

CHAPTER 4: DESIGN, METHODOLOGY AND DATA ANALYSIS

4.1 Design and study population

This was a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic. The study population consisted of patients selected from the Charlotte Maxeke Johannesburg Academic Hospital Asthma Clinic. Approval to conduct the study was obtained from the University of the Witwatersrand Human Research Ethics committee (Protocol Number M060401 (Appendix 3)), which included the need for informed consent from the patients. Witten informed consent was obtained from both the participants and healthy volunteers. The subject, the investigator and a witness signed the agreement prior to the performance of any study procedures or evaluations (Appendix 4 and 5).

Cases were identified by screening the files of over 3000 patients attending the asthma clinic over a two-year period and selecting those patients with asthma who met the set of inclusion and exclusion criteria detailed below. Only 76 patients met the strict set of inclusion and exclusion criteria detailed below. The reason for the exclusion of a significant percentage of the asthma population screened was that they did not meet the strict predefined criteria chosen to reduce the statistical “noise” and confounding bias from the inclusion of questionable factors that might dilute the final data analysis. A group of healthy volunteers (i.e. colleagues and staff of the hospital and medical school) were recruited as controls, and subjected to the same procedures as the patient cohort. The purpose for including a group of healthy volunteers was for quality assurance purposes in assessing the performance of the analyte testing kits used in this thesis. The following set of inclusion and exclusion criteria were used for the enrolment of patients screened for inclusion into the study. These criteria were modified from the ATS consensus statement on FeNO analysis (334).

4.2 Inclusion criteria

4.2.1 Patients with an established clinical diagnosis of asthma who met the current Global Initiative for Asthma (GINA) guideline diagnosis of asthma (i.e. exhibiting both a characteristic pattern of respiratory symptoms and variable expiratory airflow limitation) (8).

4.2.2 Male or female patients over the age of 16 years.

4.2.3 Written informed consent.

4.3 Exclusion criteria

4.3.1 Any patient who declined or withdrew informed consent.

4.3.2 Patients with gastroesophageal reflux disease (as it affects FeNO levels (334)).

4.3.3 History of smoking in the past year.

4.3.4 Accumulative smoking history of five pack years or greater.

4.3.5 Patients recovering from a recent respiratory tract infection (within the last four weeks) or patients with active tuberculosis (TB) or post TB bronchiectasis.

4.3.6 Patients with lung cancer, bronchiectasis, sarcoidosis, lung fibrosis, pulmonary hypertension, idiopathic interstitial pneumonia or other active infective or non-infective pulmonary diseases including significant environmental biomass exposure.

4.3.7 Patients with a chest radiograph with evidence of clinically significant abnormalities not believed to be due to asthma.

4.3.8 Patients with clinically significant or poorly controlled cardiovascular, psychiatric, renal, immunological, rheumatologic, endocrine (including uncontrolled diabetes mellitus) and haematological abnormalities.

4.3.9 Patients with a body mass index (BMI) > 30kg/m^2 (obesity may be a confounding factor in the measurement of serum inflammatory biomarkers (335)).

4.3.10 Male patients with a waist circumference >102cm and female patients with a waist circumference > 88cm (central obesity may be a confounding factor in the measurement of serum inflammatory biomarkers (335)).

4.3.11 Patients with any active malignancies.

4.3.12 Patients who had undertaken strenuous activity one hour prior to assessment (affects FeNO levels and lung function assessment in patients with exercise-induced asthma (295, 334)).

4.3.13 Patients who used short acting β2 agonist within the last 4 hours (affects FeNO levels (334)).

4.3.14 Patients who had a full meal within two hours prior to assessment (affects FeNO levels (334)).

4.3.15 Patients on long-term oxygen therapy or continuous positive airway pressure (CPAP) support.

4.3.16 Patients unable to comply with study procedures.

4.3.17 Patients with a history of psychiatric illness, intellectual deficiency or any condition that would limit the validity of the informed consent document.

4.4 Detailed methodology

4.4.1 The investigator examined all the patients, and made a clinical assessment of the patient's asthma control. This assessment was made independently of the use of any of the other objective parameters (e.g. standardised questionnaires or spirometry values) described in this study. This independent assessment was referred to as the Physicians Asthma Rating (AST).

The AST was given a numerical value, based on the investigators subjective clinical perception of the level of the patient's asthma control, in order to facilitate comparisons with the other standard parameters of asthma control used. For the purposes of the AST, a score of 1 was assigned to a patient deemed to be totally controlled, 2 to a patient that was well-controlled and 3 to a patient whose asthma was uncontrolled. The results of the spirometry and IOS tests performed, as described below, were withheld from the investigator at the time of the clinical assessment of the patient so as not to influence the AST rating.

4.4.2 Participants then completed the internationally validated questionnaire, the ACQ (Appendix 1). This questionnaire developed by Juniper *et al.* (58) consists of seven domains. The first five domains are questions focused specifically on the symptoms the patients had experienced over the last week, and the sixth is how often the patients used their reliever therapy. The seventh part of the ACQ is a categorical value determined from the percentage predicted FEV1 (Appendix 1).

4.4.3 After completion of the ACQ and AST, the patient's current medication was documented, and they were directed to the lung function laboratory. The patients were introduced to a trained asthma care sister who proceeded to complete the ACT test (Appendix 2) (61).

4.4.4 Following the completion of the ACT, the FeNO level was measured with a NIOX MINO air inflammation monitor manufactured by Aerocrine, Solana Sweden. Airflow was measured at 50 ml/sec and regulated by a built-in flow regulator, as per the American Thoracic Society (ATS) guidelines for online FeNO measurements (334). The instrument was calibrated daily and measurements were done in accordance with the ATS recommendations (334). A repeat procedure was performed 5 minutes later to eliminate

technical error. An average of the two FeNO levels was used for statistical analysis. To prevent the nitric oxide (NO) from being washed out from the airways by repeated spirometric assessments and the use of bronchodilators, the FeNO assessment was performed as the first test in the lung function laboratory.

4.4.5 All subsequent tests were performed in the lung function laboratory at the Charlotte Maxeke Johannesburg Academic Hospital. Qualified lung function technologists performed all the investigation carried out in the laboratory. At the laboratory the patient's age, height, weight and waist circumference were documented. Thereafter, all patients completed the spirometry and impulse oscillometry (IOS) tests. The spirometry and IOS were both repeated 15 minutes post inhalation of a short acting β2 agonist.

Spirometry was done on Jaeger (Hochberg, Germany) spirometers with daily calibrations according to the ATS guidelines (295, 300). IOS was measured using the Jaeger Master-Screen Impulse Oscillometry System (Hochberg, Germany), and calibrated daily according to the ATS guidelines (300).

The values of the spirometric and IOS measurements in the current cohort of patients were reported as the percentage (%) of the predicted value. The reference values used to determine the percentage predicted for spirometry values measured, were obtained from the European Community for Coal and Steel (ECCS) population database (295, 300). Importantly, all non-Caucasian patients in the current study population, a correction factor of 10% less than the reference values obtained from the ECCS population database was applied. The application of this correction factor has been described and endorsed by the South African Thoracic Society (336). The reference values used to determine the percentage predicted for IOS values measured were provided by Jaeger (Hochberg, Germany). Currently, in South Africa, as there are no guidelines for the measurement and interpretation of IOS data in a local cohort of patients, the same correction factor used for spirometry was applied to the IOS predicted values.

All the spirometric data reported were interpreted in accordance with the ATS/ERS consensus document on "interpretative strategies for lung function tests" published by Pellegrino *et al*. (301). This consensus document defining lung function parameters below

the fifth percentile of the predicted value (i.e. below the lower limit of normal (LLN)) or values below 80% of predicted as being abnormal. The document also defines a post-bronchodilator increase in the FEV1 ≥ 12% and ≥ 200 ml as indicative of a positive bronchodilator response indicating airway reversibility (i.e. bronchial airway hyper-reactivity (BAH)).

4.4.6 After completing the tests in the lung function laboratory, all the patients had one ethylenediaminetetraacetic acid (EDTA) tube and five serum separator tubes (SST) of blood collected. The blood in the SST tubes was centrifuged for 10 minutes at 1,300 × g to isolate the serum. The sera were then stored at -70 ºC until processing.

The serum biomarkers measured were as follows:

4.4.6.1 SAAP and CRP were measured via a bead bioplex assay (Appendix 6).

4.4.6.2 Cytokines and chemokines were measured using a suitable protein multiplex assay technology i.e. a bioplex assay (baseline cytokine and chemokine references ranges were pre-determined prior to purchasing the assays, by the company supplying the assays) (Appendix 7).

4.4.6.3 Serum total IgE levels were measured using a solid phase, chemiluminescent immunometric assay (Appendix 8).

4.4.6.4 Serum leukotriene levels were measured using enzyme-linked immunosorbent assay (ELISA) methodology. The methodology for the measurement of serum leukotriene levels was developed and optimised by the investigator, prior to the actual measurement of the leukotriene levels. The detailed methodology of this optimisation process is described in Appendix 9.

4.4.6.5 Serum TGFβ and VEGF were measured via bead bioplex assay (Appendix 10).

All the investigations including serum proteins measurements, FeNO and lung function tests were measure in duplicate. This was performed to avoid the loss of any analyte measurements

due to technical factors as well as to eliminate any technical errors that might have occurred with the initial tests. Average values of the two measurements were used for reporting purposes.

All the biomarkers were measured via microplate-based multiplex immunoassay technology. Multiplex technology uses microscopic microspheres (beads), each with a different colour code (spectral address) to permit discrimination of different proteins that are measured simultaneously. Each of the beads in the assay were coupled to specific antibodies targeted against different protein epitopes. After preparatory steps, the beads with their individual antibodies were further coupled with different ratios of two fluorophores, which allowed for each of different beads to emit a unique fluorescent light signal. This process enabled the beads to be differentiated and quantified by the intensity of the fluorescent light (i.e. the fluorescent intensity unit) they emit. Patient's serum was then immersed into wells containing the beads containing bound antibodies that bind specific protein epitopes within the serum (i.e. the cytokine or chemokine of interest). A bead reader (i.e. the BioPlex™ reader) then used laser technology to classify the beads according to their spectral addresses assigned, as per the manufacturer's information sheet, thus identifying the specific protein bound to the bead. The bead reader quantified the concentration of the protein by measuring the fluorescent intensity of the light emitted. In this study, the Millipore human cardiovascular disease panel 2 (HCVD2 – 62 BK) was used according to manufacturer's instructions to measure the serum CRP and SAAP, and the Bioplex pro™ cytokine, chemokine and growth factor assay (Bio-Rad M500AD1ZL6) to measure the cytokine levels. A detailed description of the methodology of the preparation and measurement of the analytes is documented in Appendix 6 and 7.

The levels of all biomarkers measured via the bioplex assays were reported as fluorescent intensity units (FU). When the level of a biomarker was below the lower limit of detection, it was assigned half the value of the lowest limit of detection. When the level of a biomarker was above the upper limit of detection, it was assigned double the value of the uppermost limit of detection. These adjustments for outlying data have been verified in the literature as an acceptable method for data correction (337, 338), and are recommended by the manufacturers of the analyte kits as well.

The reason for reporting in fluorescent intensity units (FU) as opposed to standard international units (SI) was that fluorescent intensity is the actual measurement (raw data) obtained from the

microplate-based multiplex immunoassay system. By using the raw data in FU, as opposed to diluting the data with several correction steps using pre-defined manufacturer provided control samples to determine the equivalent SI units, the statistical analysis and results obtained were improved (Appendix 6 and 7).

The reason for this is that the conversion of FU values to SI units required a computerised mathematical correction model as recommended by the manufacturer of the biomarker kits. However, the correction algorithm is not linear but it is a logistic mathematical correction algorithm utilizing a sigmoid shaped curve. Hence, the conversion of actual FU values to SI units would have resulted in some of the values of the analytes measured being assigned the same SI unit if the variations in the FU measured was minor. For example, analytes with FU values measured between 5 and 15 FU may have all been allocated the same SI unit of 10 pg based on the mathematical algorithm. Thus, if a significant number of the patients had a biomarker level that fell within this band of 5 to 15 FU, they would have been all allocated the same SI unit of 10 pg. This conversion, by estimating the value of the SI unit based on a logistic mathematical algorithm would have resulted in the unintentional creation of categorical values in SI units. These categorical values would have decreased the spread of the SI units, and made the statistical analysis more difficult by diluting and combining different FU values into a single SI unit.

4.5 Assessment of asthma control

For the purposes of standardisation within this thesis, all patients completed the Asthma Control Questionnaire (ACQ), which was used as the basis for the assessment of asthma control in the entire cohort of patients studied (Appendix 1) (58). Based on the publication by Juniper *et al.* (59), each patient was classified according to their composite ACQ score as being either controlled (CC), defined by an ACQ of < 1.5; or uncontrolled (UC), defined by an ACQ of ≥ 1.5. For a better appraisal of the controlled cohort of patients, as validated by Juniper *et al*. (59), the patients classified as controlled were sub-classified according to their composite ACQ score into totally controlled (TC), defined by an ACQ < 0.75; or well-controlled (WC) defined by an ACQ of between 0.75 and 1.5. Hence, for the statistical analyses of the data undertaken in the individual chapters described in this thesis, the patients were characterised into two groups:

Group 1: For the initial/primary analysis, the evaluation compared patients whose asthma was controlled versus those not controlled, as defined by the ACQ:

- controlled (CC) - patients with ACQ $\leq$ 1.5
- uncontrolled (UC) - patients with ACQ > 1.5

Group 2: For the subsequent/secondary analysis, the evaluation compared patients whose asthma was totally controlled, well-controlled or uncontrolled, as defined by the ACQ:

- totally controlled (CC) - patients with ACQ $\leq$ 0.75
- well-controlled (WC) - patients with ACQ between 0.75 and 1.5
- uncontrolled (UC) - patients with ACQ > 1.5

In all the studies the evaluation was done first for **group 1** analysis (i.e. primary/initial analysis: between controlled and uncontrolled asthmatic patients) and thereafter, the **group 2** analysis (i.e. subsequent/secondary analysis: between totally controlled, well-controlled and uncontrolled asthmatic patients). The reason for choosing the ACQ as the gold standard in this thesis was that the categorical cut-offs for asthma control were well defined and robust (59). However, potential limitations in using the ACQ is that might it not necessarily be the gold standard in assessment of asthma control but rather it may just be identifying a phenotype of asthmatic patients whose control can be accurately categorised by the ACQ.

4.6 Data analysis

All the data analyses were performed as recommended by Professor Piet Becker, a Chief Specialist Scientist at the South African Medical Research Council, and interpreted together with him.

In order to calculate the sample size of patients needed to undertake this investigation, the following assumptions were made. It was assumed that approximately 40% of all patients attending the adult asthma clinic, and hence included into the study, would have controlled asthma. This assumption was supported by data from a recent publication from asthma clinics in South Africa undertaken by Greenblatt *et al.* (339). They reported that 39% of patients at academic hospital respiratory clinics around South Africa assessed themselves as having controlled asthma defined by the ACT $\geq$ 20.

It was further assumed that all of the patients with uncontrolled asthma would have airway inflammation. Using an alpha error of 5% and based on the above assumptions, a sample size of 76 patients was estimated to give 80% power to detect the presence of persistent inflammation within the group of so-called “controlled” adult asthmatic patients. This sample size was also determined to have approximately 80% power to show the presence of persistent inflammation in 100% of the poorly controlled adult asthmatic patients.

The Shapiro-Wilks normality test indicated that the data reported was not normally distributed; hence, non-parametric statistical tests were used for all the analyses performed. The results were reported in both a table format, as well as graphically using a box-and-whisker plot where applicable. The results were represented in a dual format for ease of reference and interpretation. The results included in the tables were reported as medians together with the 95 percent (%) confidence intervals (CI). The box-and-whisker plot displayed the data using the standard five number summary: top and bottom boundary of the box indicating the 75th and 25th percentiles, the line within the box depicted the median and the whiskers represented the minimum and maximum values measured.

Results reported in the tables that were statistically significant are highlighted in bold black text with an asterisk *, whereas those results that had a clinically significant correlation are highlighted in bold black text with a double asterisk **, for easy recognition. The corresponding p or Rho (r) values are represented in the associated figures in **red**. In Chapter 6, results reported in the tables that represent values relating to small airway parameters are highlighted in cyan (…..) for easy recognition.

All the laboratory investigations, measured as continuous variables, were compared to the categories of asthma control, first for **group 1** and then for **group 2**. For the patients categorised into **group** 1 (i.e. controlled versus uncontrolled asthmatic patients)**,** the association between the variable measured and the level of asthma control was analysed using the Wilcoxon rank sum test. For the comparisons of matched variables within the **group 1** cohort (i.e. the comparison of pre-bronchodilator versus the post-bronchodilator values of the same patient within the controlled group and the uncontrolled group), the Wilcoxon Signed-Rank Test using paired observations was applied.

For the patients categorised into **group 2** (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Kruskal-Wallis one-way ANOVA test. The Bonferroni correction was applied to the analysis of the **group 2** cohort to control for multiplicity.

In all testing a p-value < 0.05 was considered significant, except where the Bonferroni correction was applied.

A Spearman's rank correlation was used to assess the relationship between the ACQ and all the variables measured. For the interpretation of the Spearman's rank correlation, a $r < \pm 0.4$ was reported as a weak correlation, whereas a $r > \pm 0.6$ was reported as a strong correlation and a $r > \pm 0.8$ was reported as a very strong correlation. The reason being that the closer the r is to +1.0 or -1.0, the greater is the strength of a linear relationship existing between the two parameters being compared. The calculation of r^2, known as the coefficient of determination, was reported to quantify the "goodness of the fit" between the parameters being compared. The reason for applying this method of reporting, illustrated by the following example, is that for a result indicating $r = 0.5$ ($p < 0.05$), $r^2 = 0.25$, implying only 25% of the variability in the dependant variable (X) is explained or predictable by the variance in the independent variable (Y). The coefficient of determination (r^2) is the fraction of the variance in the two variables that is "shared". For example, if $r^2 = 0.25$, then 25% of the variance in X can be explained by variation in Y. Likewise, 25% of the variance in Y can be explained by variation in X. Thus, 25% of the variance is shared between X and Y. Therefore, in the above example even though correlation maybe statistically significant, with a p value of < 0.05, the result is not of any clinical relevance as the strength of the relationship between the two variables is weak (340, 341).

For data that was recorded as a categorical variable (e.g. ACT levels < or > 19) and those laboratory measurements that were recorded as a categorical variable (e.g. FeNO levels < or > 25 ppm), the analysis was undertaken using the Pearson's chi-squared test, and if the frequency was ≤ 5, a Fisher's exact test (two-tailed) test was used.

Analysis of the association between the medications used by the patients, and the categories of asthma control as defined by the ACQ, was performed using the Pearson's chi-squared test and if the frequency was ≤ 5, a Fisher's exact test (two-tailed) test was used. Data analysis of the association between the medications used by the patients, and the levels of the biomarkers measured, was performed using the Wilcoxon rank sum test, as this data was recorded and analysed as a continuous variable.

The relationship between asthma control as defined by the ACQ, and levels of biomarkers grouped into Th1 predominant cytokines or Th2 predominant cytokines, was determined using a logistic regression analysis.

The statistical analyses and graphics were performed using the SPSS software, version 12 (**SPSS**, Chicago, Illinois), Excel software (Microsoft, Albuquerque, New Mexico) and GraphPad Software Inc. (La Jolla, California).

4.7 Layout of the presentation

The following chapters will document the specific methods, results and conclusions of the individual studies. Although the methods and statistical analysis have been described in detail in Chapter 4, there is by necessity a repetition of this information within each of the individual chapters below, to highlight the methods and statistical test applied for ease of reference.

Chapter headings are as follows:

Chapter 5: Defining a group of asthmatic patients at an outpatient, referral asthma clinic and characterising the level of asthma control.

Chapter 6: Appraisal of small airway calibre measured by spirometry and IOS in comparison to categories of asthma control as defined by the ACQ.

Chapter 7: Evaluation of the utility of serum biomarkers in assessing airway inflammation in comparison to categories of asthma control as defined by the ACQ. Biomarkers include:

7.1) Nonspecific markers of systemic inflammation i.e. CRP and SAAP

7.2) Th1 vs Th2 cytokine profiles

Chapter 8: Evaluation of the utility of FeNO, chemoattractants - RANTES and eotaxin, serum leukotrienes and IgE, in assessing airway inflammation when compared to categories of asthma control as defined by the ACQ. This part of the thesis needed to be preceded by the development and optimisation of a method for measuring serum leukotriene levels.

Chapter 9: Evaluation of the utility of serum biomarkers of airway remodelling and angiogenesis in assessing airway inflammation in comparison to categories of asthma control as defined by the ACQ.

A diagrammatic representation outlining the presentation of the subsequent chapters is included (Figure 4.1).

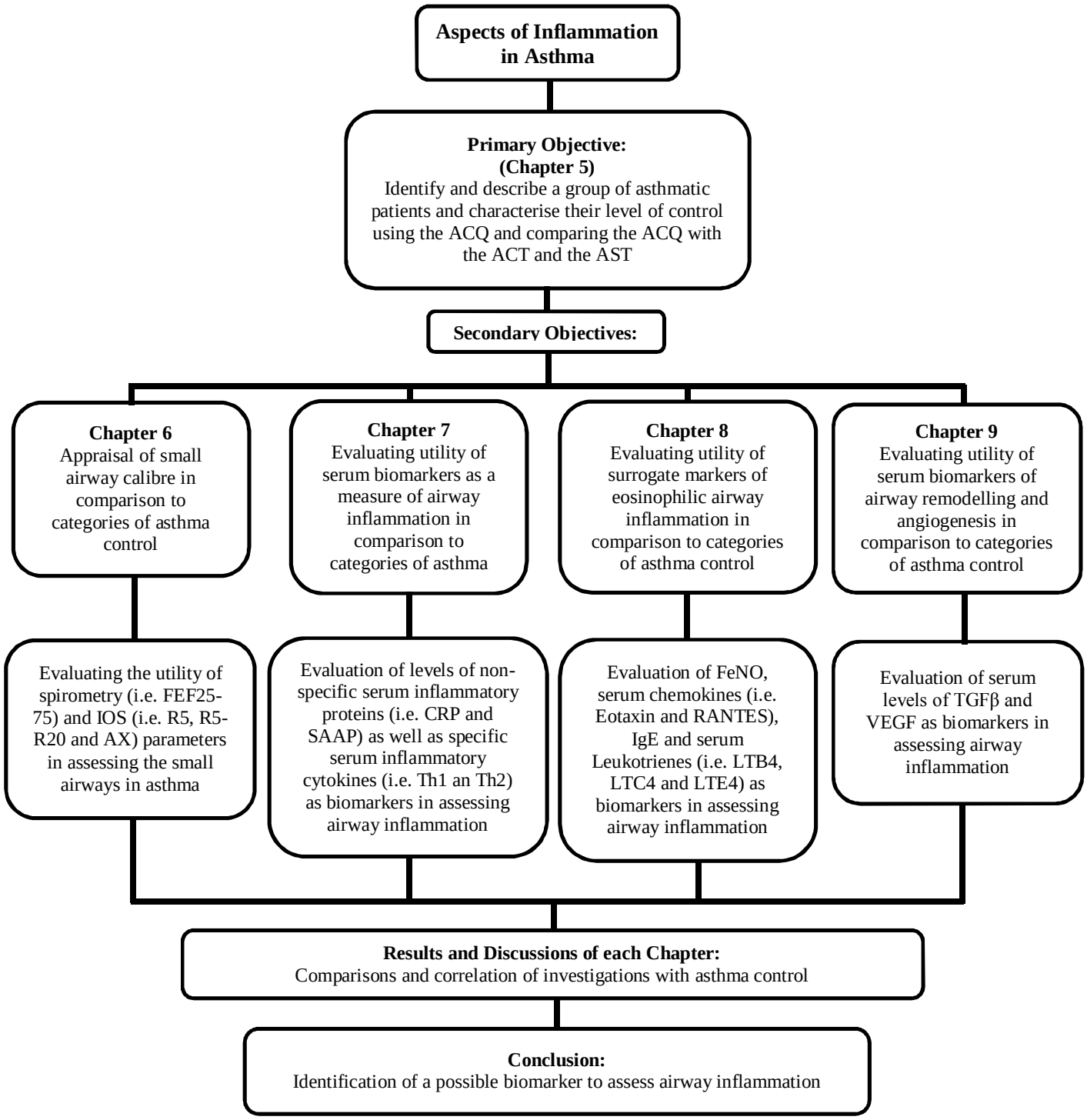

Figure 4.1: A diagrammatic representation outlining the various aspects of the thesis
ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test, AST: Physician Asthma Rating, FEF25-75: Average expired flow over the middle half of FVC, FVC: Forced vital capacity, FeNO: Fractional exhaled nitric oxide, IFNγ: Interferon γ, IgE: Immunoglobulin E, Th: T helper cell, TGFβ: Transforming growth factor β, TNFα: Tumour necrosis factor α, VEGF: Vascular endothelial growth factor.

CHAPTER 5: DEFINING A GROUP OF ASTHMATIC PATIENTS AT AN OUTPATIENT, REFERRAL ASTHMA CLINIC AND CHARACTERISING THEIR LEVEL OF ASTHMA CONTROL

5.1 Introduction

The latest Global Initiative for Asthma (GINA) guidelines recommend titrating asthma therapy to achieving a target of “asthma control” (8). In the context of asthma management, “asthma control” is a term that refers to a composite assessment of patient symptoms, reliever use, lung function tests and exacerbation rates (50). “Asthma control” is defined as the extent to which these various manifestations of asthma have been reduced or removed by treatment, and differs from the assessment of disease severity, which quantifies a departure from 'normal' (52). Initially practice guidelines for asthma management recommended optimisation of lung function and prevention of exacerbations as appropriate targets of asthma therapy, with improvement of spirometric parameters as the primary end point (342). However, with the subsequent recognition of the importance of the patient perspective, and the poor correlation between lung function tests, airway inflammation and patient symptoms, therapeutic interventions have increasingly focused on the assessment of ‘‘asthma control’’ (8, 56). The current GINA guidelines now recommend titrating asthma therapy to achieving “asthma control” (7, 8, 52, 53).

The most validated assessments of asthma control currently available, include the questionnaire developed by Juniper *et al.* (58) called the Asthma Control Questionnaire (ACQ) and the questionnaire developed by Nathan *et al*. (61) called the Asthma Control Test (ACT) (Appendix 1 and 2) (57-59, 61). One of the most significant differences between the ACQ and the ACT is that the ACT does not use spirometry as part of the assessment of control and thus can be used in a facility without access to spirometry i.e. a primary health care facility (60). However, an abridged version of the ACQ (i.e. ACQ 6), in which the spirometric assessment domain has been excluded, correlates well with the unabridged ACQ (i.e. ACQ 7) and the ACT (63, 64).

With the limited resources available within the health care sector in South Africa, the use of spirometry as a tool for assessment of asthma control is not available at most of the primary health care facilities. There is also a paucity of studies in South African adult asthmatics using any of the objective asthma questionnaires described above, especially the ACQ to assess

asthma control. One of the few such publications in a South African adult asthmatic cohort, was that by Greenblatt *et al.* (339), in which asthma control in various different clinical settings was defined using the ACT. The only other publication that included a cohort of South African adults patients, using any of the asthma control questionnaires, was the international, multicentre GOAL study by Bateman *et al*. (7), in which adult asthmatic patients had their asthma therapy titrated by the ACQ. However, this was an interventional trail with the primary objective being asthma therapy targeting asthma control.

Thus, the primary objective of this present chapter was to identify a cohort of South African asthmatic patients, and characterise their asthma control according to the ACQ. It was also important to assess the agreement between the ACQ, the ACT, the abridged ACQ (i.e. the ACQ 6) and a physician's subjective assessment of asthma control (i.e. AST) (62, 63).

5.2 Design and Study Population

This study was designed as a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic with approval to conduct the study received from the University of the Witwatersrand Human Research Ethics committee (Protocol Number M060401- Appendix 3).

5.3 Methods

A detailed description of the methodology employed in the present study has been described in Chapter 4. For the purposes of standardisation within this thesis, all patients completed the Asthma Control Questionnaire (ACQ), which was used as the basis for the assessment of asthma control in the entire cohort of patients studied (Appendix 1) (58). As indicated in Chapter 4, the population of patients studied were categorised into two groups, based on their level of control. **Group 1** consisted of the patients categorised into controlled (CC) and uncontrolled (UC), based on an ACQ level of 1.5. **Group 2** consisted of the same cohort of patients, categorised by the ACQ into totally controlled (TC) having an ACQ < 0.75, well controlled (WC) having an ACQ of between 0.75 and 1.5 and uncontrolled (UC) with an ACQ > 1.5 (59).

In all the studies, the evaluation was first for **group 1** (i.e. primary/initial analysis: controlled versus uncontrolled asthmatic patients) and thereafter, the **group 2** analysis (i.e.

subsequent/secondary analysis: totally controlled versus well controlled and versus uncontrolled asthmatic patients). Of importance in this chapter is that a subjective assessment of asthma control, the AST, was performed by the investigator, and patients completed the ACT (Appendix 2) (61).

5.4 Statistical Analysis

Methodology detailing the basic principles of the statistical analyses applied, including population size calculations, has been discussed in Chapter 4. The population of patients studied was 76 in total. The Shapiro-Wilks normality test indicated that the data reported was not normally distributed; hence, non-parametric statistical tests were used for all the analyses performed.

The results were reported in both a table format, as well as graphically using a box-and-whisker plot where applicable. The results were represented in a dual format for ease of reference and interpretation. The results included in the tables were reported as medians together with the 95 percent (%) confidence intervals (CI). The box-and-whisker plot displayed the data using the standard five number summary: top and bottom boundary of the box indicating the 75th and 25th percentiles, the line within the box depicted the median and the whiskers represented the minimum and maximum values measured.

Results reported in the tables that were statistically significant are highlighted in bold black text with an asterisk *, whereas those results that had a clinically significant correlation are highlighted in bold black text with a double asterisk **, for easy recognition. The corresponding p or Rho (r) values are represented in the associated figures in **red**.

All the laboratory investigations, measured as continuous variables, were compared to the categories of asthma control, first for **group 1** and then for **group 2**. For the patients categorised into **group** 1 (i.e. controlled versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Wilcoxon rank sum test. For the patients categorised into **group 2** (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Kruskal-Wallis one-way ANOVA test. The

Bonferroni correction was applied to the analysis of the **group 2** cohort to control for multiplicity.

In all testing a p-value < 0.05 was considered significant, except where the Bonferroni correction was applied.

A Spearman's rank correlation was used to assess the correlation between the ACQ and all the variables measured. For the interpretation of the Spearman's rank correlation, as outlined in Chapter 4, only $r > 0.6$ was reported as a having a strong correlation, even though a $r < 0.6$ may have been statistically significant with a $p < 0.05$.

Specific to this chapter, the additional following data analyses were performed. The actual ACQ and ACT values recorded were converted into categorical values. For the ACQ, the categories defined were based on composite ACQ score and were either controlled (CC) which was defined by an ACQ of < 1.5, or uncontrolled (UC) which was defined by and ACQ of ≥ 1.5. The patients classified as controlled were sub-classified according to their composite ACQ score, into totally controlled (TC) defined by an ACQ < 0.75 or well-controlled (WC) defined by an ACQ of between 0.75 and 1.5. For the ACT, the categories defined were also based on composite ACT score, with an ACT of 25 defining totally controlled asthma, an ACT between 19 and 24 defining controlled asthma and an ACT < 19 defining uncontrolled asthma. The AST was initially assessed as a numerical score, based on the investigators subjective clinical perception of the level of the patient's asthma control, in order to facilitate comparisons with the other standard parameters of asthma control used (i.e. the ACQ and ACT). For the purposes of the AST, a score of 1 was assigned to a patient deemed to be totally controlled, 2 to a patient that was well-controlled and 3 to a patient that was uncontrolled.

The agreement between the ACQ, ACT and the AST was initially assessed using the McNemar test for symmetry. It was used to assess the agreement between the ACQ classification and the physician's assessment of asthma control (i.e. the physician's asthma rating (AST)), as well as the agreement between the ACQ and the ACT classification. Further assessment of the agreement between the ACQ, ACT and AST was performed using the Fischer's exact test, as well as the Pearson's chi-squared test. A Spearman's rank correlation test was used to assess the correlation between the ACQ, the abridged ACQ (i.e. ACQ 6) and the ACT.

For data that was recorded as a categorical variable (e.g. ACT levels < or > 19), the analysis was undertaken using the Pearson's chi-squared test and if the frequency was ≤ 5, a Fisher's exact test (two-tailed) test was used. Analysis of the association between the medications used by the patient, and the categories of asthma control as defined by the ACQ, was performed using the Pearson's chi-squared test and if the frequency was ≤ 5, a Fisher's exact test (two-tailed) test was used.

The statistical analyses and graphics were performed using the SPSS software, version 12 (**SPSS**, Chicago, Illinois), Excel software (Microsoft, Albuquerque, New Mexico) and GraphPad Software Inc. (La Jolla, California).

5.5 Results

5.5.1 Demographic and clinical data

Over 3000 asthma patient files were screened and 76 patients met the strict set of inclusion and exclusion criteria described in Chapter 4. The reason for the exclusion of a significant percentage of the population screened was that they did not meet the strict predefined inclusion and exclusion criteria chosen to reduce the statistical "noise" in the final data analysis. The median age of all the patients studied was 38.9 years (95% CI 35.19 - 43.12) and there were 31 males (40.9%) and 45 females (59.1%). There was no significant difference in the assessment of the controlled versus uncontrolled asthmatic patients (i.e. **group 1** analysis) or in the **group 2** analysis (totally controlled versus well-controlled and versus uncontrolled asthmatic patients), with respect to the demographic and clinical data (i.e. age, gender, BMI and abdominal circumference) (Table 5.1; Figures 5.1, 5.2).

Table 5.1: Demographic and clinical data in patients categorized by the ACQ score

Clinical Data	Controlled n=52	Totally control n=30	Well-control n=22	Uncontrolled n=24
Age	39.8 (35.2 - 44.9)	37.9 (31.7 - 45.2)	42.5 (35.8 - 50.4)	37.1 (30.4 - 45.2)
BMI	24.0 (23.0 - 25.0)	23.9 (22.4 - 25.4)	24.1 (22.5 - 25.8)	23.8 (22.4 - 25.4)
Abd Cirm (cm)	81.6 (79.1 - 84.2)	81.6 (78.1 - 85.2)	81.7 (78.0 - 85.5)	81.5 (78.3 - 84.8)

BMI: Body mass index, Abd Cirm: Abdominal circumference, cm: Centimetre, n: Number.
The results are reported as medians together with the 95% confidence intervals (CI).

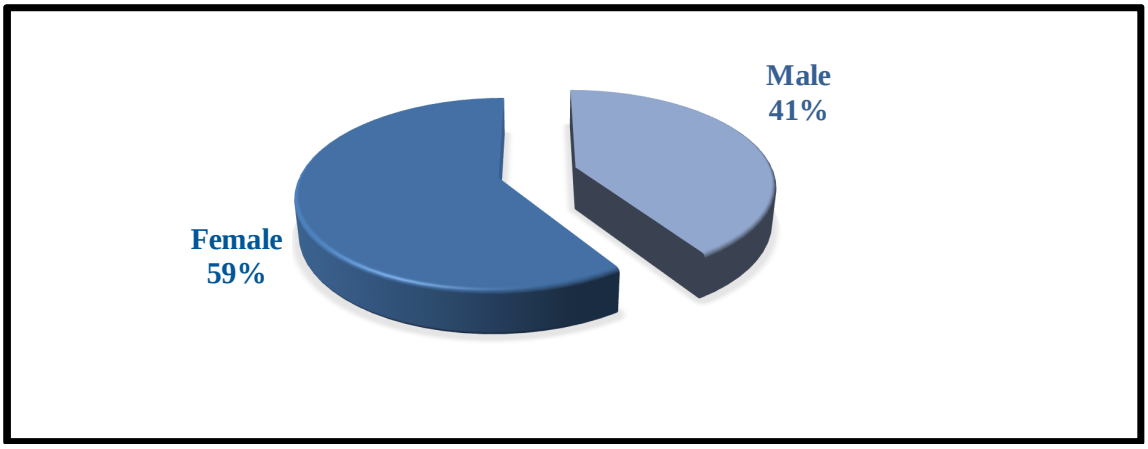

Figure 5.1: Gender distribution of the total patient cohort

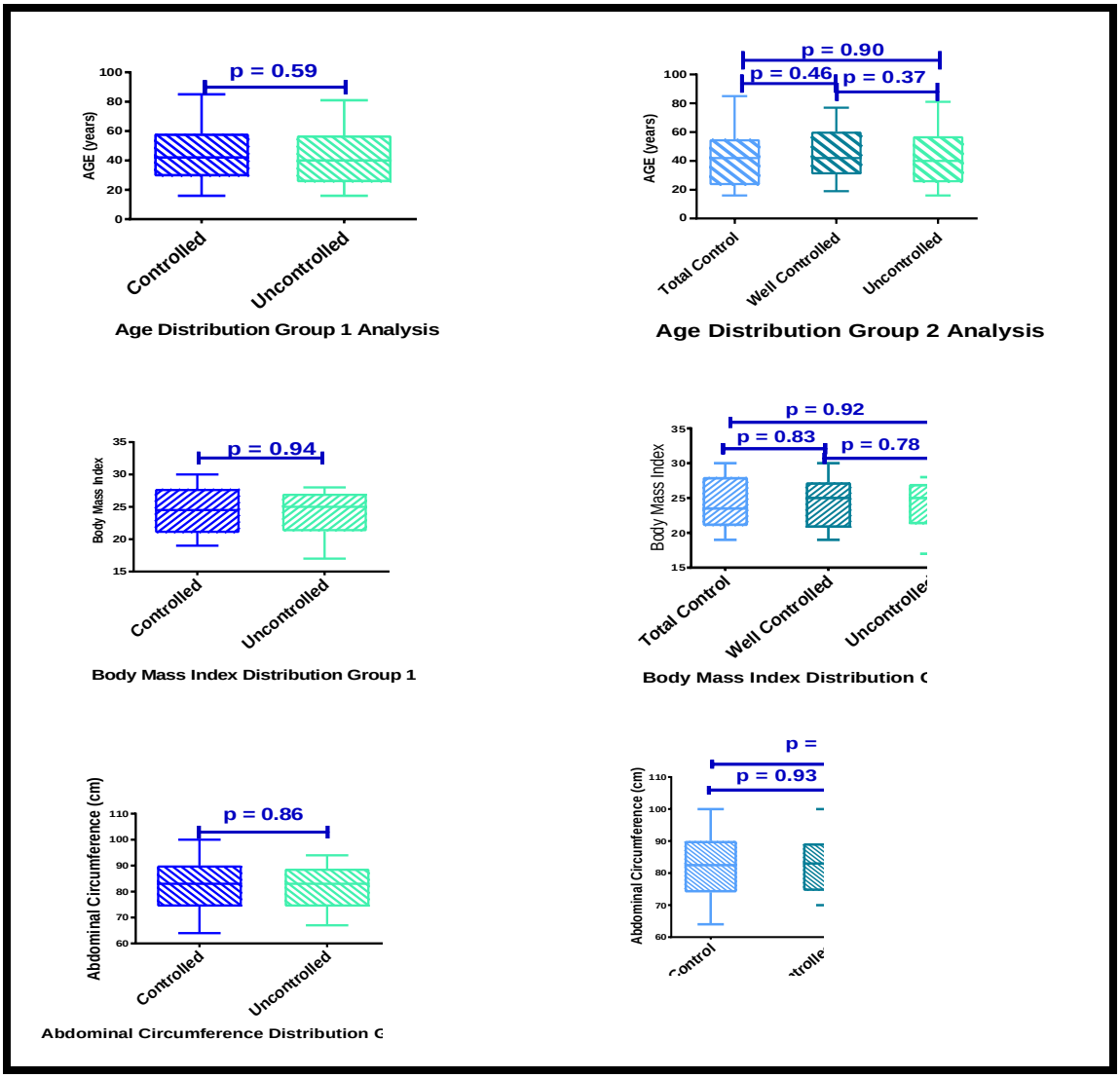

Figure 5.2: Demographic and clinical data of **group 1** (left) and **group 2** (right) patients in the different asthma control groups as defined by the ACQ
Group 1 analysis: Wilcoxon rank sum. Group 2 analysis: Kruskal-Wallis one-way ANOVA test.

5.5.2 Asthma control data

The percentage of patients who were assessed by the ACQ as being totally controlled, well-controlled or uncontrolled was 39%, 29% and 32% respectively, whereas the proportion of patients assessed by the AST as either totally controlled, well-controlled or uncontrolled was 26%, 44% and 30%. Using the ACT, 47% of the total cohort of patients were assessed as being controlled and 53% of the patients as been uncontrolled. None of the patients in this cohort, assessed by the ACT, had totally controlled asthma as defined by an ACT score of 25 (Table 5.2; Figure 5.3).

Table 5.2: Asthma control in 76 asthmatic patients as categorised by the ACQ

Asthma Control	Controlled[1]- ACQ ≤ 1.5 (CC) n=52	Totally controlled[1]- ACQ ≤ 0.75 (TC) n=30	Well-controlled[1]- 0.75 < ACQ ≤ 1.5 (WC) n=22	Uncontrolled[1]- ACQ > 1.5 (UC) n=24
ACQ[2]	0.7 (0.62 - 0.85)	0.4 (0.35 - 0.52)	1.14 (1.04 - 1.24)	2.7 (2.32 - 3.10)
ACT[3]	17.3 (16.2 - 18.5)	19.3 (17.9 - 20.8)	14.9 (13.6 - 16.3)	13.0 (11.2 – 15.0)

ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test, n: Number.
The AST was assessed as a categorical value and thus the data of the AST are not included in the table
The results are reported as medians together with the 95% confidence intervals (CI).
1: Asthma control categories defined by the ACQ score: CC: Controlled, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. The controlled group further sub classified in totally controlled and well-controlled.
2: The ACQ scores recorded in each of the asthma control categories, as defined by the ACQ, represent the actual median ACQ scores obtained when applying the ACQ questionnaire.
3: The ACT scores recorded in each of the asthma control categories, as defined by the ACQ, represent the actual median ACT scores obtained when applying the ACT questionnaire.

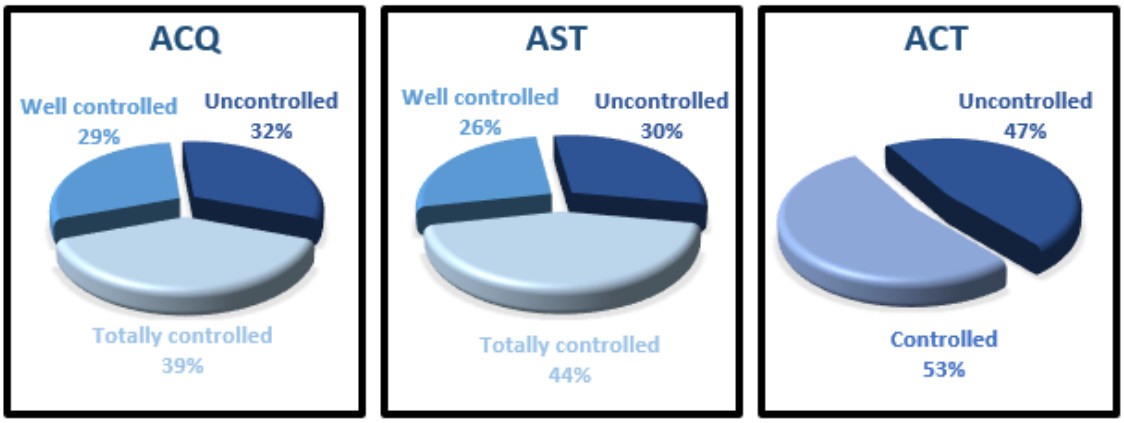

Figure 5.3: Categories of asthma control in the study population, as assessed by the ACQ, AST and ACT
AST: Physicians Asthma Rating, ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test.

There were significant differences between the ACQ scores where the controlled group of patients had significantly lower scores than the uncontrolled group of patients (**group 1** analysis), ($p < 0.0001$) (Figure 5.4). This was also true when the totally controlled group was compared with the well-controlled and uncontrolled groups (**group 2** analysis), ($p < 0.001$ and $p < 0.0001$, respectively) (Figure 5.5).

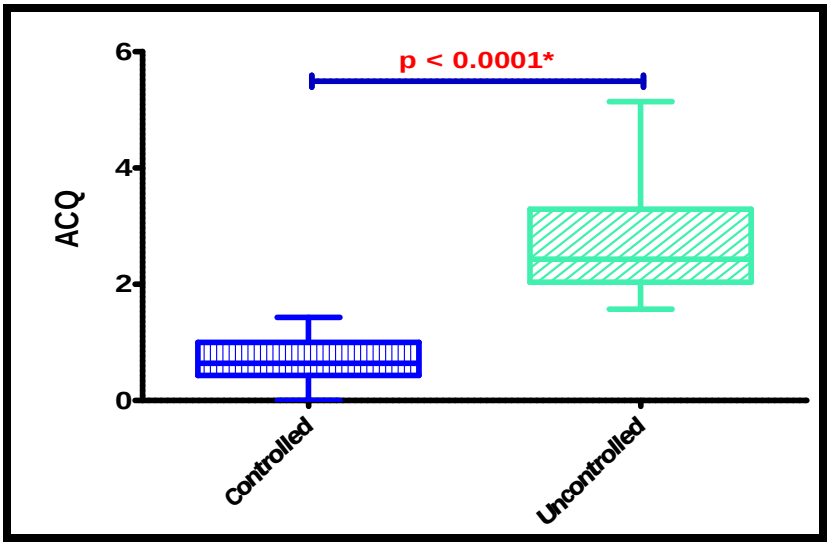

Figure 5.4: ACQ scores comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
ACQ: Asthma Control Questionnaire. Wilcoxon rank sum.

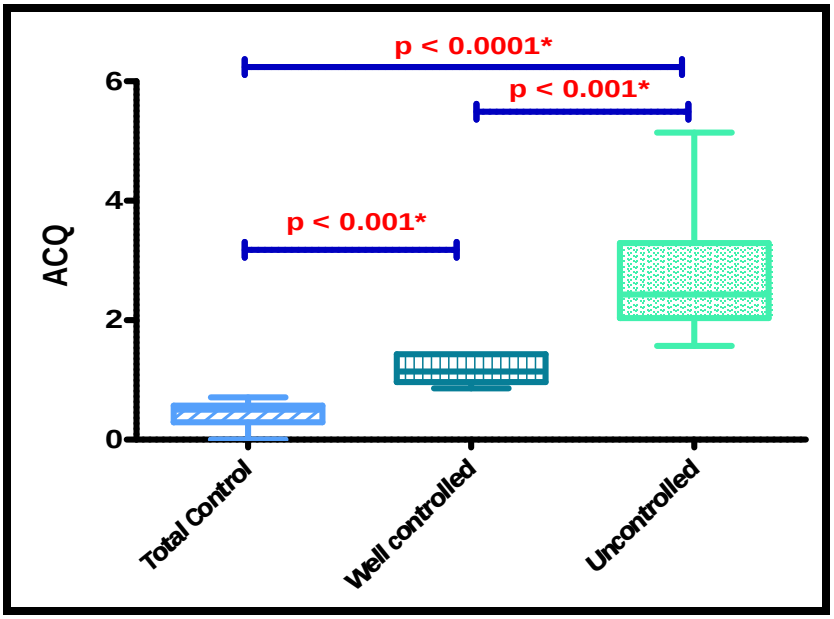

Figure 5.5: ACQ scores comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)
*p values reported as significant are depicted in **red**.
ACQ: Asthma Control Questionnaire. Kruskal-Wallis one-way ANOVA test.

5.5.3 Comparison and correlation between the ACQ, ACT and AST

There was significant agreement between the investigators independent assessment of asthma control (AST) and the ACQ classification (McNemar test for symmetry; p = 0.0326) (Table 5.3). In addition, there was also a significant agreement between the assessments of asthma control as assessed by the two most validated questionnaires, namely the ACQ and the ACT (McNemar test for symmetry; p=0.0017) (Table 5.4). The significant agreement between the ACQ, ACT and AST in their assessment of the categories of asthma control in this present cohort of patients was also confirmed with additional statistical testing (Table 5.5).

Table 5.3: Number of patients in each asthma control group when comparing the categorisation by means of the ACQ questionnaire versus the AST assessment

Physician Asthma Rating (AST)	Categories of asthma control as defined by the ACQ			Total (p value)
	TC: ACQ ≤ 0.75	WC: 0.75 < ACQ ≤ 1.5	UC: ACQ > 1.5	
Totally controlled (AST)[1]	19	1	0	20
Well-controlled (AST)[1]	10	17	6	33
Uncontrolled (AST)[1]	1	4	18	23
Total	30	22	24	**76 (p = 0.036)***

1: Categories of asthma control as determined by the investigator.
McNemar test for symmetry; * = p < 0.05.
TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled, ACQ: Asthma Control Questionnaire, AST: Physicians Asthma Rating.

Table 5.4: Number of patients in each asthma control group when comparing the categorisation by means of the ACQ versus the ACT questionnaires

Asthma Control Test (ACT)	Categories of asthma control as defined by the ACQ			Total (p value)
	TC: ACQ ≤ 0.75	WC: 0.75 < ACQ ≤ 1.5	UC: ACQ > 1.5	
Totally controlled (ACT=25)[1]	0	0	0	0
Well-controlled (ACT 20-24)[1]	25	6	5	36
Uncontrolled (ACT< 19)[1]	5	16	19	40
Total	30	22	24	**76 (p = 0.0017)***

1: Categories of asthma control as defined by the ACT score- Totally Controlled = 25, Well-controlled > 20 and ≤ 25, Uncontrolled < 19.
McNemar test for symmetry; * = p < 0.05.
TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled, ACT: Asthma Control Test, ACQ: Asthma Control Questionnaire.

Table 5.5: Association between the categories of asthma control as defined by the ACQ versus the ACT and versus the AST

Asthma Control Questionnaires	**Group 1** as defined by the ACQ[1] (Fischer's exact) p values	**Group 2** as defined by the ACQ[2] (Pearson's chi-squared) p values
ACT	**0.003***	**0.001***
AST	**0.001***	**0.001***

1: Group 1: Controlled = ACQ ≤ 1.5; Uncontrolled = ACQ > 1.5.
2: Group 2: Totally controlled = ACQ ≤ 0.75; Well-controlled = 0.75 < ACQ ≤ 1.5; Uncontrolled = ACQ > 1.5.
* = p < 0.05.
ACT: Asthma Control Test, ACQ: Asthma Control Questionnaire, AST: Physicians Asthma Rating.

There was also a significant negative correlation between the ACQ and ACT scores when analysed with a Spearman rank correlation test (Figure 5.6).

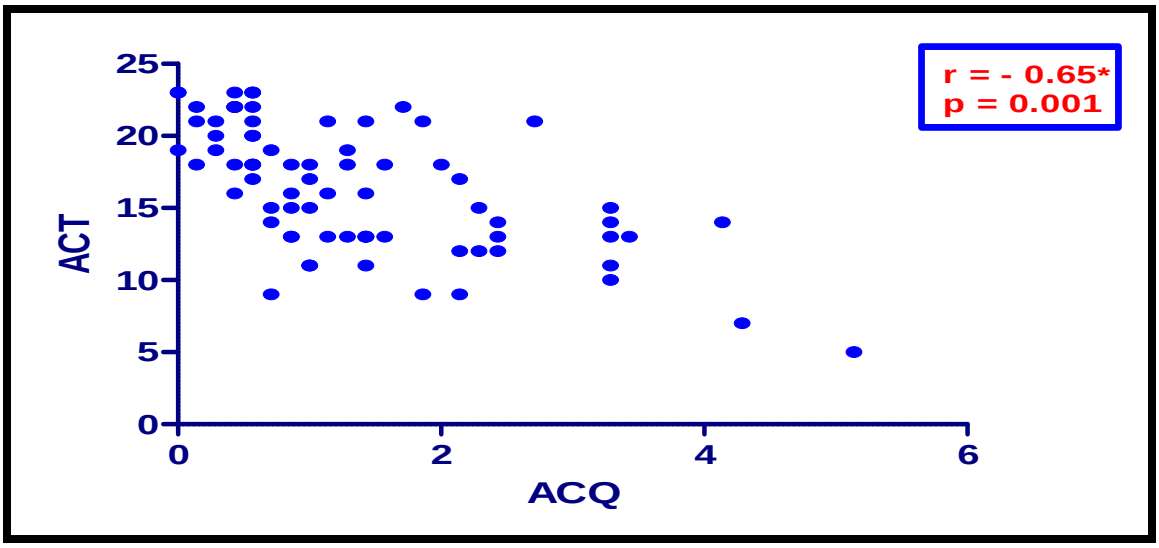

Figure 5.6: Correlation between the ACT versus the ACQ
* r values reported as a strong correlation are depicted in **red**.
ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test. Spearman rank correlation.

When the ACQ was analysed with the spirometric values omitted, there was no significant difference between the abridged ACQ (ACQ 6) and the unabridged ACQ (ACQ 7); p = 0.7 (Figures 5.7). There was also a very strong correlation between the ACQ 6 and the ACQ 7; r = 0.95, p < 0.001), thus confirming the robustness of the ACQ 6 as a test for asthma control within this cohort of patients (Figure 5.8).

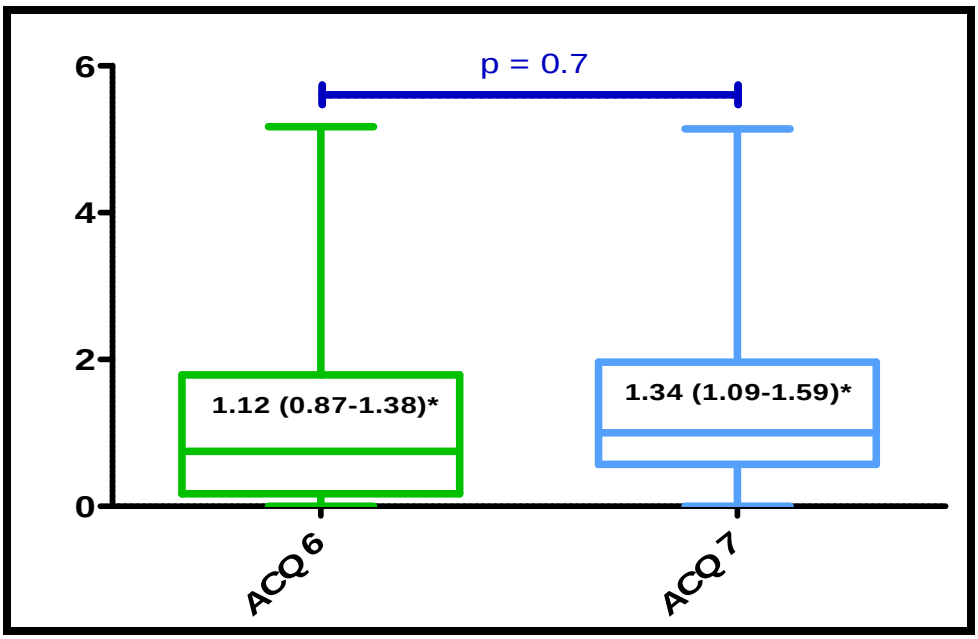

Figure 5.7: Comparison between the ACQ 6 and the ACQ 7
*Values in the figure represent the medians and the 95% CI.
ACQ: Asthma Control Questionnaire, Wilcoxon rank sum.

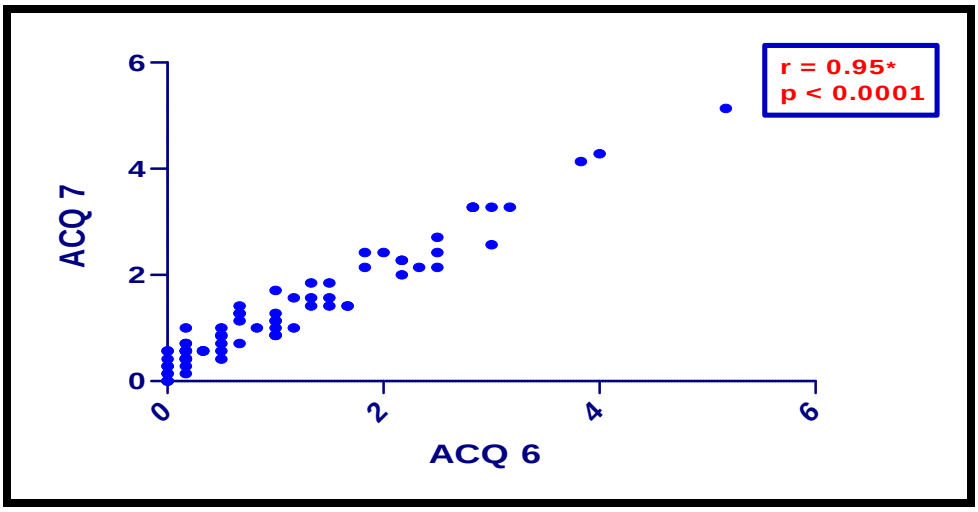

Figure 5.8: Correlation between the ACQ 6 and the ACQ 7
*r values reported as a strong correlation are depicted in **red**.
ACQ: Asthma Control Questionnaire. Spearman rank correlation.

5.5.4 Medications used and the relationship between medication and asthma control

The medication the patients were taking for their asthma was also recorded. All 76 patients were on some form of ICS. Overall, 85% (44/52) of the patients with controlled asthma (ACQ ≤ 1.5) were prescribed the moderate-dose of the inhaled corticosteroid (budesonide 400µg twice a day), and only 13% (7/52) of the patients were prescribed the high-dose inhaled budesonide

(800μg twice a day) (ICS dose category as defined by GINA) (8). Within the controlled group of patients, 10% (5/52) were prescribed oral steroids and 60% (3/5) of these patients were on a dose ≤ 7.5 mg per day. In contrast, 21% (5/24 patients) of the uncontrolled group of patients were prescribed maintenance oral steroids of 7.5 mg or less.

Overall, 57% (43/76) of all the patients included into the study were prescribed a long acting β2 agonist (LABA). In total, 54% (28/52) of the well-controlled asthmatic patients were prescribed a LABA, while 63% (15/24) of the uncontrolled group were prescribed a LABA. Only one patient in the total cohort of patients was using any form of a combination ICS-LABA therapy. None of the patients were prescribed any form of inhaled anti-muscarinic agent. Virtually all of the patients in the total cohort (99%) were receiving a short acting β2 agonist (SABA).

A methylxanthine (theophylline) was prescribed in 12% (9/76) of all the patients, 12% (6/52) within the controlled group and 12% (3/24) in the uncontrolled group. In addition, 17% (13/76) of the total cohort of patients were prescribed a leukotriene receptor antagonist (LRTA), of which 10% (5/52) were in the controlled group of patients and 33% (8/24) were within the uncontrolled group.

Nasal steroids were used by 89% (68/76) of all the patients and 30% (23/76) of the patients were prescribed antihistamines (H1 receptor blockers).

Within the total cohort, 34% (26/76) of patients were using anti-reflux medication, 18% (14/52) in the controlled group and 50% (12/24) in the uncontrolled group. A proton pump inhibitor was prescribed to 20% (15/76), while 14% (11/76) of the cohort was prescribed an H2 receptor blocker.

Figures 5.9 and 5.10 depict the cohort of patients used in the present study, categorised by their asthma control as defined by the ACQ, and the medication they were using.

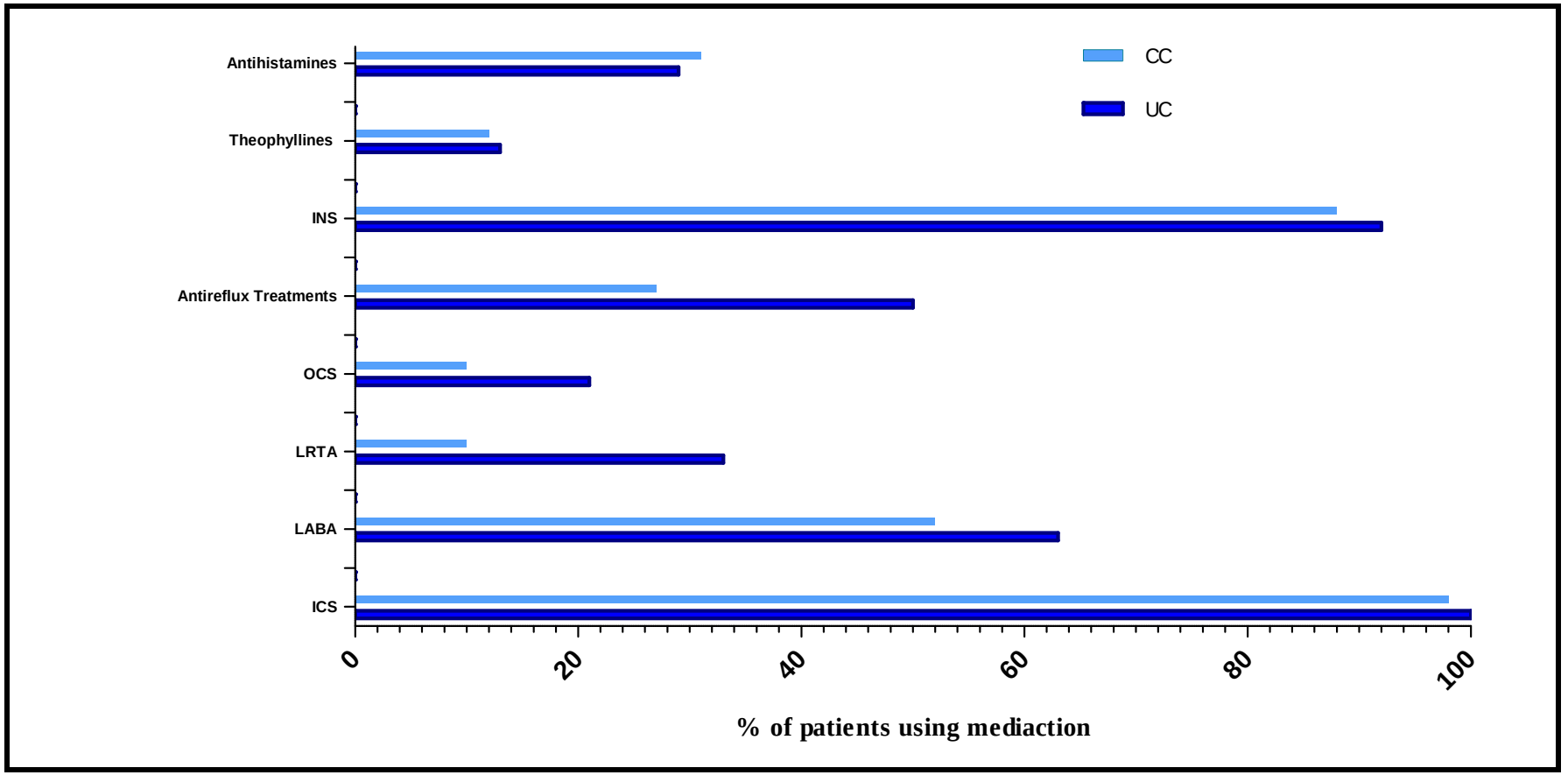

Figure 5.9: Asthma control as defined by the ACQ and the medication the patients were using
CC: Controlled, UC: Uncontrolled - as defined by the ACQ.
INS: intra-nasal corticosteroid, OCS: oral corticosteroid, LRTA: leukotriene receptor antagonist, LABA: long acting Beta 2 agonist, ICS: inhaled corticosteroid.
No data are given for the use of combined ICS-LABA as only 1 patient was on such therapy.

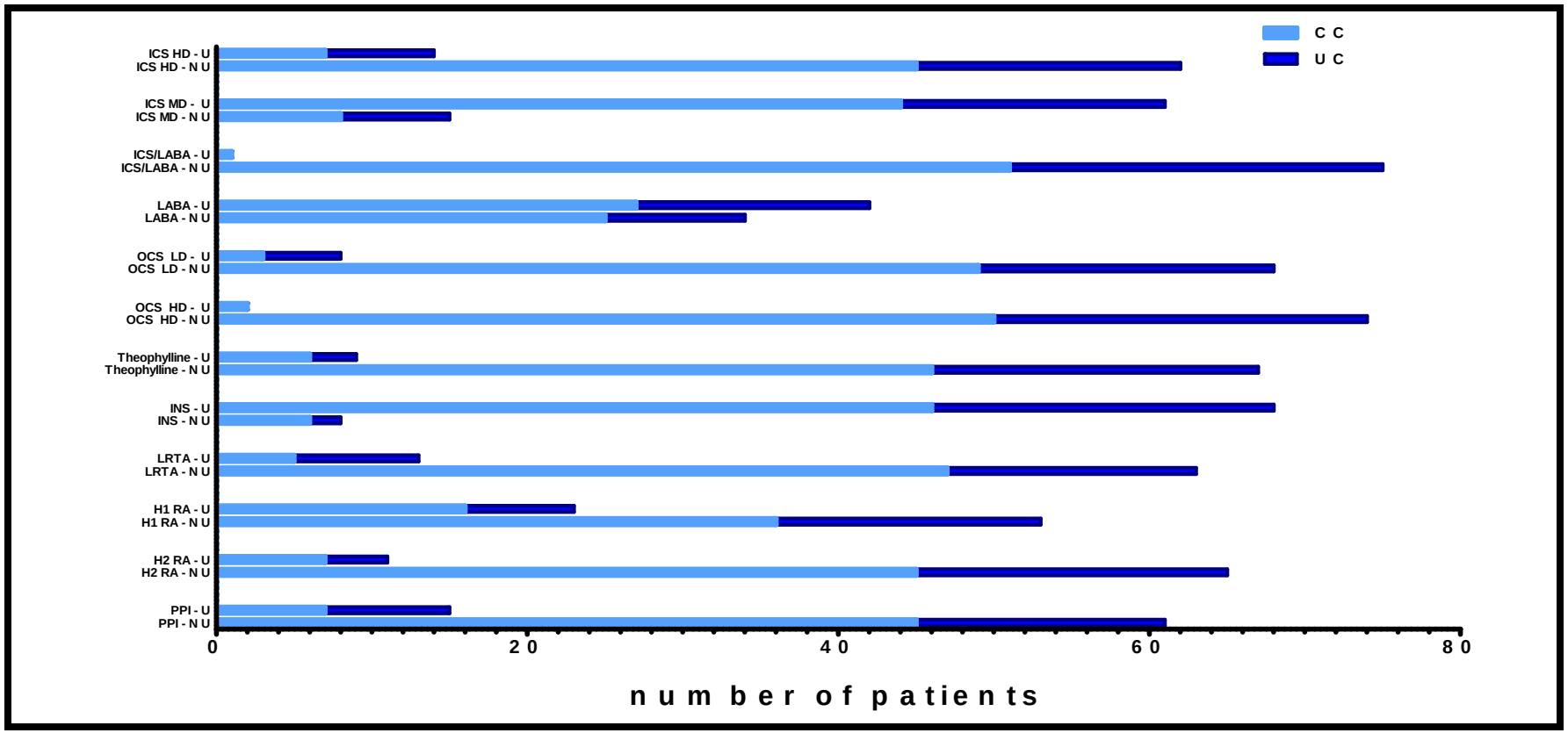

Figure 5.10: Asthma control as defined by the ACQ and the medication the patients were using or not using
CC: Controlled, UC: Uncontrolled - as defined by the ACQ, U: Using, NU: Not using.
ICS: inhaled corticosteroids, ICS HD: high-dose budesonide between 800 and 1600 µg/day, ICS MD: moderate-dose budesonide ≤ 800 µg/day, LABA: long acting Beta 2 agonist, ICS-LABA: inhaled corticosteroid - long acting β2 agonist combination therapy, OCS: oral corticosteroid, OCS HD: high-dose prednisone > 7.5 mg/day, OCS LD: low-dose prednisone ≤ 7.5 mg/day, INS: intra-nasal corticosteroid, LRTA: leukotriene receptor antagonist, H1 RA: Histamine 1 receptor blocker, H2 RA: Histamine 2 receptor blocker, PPI: proton pump inhibitor.

The patients level of asthma control was further compared to the medication the patients were prescribed in order to determine whether any of the individual medications used were associated with asthma control. For this analysis, the individual therapies were compared to the controlled and uncontrolled asthmatic patients (**group 1**) (Table 5.6). Oral prednisone usage less than 7.5 mg daily and montelukast therapy appeared to be associated with uncontrolled asthma as defined by the ACQ. Patients using oral prednisone at a dose of ≤ 7.5 mg per day, as well as those taking montelukast were significantly more likely to be uncontrolled with odds ratios of 6.58 (95% CI 1.08 - 39.91) (p = 0.018) and 4.70 (95% CI 1.26 - 17.58) (p = 0.011), respectively (Table 5.6). However, since small numbers of patients in the entire cohort were receiving these therapies (i.e. for prednisone only 7 patients and montelukast only 13 patients), and the confidence intervals were very large, the clinical significance of these findings is questionable (Table 5.6).

Table 5.6: The odds ratios (OR) of the patients having controlled versus uncontrolled asthma (**group 1**), as defined by the ACQ, in relationship to the medication used

Medication name	No. of patients using medication (%)	OR	95% CI	p value
Oral Prednisone > 7.5 mg	2 (2.6 %)	0.0	0.0 - 0.0	0.33
Oral Prednisone ≤ 7.5 mg	7 (9.2 %)	6.58	1.08 - 39.91	**0.018***
Budesonide > 800 and ≤ 1600 µg	14 (18.4 %)	2.64	0.78 - 8.94	0.11
Budesonide ≤ 800 µg	61 (80.2 %)	0.44	0.13 - 1.43	0.16
Formoterol	40 (52.6 %)	1.17	0.44 - 3.09	0.76
Salmeterol	6 (7.8 %)	2.33	0.43 - 12.81	0.32
Salbutamol	75 (98.7 %)	0.0	0.0 - 0.0	0
Fluticasone+Salmeterol	1 (1.3 %)	0.0	0.0 - 0.0	0.50
Ipratropium Bromide	0 (0 %)	0.0	0.0 - 0.0	0
Tiotropium Bromide	0 (0 %)	0.0	0.0 - 0.0	0
Montelukast	13 (17.1 %)	4.70	1.26 - 17.58	**0.011***
Nasal Steroid	68 (89.5 %)	1.43	0.26 - 7.79	0.67
Methylxanthines	9 (11.8 %)	1.10	0.25 - 4.85	0.91
Proton Pump Inhibitor	15 (19.7 %)	3.21	0.96 - 10.72	0.05
H_2 Receptor blockers	11 (14.5 %)	1.29	0.33 - 4.94	0.71
H_1 Receptor blockers	23 (30.3 %)	0.93	0.32 - 2.69	0.89

Pearson's chi-squared test. * = p < 0.05.
OR: Odds ratio, 95% CI: 95% confidence intervals, mg: milligrams, µg: micrograms, No.: number.

The same findings were documented when the above analysis was repeated using the Fisher's exact (2-tail) test (Table 5.7). Similarly, the clinical significance of these findings is questionable.

Table 5.7: The comparison of the medication prescribed in the 76 asthma patients and the level of asthma control as defined by the ACQ

Medication Name	Controlled (n=52) Pts on Rx:Pts not on Rx (% of pts on medication)	Uncontrolled (n=24) Pts on Rx:Pts not on Rx (% of pts on medication)	Fisher's exact test (p value)
Prednisone > 7.5 mg	2:50 (4)	0:24 (0)	1
Prednisone ≤ 7.5 mg	2:50 (4)	5:19 (21)	**0.029***
Budesonide > 800 and ≤ 1600 µg	7:45 (13)	7:17 (29)	0.12
Budesonide ≤ 800 µg	44:8 (85)	17:7 (71)	0.22
Formoterol	24:28 (46)	12:12 (50)	0.81
Salmeterol	3:49 (6)	3:21 (13)	0.37
Salbutamol	51:1 (98)	24:0 (100)	1
Fluticasone+Salmeterol	1:51 (2)	0:24 (0)	1
Ipratropium Bromide	0:52 (0)	0:24 (0)	0
Tiotropium Bromide	0:52 (0)	0:24 (0)	0
Montelukast	5:47 (10)	8:16 (33)	**0.019***
Nasal Steroid	46:6 (88)	22:2 (92)	1
Methylxanthines	6:46 (12)	3:21 (13)	1
Proton Pump Inhibitor	7:45 (13)	8:16 (33)	0.06
H_2 Receptor blockers	7:45(13)	4:20 (17)	0.73
H_1 Receptor blockers	16:34 (31)	7:17 (29)	1

Fisher's exact test.* = $p < 0.05$.
Pts: patients, Rx: treatment, mg: milligram, µg: microgram, n: number.

5.6 Discussion

Asthma control is defined as the extent to which the various manifestations of asthma have been reduced or removed by treatment (50). However, from a practical perspective asthma control is assessed on two domains (8).The first is the level of clinical control (i.e. day to day control) gauged from features such as symptoms and the performance of activities of daily living. Secondly, asthma control also includes the minimizing of the risk of future adverse events including loss of control, exacerbations and accelerated decline in lung function (8, 50, 51, 343).

The present study was performed to identify, categorise and describe a cohort of asthmatic patients attending a specialist referral asthma clinic and to characterise their asthma control, as defined by the ACQ, the ACT and the AST. The publication by Juniper *et al.* (59), using the GOAL cohort of patients, identified three categories of asthma control based on specific numerical scores, derived from the ACQ questionnaire. These values (i.e. ACQ < 0.75, ACQ

between 0.75 and 1.5 and ACQ > 1.5) allowed patients with asthma to be reliably classified into three categories i.e. totally controlled, well-controlled and uncontrolled (59). The reliability of the ACQ as a stratification tool has been confirmed in several publications, and was the reason for its application in this thesis (50, 65, 344). The most notable of these publications was in 2014 by Barnes *et al*. (345), in which the ACQ was recommended as a "valid and robust measure for use as a primary or secondary endpoint in future clinical trials in asthma". That publication also described the ACQ as "an instrument that allows accurate and reproducible assessment of asthma control that compares favourably with other commonly used instruments" (345).

The majority of the patients screened for inclusion into the present study were female (60% female and 40 % male). One of the reasons for this gender inequality, which ultimately served to be of no clinical significance, is probably due to the fact that female asthmatic patients may have greater health seeking behaviour than men and are potentially free during the day to go to the clinics during office hours while the men are working (346). Another possible reason for this gender inequality might also be that the incidence of asthma is higher among females than males in the adult population (347, 348).

The median age of the patients studied was 38.9 years (95% CI 35.19 - 43.12) with the median age of the patients in the controlled group being 39.8 years (95% CI 35.24 - 44.97) and in the uncontrolled group being 37.1 years (95% CI 30.56 - 45.23). The median BMI of the patients studied was 23.89 (95% CI 23.1- 24.69) and the median abdominal circumference all of the patients studied was 81.57 cm (95% CI 79.62 - 83.65) (Table 5.1; Figure 5.2). There was no difference between any of the asthma control groups for the BMI measurements or abdominal circumference measurements. The reason for excluding patients with central obesity and a high BMI was to limit the possibility of abdominal adiposity contributing to abnormalities in the levels of the biomarkers measured in the subsequent chapters (349, 350).

Currently there are two commonly used asthma control questionnaires. These are the ACQ developed by Juniper *et al*. (58) and the ACT, developed by Nathan *et al*. (61). The ACQ assesses the patient's level of control by scoring five questions based on patient's symptoms over the last week, one question on the need for reliever therapy and one on the spirometric assessment of airways (Appendix 1). The lower the average score the better the asthma control

with a score of < 0.75 reflecting total asthma control (59). The other validated asthma assessment scale is the Asthma Control Test (ACT), and conversely, the higher the ACT score the better the control (Appendix 2) (60, 61). Major differences between the ACQ and ACT are that the ACT is completed by the patient independently, assesses patient symptoms over the last month with a score of more than 19 indicative of control, and does not use spirometry parameters as part of the composite score (62).

Both tests have been extensively used in the clinical setting as well as for research purposes (345), and there are studies confirming the excellent agreement between the ACQ and ACT (51, 63). This was also observed in the present study when the ACQ was compared with the ACT ($p = 0.0017$). The median ACT in the totally controlled group as defined by the ACQ was 19.3 (17.9 - 20.8) and the median ACT in the uncontrolled group as defined by the ACQ was 13.0 (11.2 – 15.0). There was also a strong negative correlation between the ACQ and ACT in the present study ($r = -0.65$; $p = 0.001$), and this compared favourably with a previous publication by Schatz *et al*. (62), with a reported very strong negative correlation between the ACQ and ACT ($r = -0.89$; $p < 0.01$). A possible reason for the difference in the levels of correlation reported in the two studies described above (i.e. r = -065 versus r= -0.89), might have been due to a language barrier in the current cohort of patients. The ACT is completed independently by the patient and is available in several of the official Southern African languages, thus allowing the patient to understand the questions better. However, the ACQ is not available in any of the other official Southern African languages thus possibly hindering the patients' full understanding of the questions.

Another significant difference between the ACQ and ACT is the fact that the ACT does not use spirometry as part of the assessment of control. This has important implications as the ACT could be used in facilities that might not have spirometric equipment to aid in the management of patients with asthma, as is the case in most of the primary health care facilities in South Africa. To explore the additional value of the use of spirometry in improving the utility and accuracy of the ACQ, as a validated tool to assess asthma control in this present study, a further analysis was performed. The scoring of the ACQ was re-analysed with the spirometric values included (i.e. the unabridged ACQ 7) and excluded (i.e. the abridged ACQ 6) to assess the agreement between the ACQ 6 versus the ACQ 7. There was no difference between the ACQ 6 versus ACQ 7 scores ($p = 0.7$) and a very strong correlation was noted between the ACQ 6

and ACQ 7 ($r = 0.95$; $p < 0.0001$). A similar finding was noted in a systemic review and meta-analysis of the ACQ published by Jia *et al*.(63). Of note, Teeter *et al*. (351), as far back as 1998, reported that there was a poor correlation between asthma symptoms and FEV1. Hence, the documentation that spirometry adds minimal value to the assessment of asthma control, has been adopted into both the latest Global Initiative for Asthma (GINA) and the National Asthma Education and Prevention Program (NAEPP) guidelines, which do not recommend the routine use of spirometry in the assessment of asthma control (8, 352). However, spirometry remains a valuable tool in the diagnosis of asthma, in the assessment of risk of future adverse events including loss of control and future exacerbations, as well as for the monitoring of accelerated decline in lung function parameters (50).

The current NAEPP guidelines (352) also recommend that all patients with asthma have their asthma assessed by enquiring as to the level of control maintained by the patient. This should be assessed either with a validated questionnaire or by asking questions pertaining to reliever usage, day as well as nocturnal symptoms and limitation of daily activities. In line with these recommendations, the investigator also independently assessed the level of asthma control in all the patients in the present study, without using any predefined questions or having access to any of the specialised tests performed. This independent assessment was termed the physicians asthma rating (AST), and the investigator categorised the patient's asthma control as being either totally controlled, well-controlled or uncontrolled. There was a significant agreement between the investigator's independent assessment of asthma control (AST) and the ACQ (McNemar test for symmetry; $p = 0.0326$). These findings were verified with additional statistical analysis (Table 5.4).

This agreement between the ACT and a doctor's independent assessment of asthma control (referred to as the AST in the present study) was documented in another South African publication by Greenblatt *et al*. (339). This was a multicentre study which was undertaken to obtain data on asthma patients from public and private medical facilities, and included assessments of asthma control from doctors of varying qualification and experience (339). Significant association existed between the ACT score and the doctor's independent assessment of asthma control, with the median ACT scores increasing concordantly when compared with the three categories of the doctor's independent assessment of asthma control (i.e. totally controlled, well-controlled and uncontrolled) (overall and pair-wise tests, $p < 0.0001$). In that

study the mean ACT score was 12.8 where doctors assessed the patients as being ‘not controlled’ and 19.9 where doctors assessed the patients as being ‘well-controlled’.

Greenblatt *et al*. (339) further reported that combination therapy with a LABA- ICS was the only therapy that appeared to be associated with better asthma control. After controlling for the other factors, the odds of the patients’ asthma being controlled when using individual ICS and LABA inhalers was 0.34 when compared with those patients’ on combined ICS-LABA inhalers, with patients 2.94 times more likely to have controlled asthma if they were on combined ICS-LABA than on separate ICS-LABA therapy. This has been corroborated in the Gaining Optimal Asthma Control (GOAL) study by Bateman *et al*. (7), wherein a greater percentage of patients achieved asthma control more rapidly and at a lower corticosteroid dose when using a combined ICS-LABA inhaler compared to patients using ICS only (71% vs 59% ; $p < 0.001$). Unfortunately, we were unable to determine the benefit combined ICS-LABA therapy in this study as it was undertaken in the public sector and such therapy was not routinely available at that time.

In this present study, certain medications (i.e. low-dose oral prednisone and montelukast) appeared to be associated with uncontrolled asthma as defined by the ACQ (Tables 5.6, 5.7). However, because only a few of the total study population (i.e. 9% using low-dose oral prednisone and 13% using montelukast) were on these medications, and because the confidence intervals were so large, the clinical significance of these findings with respect to their association with uncontrolled asthma is questionable.

5.7 Conclusion

This present study of South African asthmatics documented that the two internationally validated asthma control questionnaires, namely the ACQ and the ACT, performed well and agreed not only with each other, but also with an independent physician’s assessment of the level of asthma control in the patients studied. The ACQ performed robustly in the assessment of control even when the spirometric measurements were omitted from the composite score (i.e. ACQ 6), enhancing its applicability as a tool in asthma management in resource poor settings, where facilities for the measurement of spirometry are limited. Unfortunately, the study was not able to confirm the value of combination LABA-ICS therapy in attaining asthma control since this form of therapy was not routinely available in the study setting.

CHAPTER 6: APPRAISAL OF AIRWAY CALIBRE MEASURED BY SPIROMETRY AND IMPULSE OSCILLOMETRY IN COMPARISON TO CATEGORIES OF ASTHMA CONTROL AS DEFINED BY THE ACQ

6.1 Introduction

Asthma is increasingly being recognised as an inflammatory airways disease affecting not only the large airways, but also the small airways (20, 297). Appraisal of the small airway in patients with asthma has always been an enigma for pulmonologists (21). Impulse oscillometry (IOS) has re-emerged as a novel method of assessing small and large airway calibre especially in patients with asthma. It measures airway impedance over a range of frequencies (i.e. 5 to 35 Hz). Resistance and reactance to sound impulses at these various frequencies provides valuable information about the nature of the airways (310). Higher frequency oscillations, such as 20 Hz, transmit signals more proximally and provide information primarily concerning the central airways. Lower frequency oscillations, such as 5 Hz, generally travel farther to the lung periphery and provide information on the entire pulmonary system. If the value of central airway resistance (R20) is subtracted from that for total airway resistance (R5) i.e. R5-R20, this difference provides a measurement of peripheral or small airway resistance (296). The parameter R5-R20 is also known as frequency-dependence of resistance (fdR).

There is no published data in South Africa on the appraisal of the small airways in the context of different categories of asthma control as defined by the ACQ. This is important as it is becoming increasingly recognised that persistent inflammation in the small airways may be present in patients whose asthma appears to be controlled, when assessed by spirometry and asthma control questionnaires (66). Hence, the next objective of this study was to use IOS to evaluate the small airways in a cohort of patients with asthma and to evaluate the relationship between IOS and standard spirometry. Furthermore, the aim was to determine whether IOS and standard spirometric measures of the small airways (i.e. FEF 25-75) demonstrated a significant association and/or correlation with asthma control as defined by the ACQ.

6.2 Design and Study Population

This study was designed as a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic with approval to conduct the

study received from the University of the Witwatersrand Human Research Ethics committee (Protocol Number M060401- Appendix 3). The specifics of the design and population cohort are the same as the study population in Chapter 5 and have been described detail in Chapter 4.

6.3 Methods

A detailed description of the methodology employed in the present study has been described in Chapter 4. For the purposes of standardisation within this thesis, all patients completed the Asthma Control Questionnaire (ACQ), which was used as the basis for the assessment of asthma control in the entire cohort of patients studied (Appendix 1) (58). As indicated in Chapter 4, the population of patients studied were categorised into two groups, based on their level of control. **Group 1** consisted of the patients categorised into controlled (CC) and uncontrolled (UC), based on an ACQ level of 1.5. **Group 2** consisted of the same cohort of patients categorised by the ACQ into totally controlled (TC) having an ACQ < 0.75, well controlled (WC) having an ACQ of between 0.75 and 1.5 and uncontrolled (UC) with an ACQ > 1.5 (59).

In all the studies, the evaluation was first for **group 1** (i.e. primary/initial analysis: controlled versus uncontrolled asthmatic patients) and thereafter, the **group 2** analysis (i.e. subsequent/secondary analysis: totally controlled versus well controlled and versus uncontrolled asthmatic patients).

Of importance in this chapter is that patients underwent lung function studies. All lung function testing, including spirometry and measurements of IOS were done in the Lung Function Laboratory of the Charlotte Maxeke Johannesburg Academic Hospital and were performed by qualified laboratory technologists. All tests done conformed to the guidelines published by the ATS/ERS (295, 300). The rationale and methodology of the application of IOS was performed as described in Chapter 2. The values of the spirometric and IOS measurements in the current cohort of patients are all reported as the percentage (%) of the predicted value. The reference values used to determine the percentage predicted for spirometry values measured were obtained from the European Community for Coal and Steel (ECCS) population database (300). The reference values for the IOS parameters used were provided by Jaeger (Hochberg, Germany). Most important is that for all non-Caucasian patients, a correction factor of 10% less than the reference values obtained from the ECCS population database was applied for the

spirometric data, as described and endorsed by the South African Thoracic Society (336). Currently, in South Africa, as there are no guidelines for the measurement and interpretation of IOS data in a local cohort of patients, the same correction factor used for spirometry was applied to the IOS predicted values. All the spirometric data reported were interpreted in accordance with the ATS/ERS consensus document on "interpretative strategies for lung function tests" published by Pellegrino *et al.* (301). Lung function parameters below the fifth percentile of the predicted value (i.e. below the lower limit of normal (LLN)) or below 80% of predicted were interpreted and reported as being abnormal. Furthermore, a post-bronchodilator increase in the FEV1 $\geq$ 12% and $\geq$ 200 ml was reported as a positive bronchodilator response indicating airway reversibility (i.e. bronchial airway hyper-reactivity (BAH)).

6.4 Statistical Analysis

Methodology detailing the basic principles of the statistical analyses applied, including population size calculations, has been discussed in Chapter 4. The population of patients studied were 76 in total. The Shapiro-Wilks normality test indicated that the data reported was not normally distributed; hence, non-parametric statistical tests were used for all the analyses performed. The results were reported in both a table format, as well as graphically using a box-and-whisker plot where applicable. The results were represented in a dual format for ease of reference and interpretation. The results included in the tables were reported as medians together with the 95 percent (%) confidence intervals (CI). The box-and-whisker plot displayed the data using the standard five number summary: top and bottom boundary of the box indicating the 75th and 25th percentiles, the line within the box depicted the median and the whiskers represented the minimum and maximum values measured.

Results reported in the tables that were statistically significant are highlighted in bold black text with an asterisk *, whereas those results that had a clinically significant correlation are highlighted in bold black text with a double asterisk **, for easy recognition. The corresponding p or Rho (r) values are represented in the associated figures in **red**. Specific to this chapter, is that results reported in the tables that represent values relating to small airway parameters are highlighted in cyan …..

All the laboratory investigations, measured as continuous variables, were compared to the categories of asthma control, first for **group 1** and then for **group 2**. For the patients categorised into **group** 1 (i.e. controlled versus uncontrolled asthmatic patients), the association between

the variable measured and the level of asthma control was analysed using the Wilcoxon rank sum test. For the comparisons of matched variables within the **group 1** cohort (i.e. the comparison of pre-bronchodilator versus the post-bronchodilator values of the same patient within the controlled group and the uncontrolled group), the Wilcoxon Signed-Rank Test using paired observations was applied. For the patients categorised into **group 2** (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Kruskal-Wallis one-way ANOVA test. The Bonferroni correction was applied to the analysis of the **group 2** cohort to control for multiplicity.

In all testing a p-value < 0.05 was considered significant, except where the Bonferroni correction was applied.

A Spearman's rank correlation was used to assess the correlation between the ACQ and all other variables measured. For the interpretation of the Spearman's rank correlation, as outlined in Chapter 4, only $r > 0.6$ was reported as a having a strong correlation, even though a $r < 0.6$ may have been statistically significant with a $p < 0.05$.

The statistical analyses and graphics were performed using the SPSS software, version 12 (**SPSS**, Chicago, Illinois), Excel software (Microsoft, Albuquerque, New Mexico) and GraphPad Software Inc. (La Jolla, California).

6.5 Results

The results reported will focus on the parameters of the small airways measured by spirometer and IOS. However, the data for the large, as well as the small airways, will be included in the tables and figures. The values of the spirometric and IOS measurements in the current cohort of patients are reported as the percentage (%) of predicted, as discussed above.

6.5.1 Spirometry data

A summary of the spirometric data for the patients in the **group 1** analysis (controlled vs uncontrolled asthmatics) are included in Tables 6.1 and 6.2. Table 6.1 representing all the pre-bronchodilator spirometric parameters, and Table 6.2 the post-bronchodilator parameters measured. The data are represented graphically as well, with Figure 6.1 depicting the large airway parameters measured, and Figure 6.2 depicting the small airway parameters measured.

Table 6.1: Pre-bronchodilator spirometric parameters (reported as percentage of predicted and FEV1/FVC ratio) comparing controlled and uncontrolled asthmatic patients **(group 1)**, and the correlation between these spirometric parameters and the ACQ score

Spirometry parameters+	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (pvalues) n=76[2]
Pre FVC	108.3 (101.5 - 114.9)	96.5 (87.4 - 105.7)	**0.02***	-0.2657 (0.0203)
Pre FEV1	81.0 (75.9 - 86.5)	57.1 (48.8 - 66.8)	**0.0001***	-0.5357 (0.0001)
Pre FEV1/FVC	62.7 (59.1 - 66.5)	51.5 (45.0 - 58.9)	**0.01***	-0.4051 (0.0003)
Pre PEFR	97.0 (90.1 - 103.9)	72.9 (62.0 - 83.9)	**0.0002***	-0.5157 (0.0001)
Pre FEF 25	56.0 (48.4 - 64.9)	29.2 (21.7 - 39.3)	**0.0001***	-0.5145 (0.0001)
Pre FEF 50	37.9 (32.3 - 44.6)	19.2 (14.0 - 26.4)	**0.0003***	-0.4964 (0.0001)
Pre FEF 75	25.3 (21.1 - 30.3)	15.4 (11.9 - 19.9)	**0.0027***	-0.4496 (0.0001)
Pre FEF 25-75	31.9 (26.9 - 37.9)	17.3 (12.7 - 23.5)	**0.0004***	-0.4914 (0.0001)

+: All spirometry parameters are reported as % of predicted unless otherwise indicated.
1: Wilcoxon rank sum test.* = $p < 0.05$.
2: Spearman rank correlation between ACQ score and other variables.
Values highlighted …….are spirometry measurement of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
FEV1: Forced expiratory volume in one second, FEV1/FVC: Percentage of the FVC expired in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF25-75: Forced expiratory flow during the middle half of the FVC, FVC: Forced vital capacity, n: Number, PEFR: Peak expiratory flow, Pre: Pre-bronchodilator.

Table 6.2: Post-bronchodilator spirometric parameters (reported as percentage of predicted and FEV1/FVC ratio) comparing controlled and uncontrolled asthmatic patients **(group 1)**, and the correlation between these spirometric parameters and the ACQ score

Spirometry parameters[+]	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (p values) n= 76[2]
Post FVC	109.1 (100.9 - 117.2)	94.8 (79.7 - 110.0)	0.05	-0.1825 (0.1145)
Post FEV1	87.9 (82.8 - 93.3)	67.2 (58.9 - 76.6)	**0.0001***	-0.5308 (0.000)
Post FEV1/FVC	66.3 (62.4 - 70.5)	56.6 (49.6 - 64.7)	**0.04***	-0.3432 (0.0024)
Post PEFR	103.5 (96.3 - 110.8)	83.8 (73.0 - 94.6)	**0.001***	-0.4795 (0.000)
Post FEF 25	62.6 (49.7 - 78.9)	39.7 (29.8 - 52.7)	**0.0014***	-0.4634 (0.000)
Post FEF 50	47.1 (40.0 - 55.3)	25.3 (18.4 - 34.8)	**0.0006***	-0.4939 (0.000)
Post FEF 75	31.1 (25.7 - 37.7)	15.5 (9.6 - 24.9)	**0.0044***	-0.4301 (0.0001)
Post FEF 25-75	40.2 (33.9 - 47.7)	22.3 (16.3 - 30.6)	**0.0009***	-0.4951 (0.000)

+: All spirometry parameters are reported as % of predicted unless otherwise indicated.
1: Wilcoxon rank sum test.* = $p < 0.05$.
2: Spearman rank correlation between ACQ score and other variables.
Values highlighted …….are spirometry measurement of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
FEV1: Forced expiratory volume in one second, FEV1/FVC: Percentage of the FVC expired in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF25-75: Forced expiratory flow during the middle half of the FVC, FVC: Forced vital capacity, n: Number, PEFR: Peak expiratory flow, Post: Post-bronchodilator.

There was a significant difference when the controlled group was compared with the uncontrolled group of patients for all the pre-bronchodilator spirometric parameters, with the controlled group having significantly higher values than the uncontrolled group (Table 6.1; Figures 6.1, 6.2). For the small airways, as assessed by the FEF 25-75, the measured values in the controlled group at baseline (i.e. pre-bronchodilation) were low at 31.9% (95% CI 26.9 - 37.9), suggesting a significant airflow limitation in those patients assessed as being controlled by the ACQ. This finding was mirrored in the uncontrolled group with the pre-bronchodilator median FEF 25-75 being 17.3% (95% CI 12.7- 23.5); $p = 0.0004$ compared with the controlled group (Table 6.1; Figure 6.2).

These findings were similar for the post-bronchodilator analysis with the controlled patients having higher spirometric parameters when compared with the uncontrolled patients (Table 6.2; Figures 6.1, 6.2). Importantly, the post-bronchodilator FEF 25-75 was also markedly reduced in the controlled group of asthmatics with a median of 40.2% (95% CI 33.9 - 47.7), which was still significantly higher than the uncontrolled group of asthmatics post-bronchodilator with FEF 25-75 median of 22.3% (95% CI 16.3 - 30.6); $p = 0.0009$ (Table 6.2; Figure 6.2).

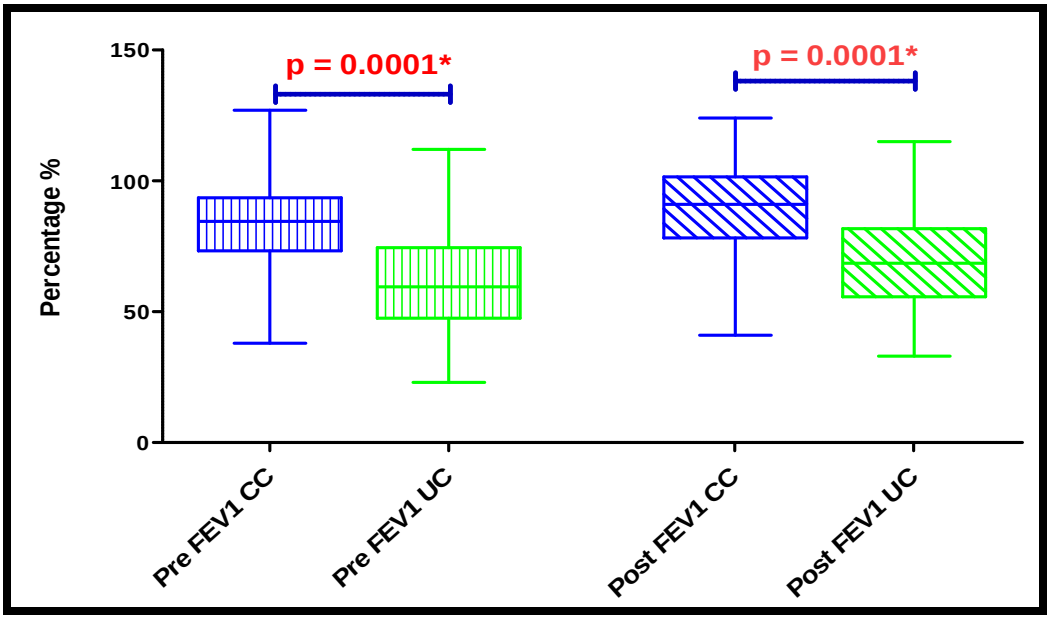

Figure 6.1: Pre- and post-bronchodilator spirometric values, as a percentage of predicted, for **FEV1** (large airways) comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
FEV1: Forced expiratory volume in one second, Pre: Pre-bronchodilator, Post: Post-bronchodilator, CC: Controlled, UC: Uncontrolled, %: Percentage of predicted. Wilcoxon rank sum test.

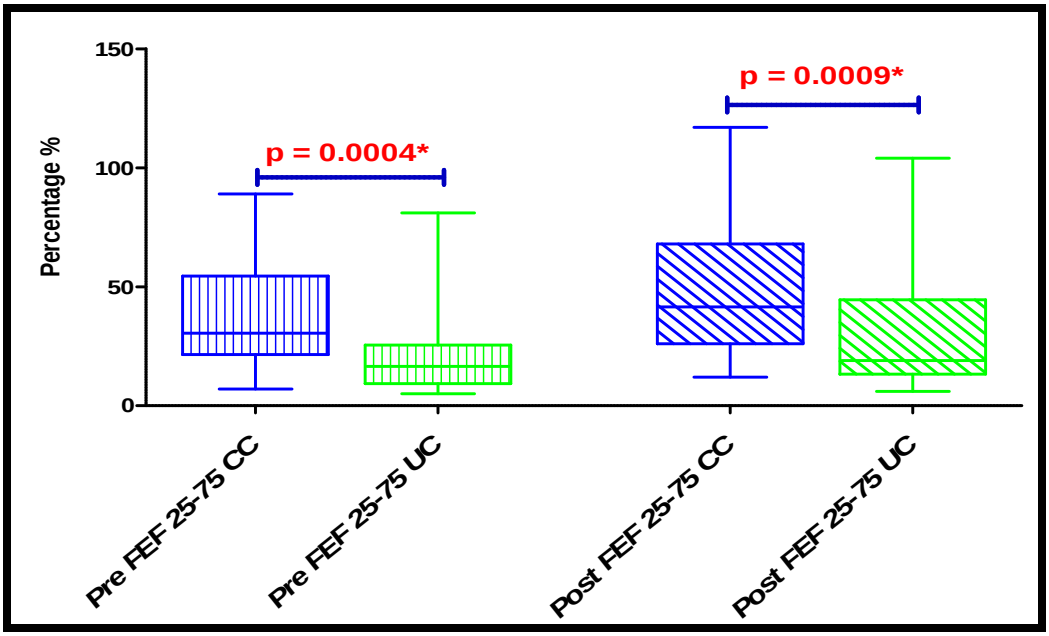

Figure 6.2: Pre- and post-bronchodilator spirometric values, as a percentage of predicted, for **FEF 25–75** (small airways) comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
FEF 25-75: Forced expiratory flow during the middle half of the FVC, Pre: Pre-bronchodilator, Post: Post-bronchodilator, CC: Controlled, UC: Uncontrolled, %: Percentage of predicted. Wilcoxon rank sum test.

Changes in the spirometric parameters measured post-bronchodilation (i.e. reversibility - %Δ) were significantly greater in the uncontrolled group when compared with the controlled group (**group 1** analysis) for FEV1, but not for FEF 25-75. The FEV1 percentage predicted change for the controlled group was a median of 6.5% (95% CI 4.2 - 10.1) and for the uncontrolled group was a median of 14.3 % (95% CI 10.1- 20.3); p = 0.0082 (Table 6.3; Figure 6.3). This was not evident for the small airways with no significance difference in post-bronchodilator change in FEF 25-75, when comparing the controlled group with a median FEF 25-75 of 29% (95% CI 5.25 - 47.5) with the uncontrolled group having a median FEF 25-75 of 31% (95% CI 19.25- 58.25); p = 0.7. However, although the change post-bronchodilation in the parameters measuring the small airways was not significantly different in the controlled and uncontrolled groups, it was clinically relevant because there was a considerable absolute post-bronchodilator reversibility in both the controlled and uncontrolled groups of asthmatic patients in the small airways (FEF 25-75). The absolute post-bronchodilator reversibility in the controlled and uncontrolled groups was a percentage change of 29% and 31% respectively (Table 6.3; Figure 6.3).

Table 6.3: Percentage change post-bronchodilator spirometric parameters comparing controlled and uncontrolled asthmatic patients **(group 1)** and the correlation between these spirometric parameters and the ACQ score

Spirometry parameters	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (p values) n= 76[2]
FEV1 %Δ	6.5 (4.2 - 10.1)	14.3 (10.1 - 20.3)	**0.0082***	0.3685 (0.0011)
PEFR %Δ	5.5 (3.6 - 8.5)	12.9 (8.7 - 19.2)	**0.0061***	0.3922 (0.0005)
FEF 25 %Δ	20.2 (15.8 - 25.8)	33.1 (24.4 - 45.0)	**0.0186***	0.3104 (0.0064)
FEF 50 %Δ	24.4 (18.7 - 31.7)	25.1 (16.3 - 38.7)	0.8933	0.0507 (0.6634)
FEF 75 %Δ	16.2 (8.9 - 29.4)	5.5 (1.3 - 23.6)	0.5094	-0.0767 (0.5099)
FEF 25-75 %Δ	29 (5.25 - 47.5)	31 (19.25 - 58.25)	0.6998	0.0585 (0.6158)

1: Wilcoxon rank sum test.* = $p < 0.05$
2: Spearman rank correlation between ACQ score and other variables.
Values highlighted …….are spirometry measurement of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
FEV1: Forced expiratory volume in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF25-75: Forced expiratory flow during the middle half of the FVC n: Number, Δ: % Change post bronchodilator, %: Percentage of predicted.

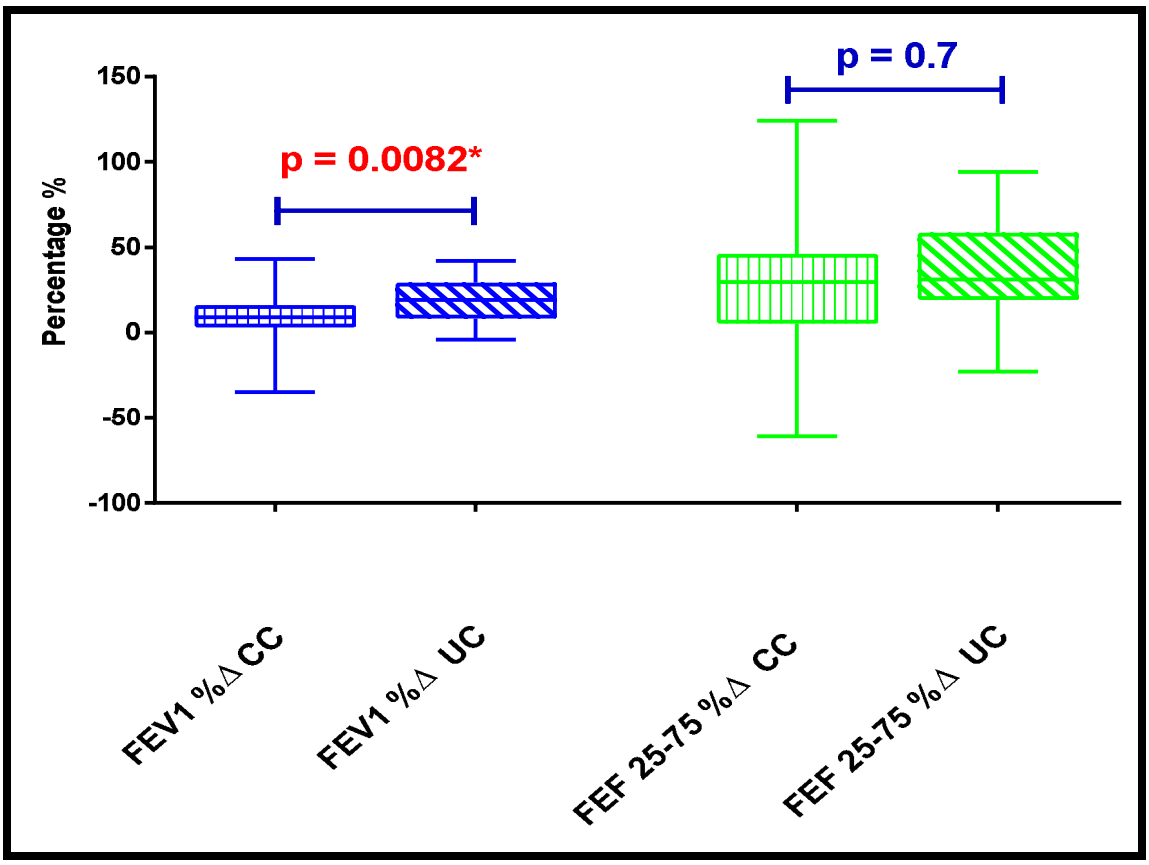

Figure 6.3: Percentage change post-bronchodilator spirometric parameters, as a percentage of predicted, for **FEV1 and FEF 25-75** comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
FEF 25-75: Forced expiratory flow during the middle half of the FVC, FEV1: Forced expiratory volume in one second, CC: Controlled, UC: Uncontrolled, %Δ: % Change post-bronchodilator. Wilcoxon rank sum test.

Furthermore, for the small airways as assessed by the FEF 25-75, there was a significant difference in the individual patient paired comparisons (i.e. for the same patient), with the pre-bronchodilator values significantly lower when compared with the post-bronchodilator values for the patients in the uncontrolled group (p = 0.01). This was not evident for the comparison between the pre-bronchodilator and the post-bronchodilator values in the controlled group (p = 0.07) (Figure 6.4). For the large airways, there was also a difference in individual patient paired comparisons (i.e. for the same patient) for both the controlled and uncontrolled group of patients, with the FEV1 pre-bronchodilator values being significantly higher when compared with the post-bronchodilator FEV1 values (p = 0.01) (Figure 6.4).

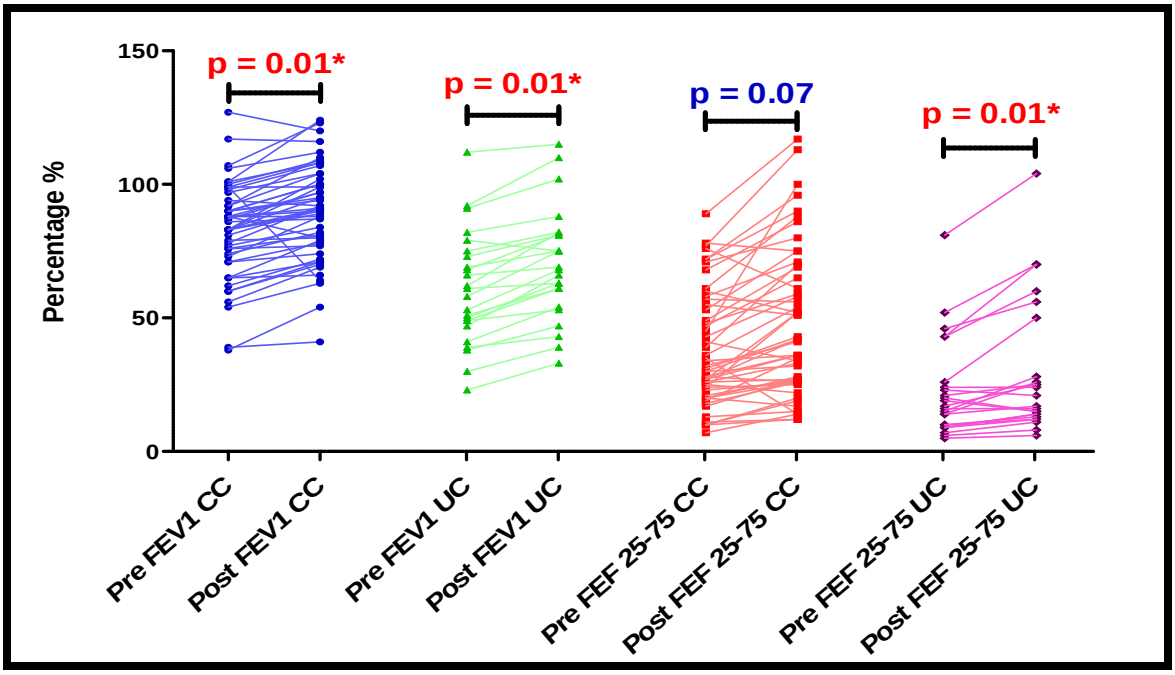

Figure 6.4: Pre-bronchodilator versus post-bronchodilator spirometric parameters, as percentage of predicted, for **FEV1 and FEF 25-75** comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
FEV1: Forced expiratory volume in one second, FEF 25-75: Forced expiratory flow during the middle half of the FVC, Pre: Pre-bronchodilator, Post: Post-bronchodilator, CC: Controlled, UC: Uncontrolled, %: Percentage of predicted. Wilcoxon Signed-Rank Test.

Despite these significant differences in the comparison of the controlled group with the uncontrolled group reported above, there was a poor correlation between the ACQ score and all specific spirometric measurements of airway calibre, including the measurements of the small airways (Figure 6.5).

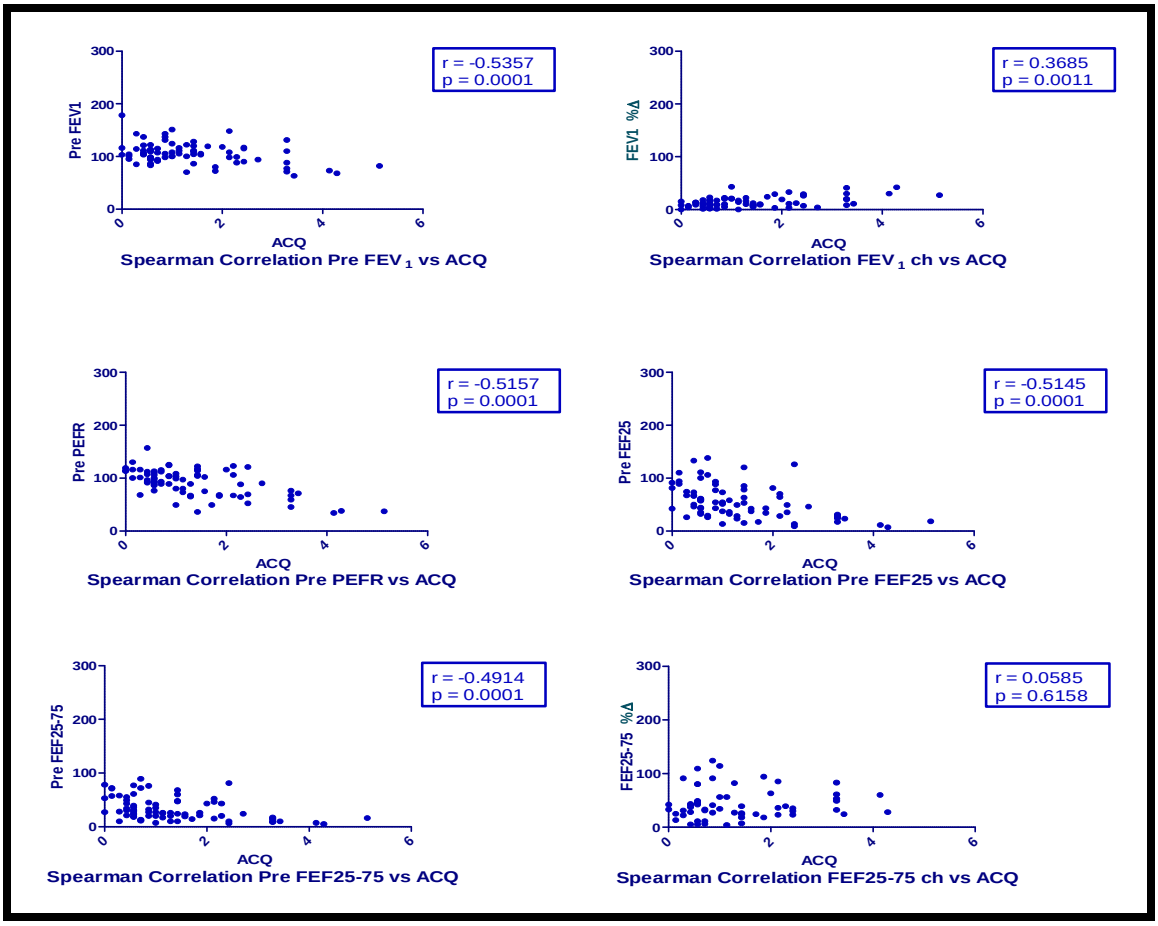

Figure 6.5: Correlation between the spirometry parameters and the ACQ score in the study population

FEV1: Forced expiratory volume in one second, PEFR: Peak expiratory flow, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF25-75: Forced expiratory flow during the middle half of the FVC, %Δ: % Change post-bronchodilator, ACQ: Asthma Control Questionnaire, Pre: Pre-bronchodilator, Post: Post-bronchodilator. Spearman rank correlation.

For the **group 2** analysis (i.e. totally controlled (TC) versus well-controlled (WC) versus uncontrolled (UC) asthmatic patients), spirometric parameters of small airways measured pre- and post-bronchodilator (i.e. FEF 25-75) were significantly higher in the totally controlled and well-controlled groups when compared with the uncontrolled group of asthmatic patients (Tables 6.4, 6.5; Figures 6.6, 6.7). However, when the totally controlled group was compared with the well-controlled group, there was no significant difference documented. This data analysis for the **group 2** patient cohort was similar to the data analysis for the **group 1** patient cohort in that the pre-bronchodilator values of the small airways (i.e. FEF 25-75) were significantly lower in the totally controlled, well-controlled and uncontrolled groups when assessed as the percentage predicted.

Table 6.4: Pre-bronchodilator spirometric parameters (reported as percentage of predicted and FEV1/FVC ratio) comparing totally controlled, well-controlled and uncontrolled asthmatic patients **(group 2)**

Spirometry parameters+	Totally controlled n=30	Well-controlled n=22	Uncontrolled n=24	Totally controlled vs Well-controlled[1] (p values)	Totally controlled vs Uncontrolled[1] (p values)	Well control vs Uncontrolled[1] (p values)
Pre FVC	105.0 (94.8 - 115.2)	111.7 (104.4 -120.9)	96.5 (87.4 - 105.7)	0.17	0.09	**0.01***
Pre FEV1	83.7 (78.9 - 88.7)	81.0 (67.5 - 89.1)	57.1 (48.8 - 66.8)	0.81	**0.0001***	**0.004***
FEV1/FVC	65.9 (61.4 - 70.8)	58.9 (53.0 - 64.8)	51.5 (45.0 - 58.9)	0.08	**0.004***	0.15
Pre PEFR	99.7 (90.3 - 109.1)	93.4 (82.7 - 104.0)	72.9 (62.0 - 83.9)	0.44	**0.0002***	**0.01***
Pre FEF 25	62.3 (52.3 - 74.3)	48.4 (37.5 - 62.5)	29.2 (21.7 - 39.3)	0.1335	**0.0001***	**0.0083***
Pre FEF 50	42.6 (34.6 - 52.3)	32.4 (24.9 - 42.2)	19.2 (14.0 - 26.4)	0.1071	**0.0002***	**0.0118***
Pre FEF 75	28.0 (21.9 - 35.8)	22.0 (16.7 - 29.0)	15.4 (11.9 - 19.9)	0.18	**0.0017***	0.0543
Pre FEF 25-75	35.7 (28.5 - 44.7)	27.3 (20.8 - 36.0)	17.3 (12.7 - 23.5)	0.10	**0.0003***	**0.0156***

+: All spirometry parameters are reported as % of predicted unless otherwise indicated.
1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
Values highlighted …….are spirometry measurements of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
FEV1: Forced expiratory volume in one second, FEV1/FVC: Percentage of the FVC expired in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF25-75: Forced expiratory flow during the middle half of the FVC, FVC: Forced vital capacity, n: Number, PEFR: Peak expiratory flow, Pre: Pre-bronchodilator.

Table 6.5: Post-bronchodilator spirometric parameters (reported as percentage of predicted and FEV1/FVC ratio) comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Spirometry parameters+	Totally controlled n=30	Well-controlled n=22	Uncontrolled n=24	Totally controlled vs Well-controlled[1] (p values)	Totally controlled vs Uncontrolled[1] (p values)	Well control vs Uncontrolled[1] (p values)
Post FVC	104.5 (91.4 - 117.6)	115.2 (107.2 - 123.2)	94.8 (79.7 - 110.0)	0.12	0.25	0.02
Post FEV1	90.7 (85.7 - 96.1)	84.2 (74.6 - 95.0)	67.2 (58.9 - 76.6)	0.40	**0.0001***	**0.01***
Post FEV1/FVC	69.7 (64.7 - 75.1)	62.0 (56.1 - 68.7)	56.6 (49.6 – 64.7)	0.06	**0.0188***	0.30
Post PEFR	110.6 (103.9 - 117.3)	93.8 (79.6 - 108.1)	83.8 (73.0 - 94.6)	0.07	**0.0001***	0.09
Post FEF 25	75.8 (64.1 - 89.5)	48.3 (29.2 - 79.7)	39.7 (29.8 - 52.7)	0.0769	**0.0003***	0.0843
Post FEF 50	53.7 (43.7 - 66.0)	39.3 (30.3 - 51.0)	25.3 (18.4 - 34.8)	0.0529	**0.0003***	0.0312
Post FEF 75	36.3 (28.0 - 47.1)	25.2 (19.1 - 33.4)	15.5 (9.6 - 24.9)	0.0614	**0.0025***	0.0749
Post FEF 25-75	46.7 (37.5 - 58.1)	32.9 (25.2 - 42.8)	22.3 (16.3 - 30.6)	0.0389	**0.0003***	0.0442

+: All spirometry parameters are reported as % of predicted unless otherwise indicated.
1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
Values highlighted ……. are spirometry measurements of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
FEV1: Forced expiratory volume in one second, FEV1/FVC: Percentage of the FVC expired in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF 25-75: Forced expiratory flow during the middle half of the FVC, FVC: Forced vital capacity, n: Number, PEFR: Peak expiratory flow, Post: Post-bronchodilator.

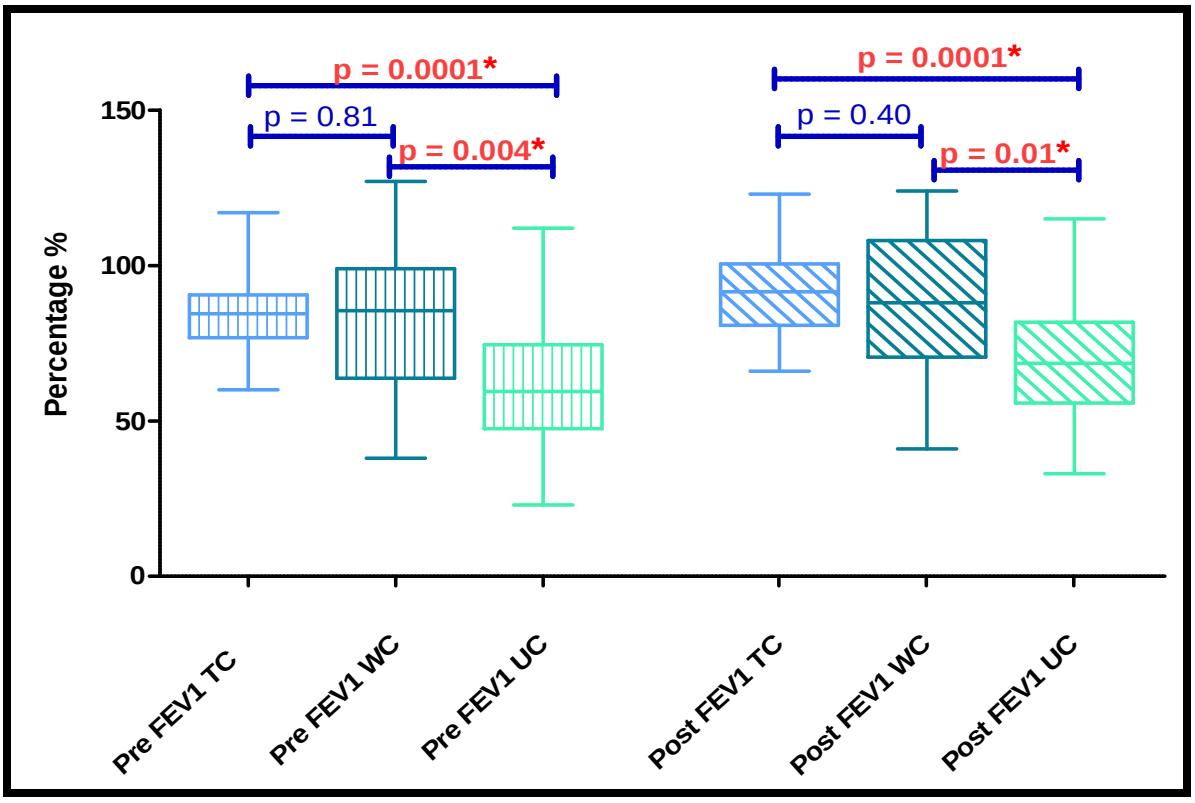

Figure 6.6: Pre- and post-bronchodilator spirometric values, as a percentage of predicted, for **FEV1 (large airways)** comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

*p values reported as significant are depicted in **red**.

FEV1: Forced expiratory volume in one second, Pre: Pre-bronchodilator, Post: Post-bronchodilator TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled, %: Percentage of predicted. Kruskal-Wallis one-way ANOVA test.

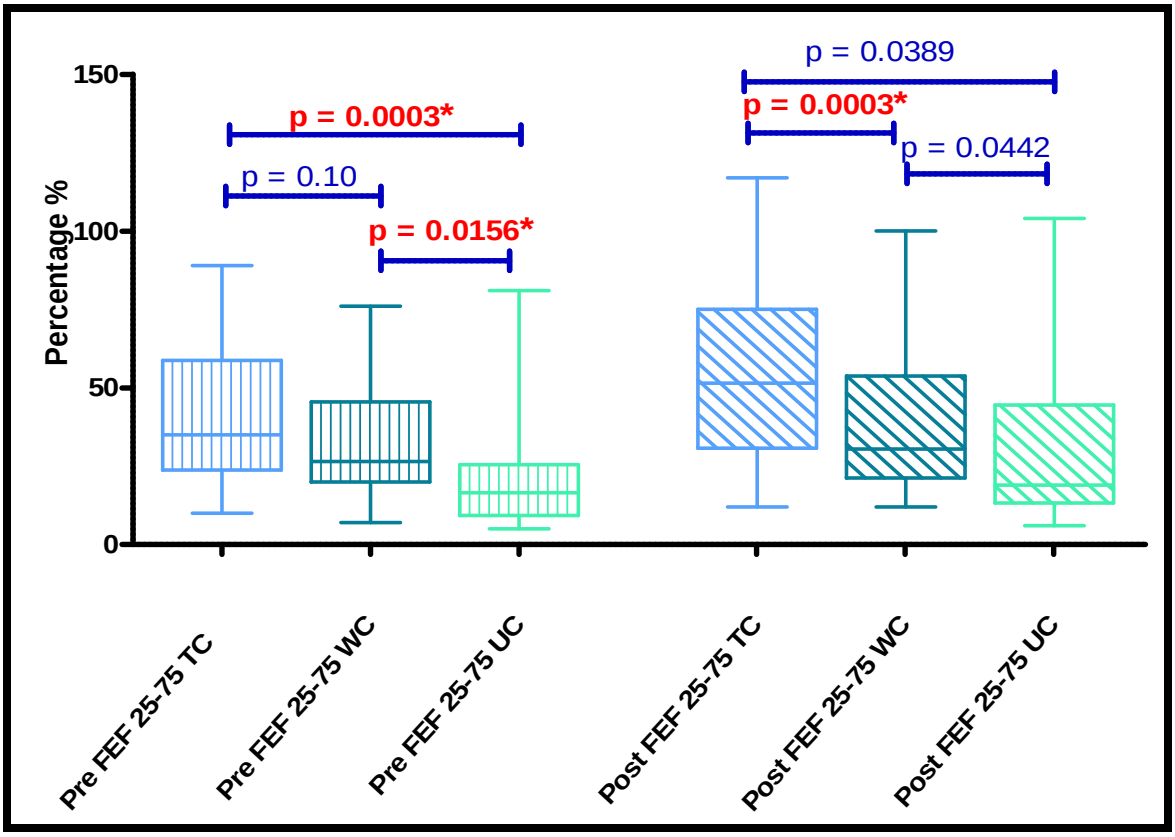

Figure 6.7: Pre- and post-bronchodilator spirometric values, as a percentage of predicted, for **FEF 25-75 (small airways)** comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)
*p values reported as significant are depicted in **red**.
FEF: Forced expiratory flow related to a specific portion of the FVC, Pre: Pre-bronchodilator, Post: Post-bronchodilator TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled, %: Percentage of predicted. Kruskal-Wallis one-way ANOVA.

For the **group 2** analysis, changes in spirometric parameters post-bronchodilation (i.e. reversibility - %Δ) are summarised in Table 6.6 and Figure 6.8. There were no significant differences in any of these parameters measured. Nonetheless, similar to the **group 1** analysis, FEF 25-75 in the totally controlled, well-controlled and uncontrolled asthmatic patients demonstrated an absolute post-bronchodilator reversibility of 32%, 26.5% and 31% respectively, confirming the presence of bronchial airway hyper-reactivity (BAH) in the small airways (Table 6.6), hence, making this finding clinically relevant.

Table 6.6: P**ercentage change post-bronchodilator** spirometric parameters comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Spirometry parameters	Totally controlled n=30	Well-controlled n=22	Uncontrolled n=24	Totally controlled vs Well-controlled[1] (p values)	Totally controlled vs Uncontrolled[1] (p values)	Well control vs Uncontrolled[1] (p values)
FEV1 %Δ	5.3 (3.0 - 9.2)	8.6 (4.1 - 18.0)	14.3 (10.1 - 20.3)	0.0537	**0.0016***	0.222
PEFR %Δ	4.6 (2.7 - 8.0)	7.0 (3.3 - 14.9)	12.9 (8.7 - 19.2)	0.0703	**0.0017***	0.1526
FEF 25 %Δ	16.7 (11.9 - 23.4)	26.2 (18.4 - 37.4)	33.1 (24.4 - 45.0)	0.0723	**0.0048***	0.286
FEF 50 %Δ	37.6 (15.0 - 31.0)	28.8 (19.4 - 42.7)	25.1 (16.3 - 38.7)	0.3081	0.5953	0.6841
FEF 75 %Δ	14.4 (6.1 - 34.1)	18.9 (7.9 - 45.3)	5.5 (1.3 - 23.6)	0.9852	0.6011	0.5231
FEF 25-75 %Δ	32 (9.75 - 47.25)	26.5 (11.5 - 56)	31 (19.25 - 58.25)	0.463	0.9099	0.5453

1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
Values highlighted ……. are spirometry measurements of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
FEV1: Forced expiratory volume in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, FEF25-75: Forced expiratory flow during the middle half of the FVC n: Number, Δ: % Change post-bronchodilator, %: Percentage of predicted.

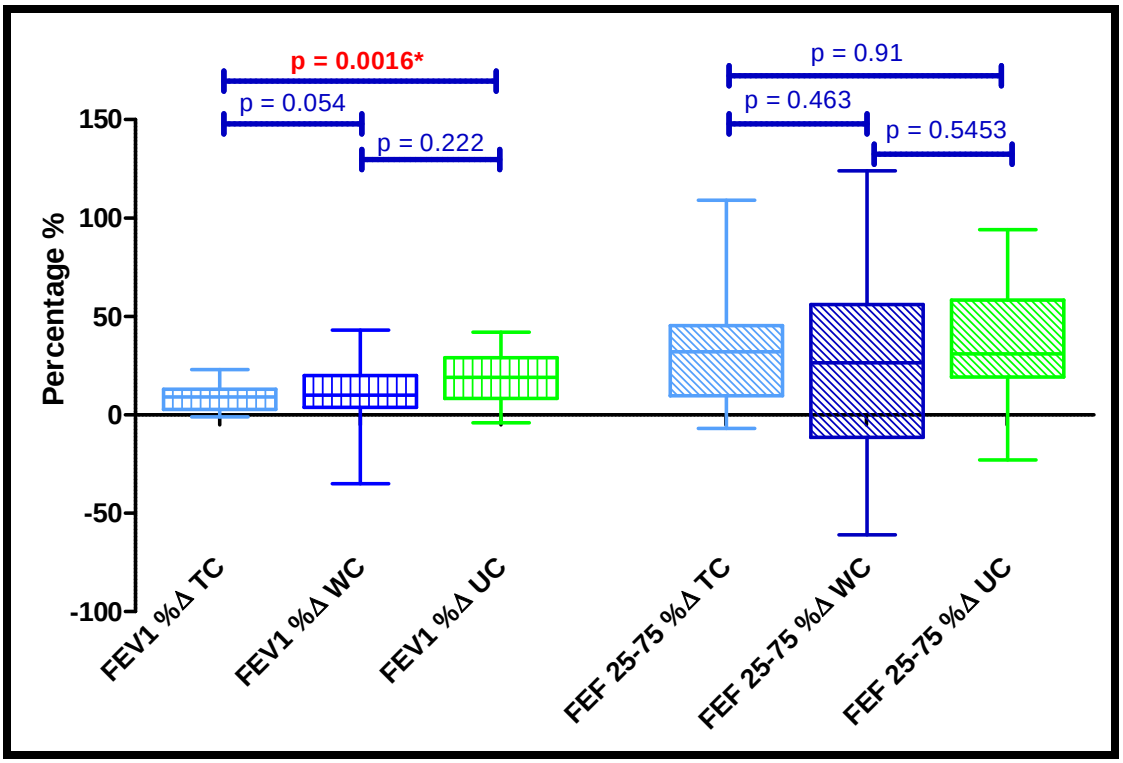

Figure 6.8: Percentage change post-bronchodilator in spirometric parameters for **FEV1 and FEF 25-75** comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)
*p values reported as significant are depicted in **red**.
FEV1: Forced expiratory volume in one second, FEF: Forced expiratory flow related to a specific portion of the FVC, %Δ: % Change post-bronchodilator, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled, %: Percentage of predicted. Kruskal-Wallis one-way ANOVA.

6.5.2 Impulse oscillometry (IOS) data

A summary of the IOS data for the patients in the **group 1** analysis (controlled vs uncontrolled asthmatics) are included in Tables 6.7 and 6.8. Table 6.7 representing all the pre-bronchodilator IOS parameters measured, and Table 6.8 the post-bronchodilator IOS parameters measured. The important parameters that are derived from IOS, relating to the small airways, include R5 (total airway resistance), R20 (central airway resistance), R5-R20 (small airway resistance) and AX (reactance area - Goldman's triangle), and these data will be elaborated in the accompanying text. They are represented graphically with Figure 6.9 depicting the pre-bronchodilator data of R5, R20 and R5-R20, Figure 6.10 depicting the post-bronchodilator data of R5, R20 and R5-R20 and Figure 6.11 depicting the pre- and post-bronchodilator data of AX.

The pre-bronchodilator values for the R5 for the controlled group, with a median of 147.1% (95% CI 135.2 - 159.9), was significantly lower when compared with the uncontrolled group with a median of 202.3% (95% CI 175.0 - 233.9); p = 0.0004 (Table 6.7; Figure 6.9). Similarly, for R5 post-bronchodilation, the controlled group with a median of 120.6% (95% CI 112.4 - 129.4) was significantly lower when compared with the uncontrolled group with a median of 157.7% (95% CI 136.1 - 182.7); p = 0.0053 (Table 6.8; Figure 6.10).

The pre-bronchodilator values for the frequency dependence of resistance (R5-R20) for the controlled group with a median of 24.6% (95% CI 22.2 - 27.3) was significantly lower when compared with the uncontrolled group with a median of 33.5% (95% CI 28.9 - 38.9); p = 0.0026 (Table 6.7; Figure 6.9). Similarly, for R5-R20 post-bronchodilation, the controlled group with a median of 18.5% (95% CI 16.2- 21.0) was significantly lower when compared with the uncontrolled group with a median of 23.1% (95% CI 18.7 - 28.6); p = 0.042 (Table 6.8; Figure 6.10).

The pre-bronchodilator values for the reactance area (AX) for the controlled group with a median of 1.2% (95% CI 1.0 - 1.5) was significantly lower when compared with the uncontrolled group with a median of 2.8% (95% CI 1.8 - 4.4); p = 0.0013 (Table 6.7; Figure 6.11). Similarly, for AX post-bronchodilation, the controlled group with median of 0.7% (95% CI 0.6% - 0.9%) was significantly lower when compared with the uncontrolled group with a median 1.5% (95% CI 0.9 - 2.3); p = 0.042 (Table 6.8; Figure 6.11).

In summary, the **group 1** analysis (i.e. controlled (CC) versus uncontrolled (UC) asthmatic patients) of the IOS tests revealed that the airway resistance in the smaller airways was significantly higher in the uncontrolled asthmatic patients when compared with the controlled asthmatic patients, both pre- and post-bronchodilation. However, the correlations between the IOS parameters measured and the ACQ score were, at best, moderate and some were poor (Tables 6.7, 6.8).

Table 6.7: Pre-bronchodilator IOS parameters (reported as percentage of predicted) comparing controlled and uncontrolled asthmatic patients (**group 1**) and the correlation between these IOS parameters and the ACQ score

IOS parameters[+]	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (p-values) n= 76[2]
Pre R5	147.1 (135.2 - 159.9)	202.3 (175.0 - 233.9)	**0.0004***	0.40 (0.01)
Pre R20	127.1 (118.1 - 136.8)	153.0 (136.3 - 171.7)	**0.0125***	0.27 (0.02)
Pre R5 - R20	24.6 (22.2 - 27.3)	33.5 (28.9 - 38.9)	**0.0026***	0.36 (0.01)
Pre R35	169.4 (158.3 - 181.3)	197.9 (178.5 - 219.5)	**0.0097***	0.27 (0.02)
Pre Z5	157.5 (144.1 - 172.0)	222.9 (189.1 - 262.8)	**0.0005***	0.41 (0.01)
Pre X5	814.7 (623.8 - 1063.8)	1188.4 (790.0 - 1787.7)	0.1372	0.28 (0.02)
Pre X20	44.3 (30.1 - 65.1)	117.5 (56.2 - 246.0)	**0.0038***	0.35 (0.01)
Pre X35	43.0 (33.5 - 55.3)	45.5 (35.5 - 58.4)	0.6741	-0.13 (0.28)
Pre AX	1.2 (1.0 - 1.5)	2.8 (1.8 - 4.4)	**0.0013***	0.43 (0.01)
Pre res freq	22.9 (21.2 - 24.7)	30.0 (25.4 - 35.4)	**0.0033***	0.43 (0.01)

+: All IOS parameters are reported as % of predicted unless otherwise indicated.
1: Wilcoxon rank sum test.* = $p < 0.05$
2: Spearman rank correlation between ACQ score and other variables.
Values highlighted ……. are IOS measurement of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, R35: Airway resistance at 35Hz, Z5: Respiratory impedance at 5 Hz, Hz: Hertz, X5: Peripheral capacitive reactance, X20: Reactance at 20Hz, X35: Reactance at 35Hz, V: Flow, AX: Reactance area, res freq: Resonant frequency, n: Number, Pre: pre-bronchodilator.

Table 6.8: Post-bronchodilator IOS parameters (reported as percentage of predicted) comparing controlled and uncontrolled asthmatic patients (**group 1**), and the correlation between these IOS parameters and the ACQ score

IOS parameters[+]	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (p-values) n= 76[2]
Post R5	120.6 (112.4 - 129.4)	157.7 (136.1 - 182.7)	**0.0053***	0.37 (0.01)
Post R20	113.2 (105.6 - 121.4)	137.5 (123.6 - 153.1)	**0.0082***	0.31 (0.01)
Post R5 - R20	18.5 (16.2 - 21.0)	23.1 (18.7 - 28.6)	**0.0420***	0.22 (0.06)
Post R35	160.4 (149.4 - 172.3)	195.2 (167.8 - 227.1)	**0.0192***	0.29 (0.01)
Post Z5	128.8 (120.0 - 138.3)	168.3 (144.1 - 196.5)	**0.0068***	0.37 (0.01)
Post X5	639.2 (490.4 - 833.1)	789.1 (534.5 - 1165.1)	0.4341	0.19 (0.11)
Post X20	45.9 (35.6 - 59.3)	83.3 (49.4 - 140.4)	**0.0802***	0.07 (0.53)
Post X35	68.5 (58.3 - 80.5)	33.5 (21.4 - 52.4)	**0.0005***	-0.39 (0.01)
Post AX	0.7 (0.6 - 0.9)	1.5 (0.9 - 2.3)	**0.0042***	0.34 (0.01)
Post res freq	17.1 (15.6 - 18.7)	23.5 (19.5 - 28.3)	**0.0031***	0.38 (0.01)

+: All IOS parameters are reported as % of predicted unless otherwise indicated.
1: Wilcoxon rank sum test.* = $p < 0.05$
2: Spearman rank correlation between ACQ score and other variables.
Values highlighted ……. are IOS measurement of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, R35: Airway resistance at 35Hz, Z5: Respiratory impedance at 5 Hz, Hz: Hertz, X5: Peripheral capacitive reactance, X20: Reactance at 20Hz, X35: Reactance at 35Hz, V: Flow, AX: Reactance area, res freq: Resonant frequency, n: Number, Post: Post-bronchodilator.

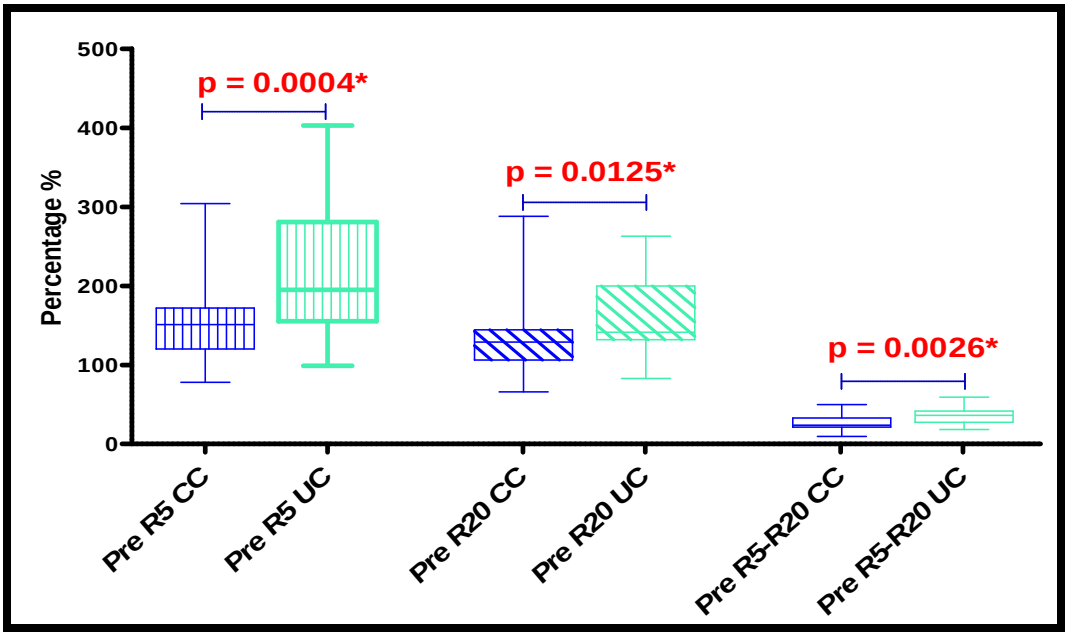

Figure 6.9: Pre-bronchodilator parameters for IOS resistance (R5, R20 and R5-R20), as percentage of predicted, comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways CC: Controlled, UC: Uncontrolled, Pre: Pre-bronchodilator. Wilcoxon rank sum test.

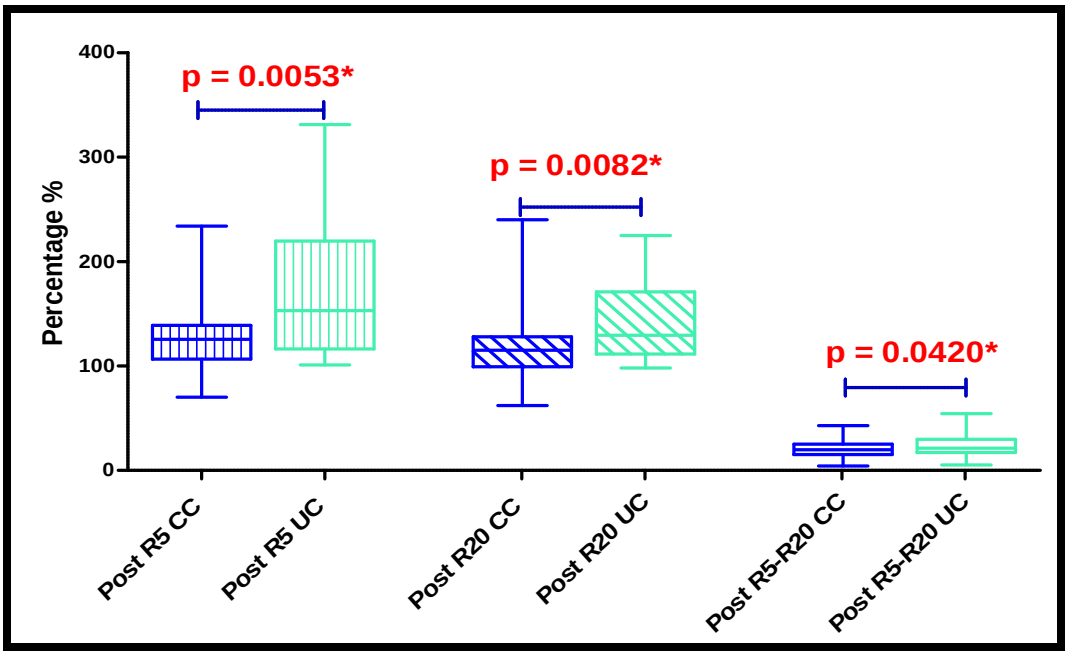

Figure 6.10: Post-bronchodilator parameters for IOS resistance (R5, R20 and R5-R20), as percentage of predicted, comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways CC: Controlled, UC: Uncontrolled, Pre: Pre-bronchodilator. Wilcoxon rank sum test.

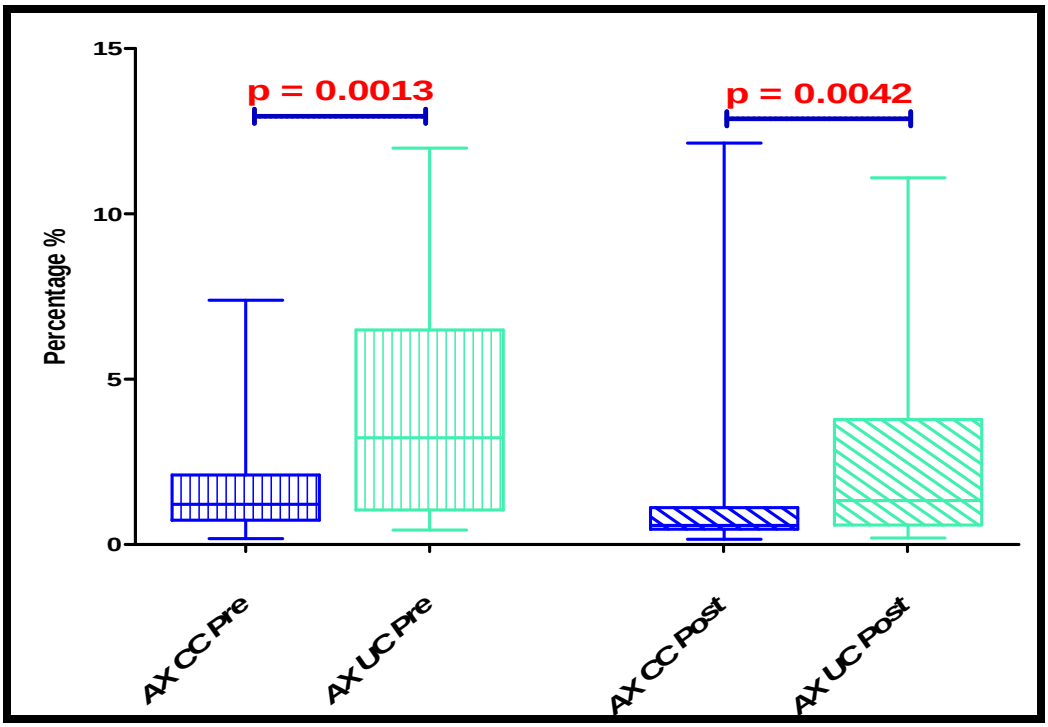

Figure 6.11: Pre- and post-bronchodilator values for IOS AX, as percentage of predicted, comparing controlled and uncontrolled asthmatic patients (**group 1**)
* p values reported as significant are depicted in **red**.
AX: Reactance area, CC: Controlled, UC: Uncontrolled, Pre: Pre-bronchodilator, Post: Post-bronchodilator, %: Percentage of predicted. Wilcoxon rank sum test.

For the **group 1** analysis of parameters measuring changes in airway resistance post-bronchodilator (i.e. reversibility - %Δ), there was no differences in any of the parameters measured, when comparing controlled patients to uncontrolled patients (Table 6.9; Figure 6.12).

Table 6.9: Percentage change post-bronchodilator IOS parameters comparing controlled and uncontrolled asthmatic patients **(group 1)** and the correlation between these IOS parameters and the ACQ score

IOS parameters	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (p-values) n= 76[2]
R5 %Δ	13.0 (9.8 - 17.1)	16.9 (11.2 - 25.6)	0.1174	0.13 (0.28)
R20 %Δ	8.8 (6.1 - 12.7)	6.9 (3.3 - 14.2)	0.6626	-0.17 (0.14)
R5 - R20 %Δ	18.3 (12.5 - 26.6)	17.6 (10.6 - 29.2)	0.9819	0.03 (0.79)
R35 %Δ	7.0 (4.5 - 10.8)	5.1 (2.5 - 10.3)	0.3059	-0.12 (0.31)
zbei5 %Δ	10.7 (6.3 - 17.9)	16.1 (7.9 - 32.7)	0.05	0.19 (0.09)
X5 %Δ	13.9 (8.3 - 23.1)	28.1 (19.2 - 40.9)	0.05	0.23 (0.04)
X20 %Δ	124.7 (85.2 - 182.5)	90.2 (51.5 - 157.8)	0.1402	-0.19 (0.12)
X35 %Δ	57.4 (35.7 - 92.4)	63.0 (39.0 - 101.8)	0.2934	0.19 (0.11)
AX %Δ	28.0 (17.3 - 45.4)	18.7 (6.0 - 58.//4)	0.7584	0.08 (0.52)
Res freq %Δ	19.1 (14.9 - 24.50)	7.1 (2.2 - 23.2)	0.4244	-0.12 (0.37)

1: Wilcoxon rank sum test.* = $p < 0.05$
2: Spearman rank correlation between ACQ score and other variables.
Values highlighted ……. are IOS measurement of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, R35: Airway resistance at 35Hz, zbei5: respiratory impedance at 5 Hz, Hz: Hertz, X5: peripheral capacitive reactance, X20: reactance at 20Hz, X35: Reactance at 35Hz, V: Flow, AX: Reactance area, res freq: Resonant frequency, n: Number, Δ: % Change post-bronchodilator,%: Percentage of predicted.

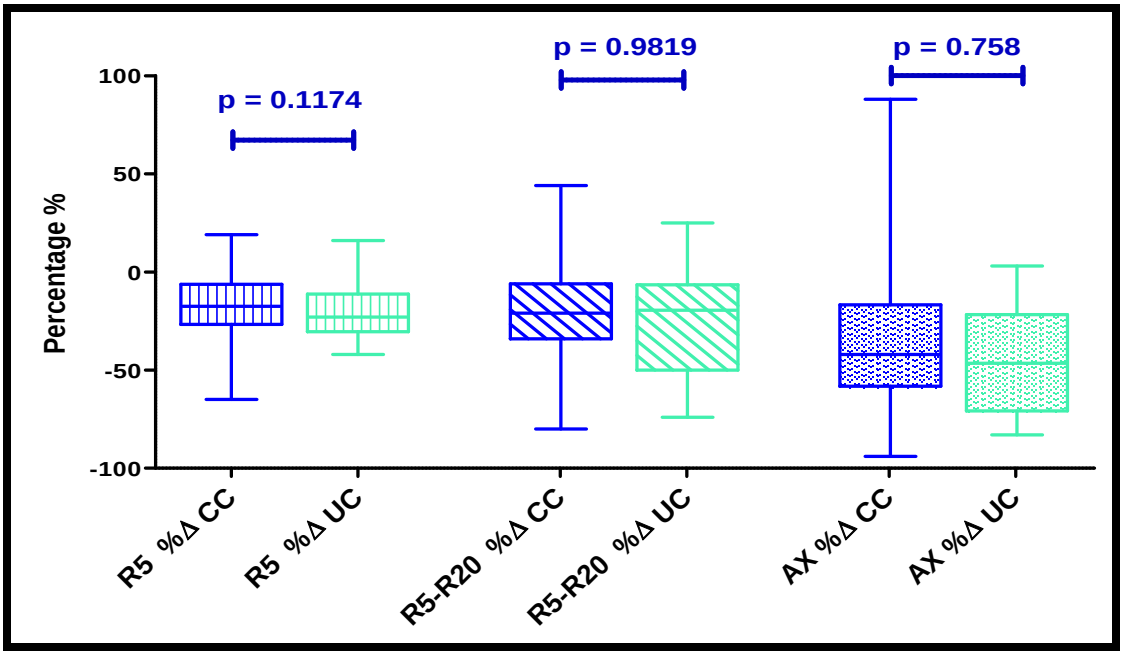

Figure 6.12: Percentage change post-bronchodilator for IOS parameters of the small airways (R5, R5-R20 and AX) comparing controlled and uncontrolled asthmatic patients (**group 1**)
R5: Total airway resistance, R5-R20: resistance of the small airways, AX: Reactance area, CC: Controlled, UC: Uncontrolled, %: Percentage of predicted, %Δ: % Change post-bronchodilator. Wilcoxon rank sum test.

For the small airways as assessed by IOS (i.e. R5 and R5-20), there was a significant difference in the individual patient paired comparisons (i.e. for the same patient), with the pre-bronchodilator values for both R5 and R5-20 significantly higher when compared with the post-bronchodilator values, for the patients in both the controlled and uncontrolled groups (p = 0.001) (Figure 6.13).

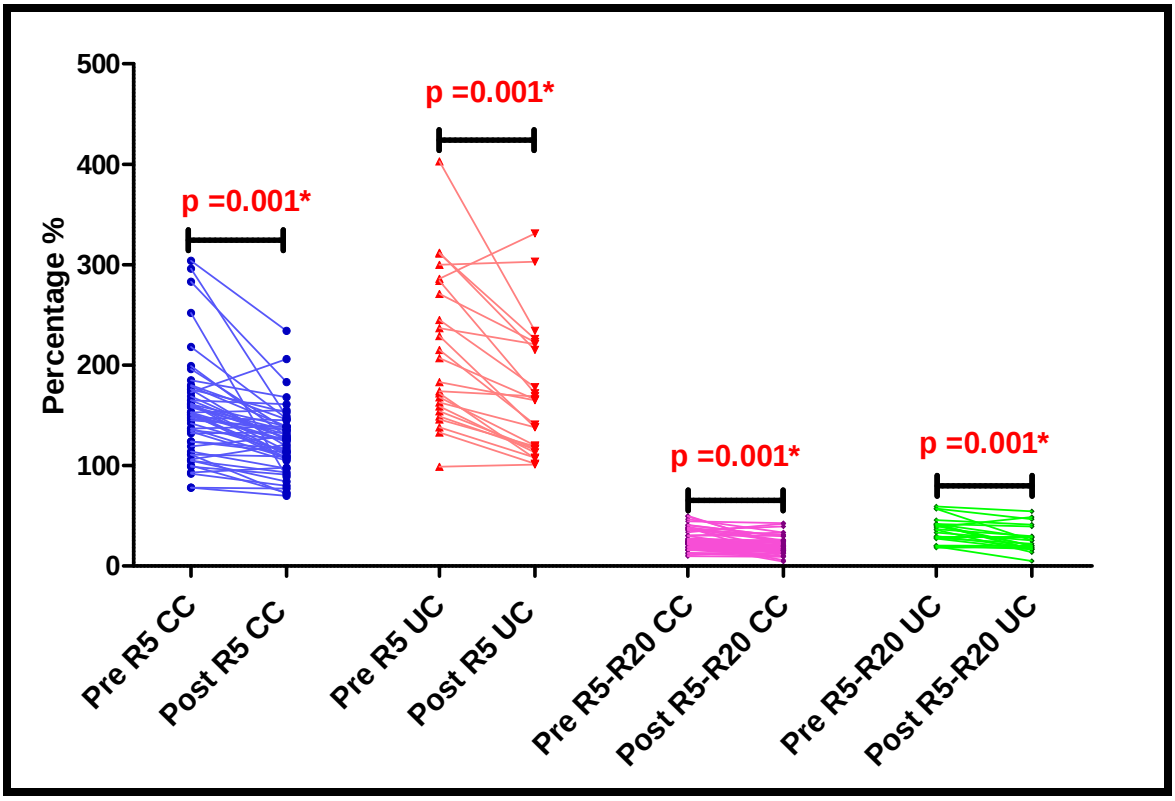

Figure 6.13: Comparison of pre-bronchodilator versus post-bronchodilator IOS resistance parameters (R5, R20 and R5-R20) as percentage of predicted, in controlled and uncontrolled asthmatic patients

* p values reported as significant are depicted in **red**.

R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways CC: Controlled, UC: Uncontrolled, Pre: Pre-bronchodilator, Post: Post-bronchodilator, %: Percentage of predicted. Wilcoxon Signed-Rank Test.

Similar to the spirometry data discussed above, there was a poor correlation between ACQ score and the specific IOS parameters of airway calibre, including measurements of the small airways (Tables 6.7, 6.8, 6.9; Figure 6.14).

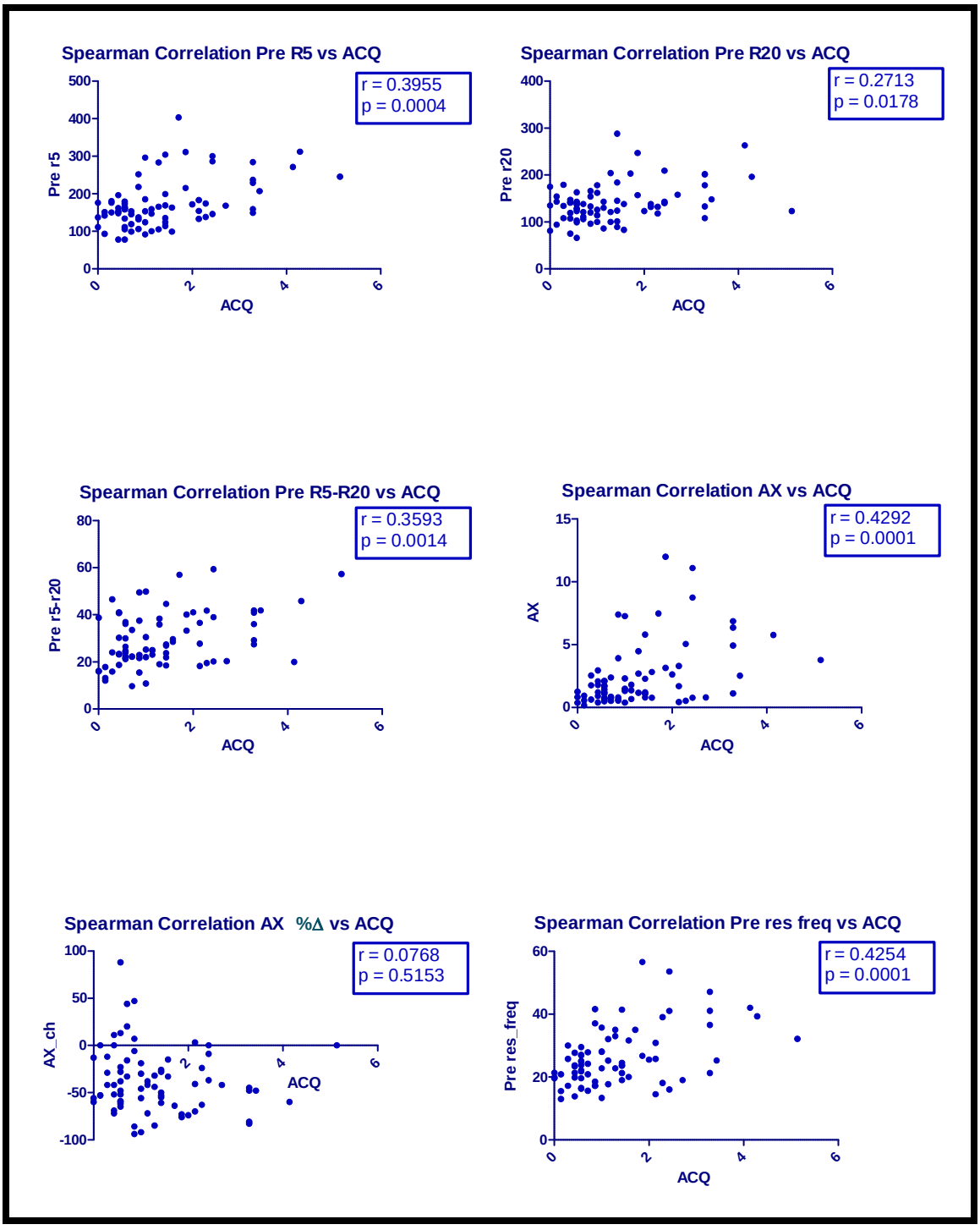

Figure 6.14: Correlation between IOS parameters and the ACQ score in the study population
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, AX: Reactance area, %Δ: % Change post-bronchodilator, res freq: Resonant frequency, ACQ: Asthma Control Questionnaire, Pre: Pre-bronchodilator, Post: Post-bronchodilator. Spearman rank correlation.

As shown in Table 6.10 and Figures 6.15 and 6.16, for the comparison between totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2** analysis), the main pre-bronchodilator IOS parameters measured (i.e. R5, R20, R5-R20 and AX) were significantly lower in the totally controlled group when compared with the uncontrolled group.

Similarly, as shown in Table 6.11 and Figures 6.16 and 6.17, for the comparison between totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2** analysis), the main post-bronchodilator IOS parameters measured (i.e. R5, R20, R5-R20 and AX) were also significantly lower in the totally controlled group when compared with the uncontrolled group.

These lower values measured by IOS in the totally and well-controlled asthmatic groups actually reflect lower airway resistance, and thus less airway bronchoconstriction in the totally controlled asthmatic patients as defined by the ACQ when compared to the uncontrolled asthmatic patients.

Table 6.10: Pre-bronchodilator IOS parameters (reported as percentage of predicted) comparing totally controlled, well-controlled and uncontrolled asthmatics (**group 2**)

IOS parameters[+]	Totally controlled n=30	Well-controlled n=22	Uncontrolled n=24	Totally controlled vs Well-controlled[1] (p values)	Totally controlled vs Uncontrolled[1] (p values)	Well-controlled vs Uncontrolled[1] (p values)
Pre R5	139.7 (127.5 - 153.1)	157.7 (134.5 - 184.9)	202.3 (175.0 - 233.9)	0.4643	**0.0003***	0.0192
Pre R20	122.9 (112.5 - 134.2)	133.2 (116.8 - 151.9)	153.0 (136.3 - 171.7)	0.541	**0.0107***	0.0903
Pre R5 - R20	23.5 (20.4 - 27.1)	26.2 (22.2 - 30.9)	33.5 (28.9 - 38.9)	0.3083	**0.0026***	0.0329
Pre R35	166.3 (153.2 - 180.5)	173.8 (153.7 - 196.5)	197.9 (178.5 - 219.5)	0.8822	**0.0119***	0.0557
Pre Z5	148.4 (134.8 - 163.3)	170.8 (144.5 - 201.9)	222.9 (189.1 - 262.8)	0.369	**0.0002***	0.0279
Pre X5	713.6 (503.4 - 1011.6)	975.9 (631.1 - 1509.1)	1188.4 (790.0 - 1787.7)	0.2359	0.065	0.5527
Pre X20	31.3 (17.9 - 55.0)	70.9 (44.1 - 114.0)	117.5 (56.2 - 246.0)	0.0653	**0.0019***	0.0748
Pre X35	46.5 (34.3 - 63.0)	38.5 (24.4 - 60.8)	45.5 (35.5 - 58.4)	0.7018	0.4727	0.9251
Pre AX	1.0 (0.8 - 1.3)	1.6 (1.1 - 2.4)	2.8 (1.8 - 4.4)	0.0849	**0.0003***	0.0671
Pre res_freq	21.3 (19.6 - 23.2)	25.1 (21.8 - 28.9)	30.0 (25.4 - 35.4)	0.068	**0.0011***	0.0951

+: All IOS parameters are reported as % of predicted unless otherwise indicated.
1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
Values highlighted ……. are IOS measurements of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, R35: Airway resistance at 35Hz, Z5: Respiratory impedance at 5 Hz, Hz: Hertz, X5: Peripheral capacitive reactance, X20: Reactance at 20Hz, X35: Reactance at 35Hz, V: Flow, AX: Reactance area, res freq: Resonant frequency, n: Number, Pre: Pre-bronchodilator.

Table 6.11: Post-bronchodilator IOS parameters (reported as percentage of predicted) comparing totally controlled, well-controlled and uncontrolled asthmatics (**group 2**)

IOS parameters[+]	Totally controlled n=30	Well-controlled n=22	Uncontrolled n=24	Totally controlled vs Well-controlled[1] (p values)	Totally controlled vs Uncontrolled[1] (p values)	Well-controlled vs Uncontrolled[1] (p values)
Post R5	116.7 (106.8 - 127.5)	126.1 (111.8 - 142.2)	157.7 (136.1 - 182.7)	0.2585	**0.0034***	0.0731
Post R20	109.7 (101.2 - 119.0)	118.1 (103.9 - 134.4)	137.5 (123.6 - 153.1)	0.5047	**0.0059***	0.0842
Post R5 - R20	17.8 (14.6 - 21.6)	19.5 (16.6 - 22.9)	23.1 (18.7 - 28.6)	0.8333	0.088	0.0654
Post R35	156.0 (143.1 - 170.0)	166.7 (146.6 - 189.4)	195.2 (167.8 - 227.1)	0.4587	**0.0141***	0.132
Post Z5	125.2 (114.4 - 137.0)	134.0 (118.7 - 151.3)	168.3 (144.1 - 196.5)	0.2825	**0.0052***	0.0731
Post X5	601.8 (413.3 - 876.2)	694.0 (468.1 - 1029.0)	789.1 (534.5 - 1165.1)	0.7529	0.3841	0.6601
Post X20	54.8 (39.0 - 76.8)	36.7 (24.7 - 54.6)	83.3 (49.4 - 140.4)	0.0401	0.3083	0.0347
Post X35	68.7 (57.6 - 81.9)	68.2 (49.7 - 93.5)	33.5 (21.4 - 52.4)	0.4699	**0.0034***	**0.0014***
Post AX	0.7 (0.5 - 1.0)	0.7 (0.6 - 0.9)	1.5 (0.9 - 2.3)	0.5349	**0.0126***	**0.0125***
Post res freq	16.5 (14.2 - 19.1)	17.9 (16.3 - 19.8)	23.5 (19.5 - 28.3)	0.4815	**0.0043***	0.0261

+: All IOS parameters are reported as % of predicted unless otherwise indicated.
1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
Values highlighted ……. are IOS measurements of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, R35: Airway resistance at 35Hz, Z5: Respiratory impedance at 5 Hz, Hz: Hertz, X5: Peripheral capacitive reactance, X20: Reactance at 20Hz, X35: Reactance at 35Hz, V: Flow, AX: Reactance area, res freq: Resonant frequency, n: Number, Post: Post-bronchodilator.

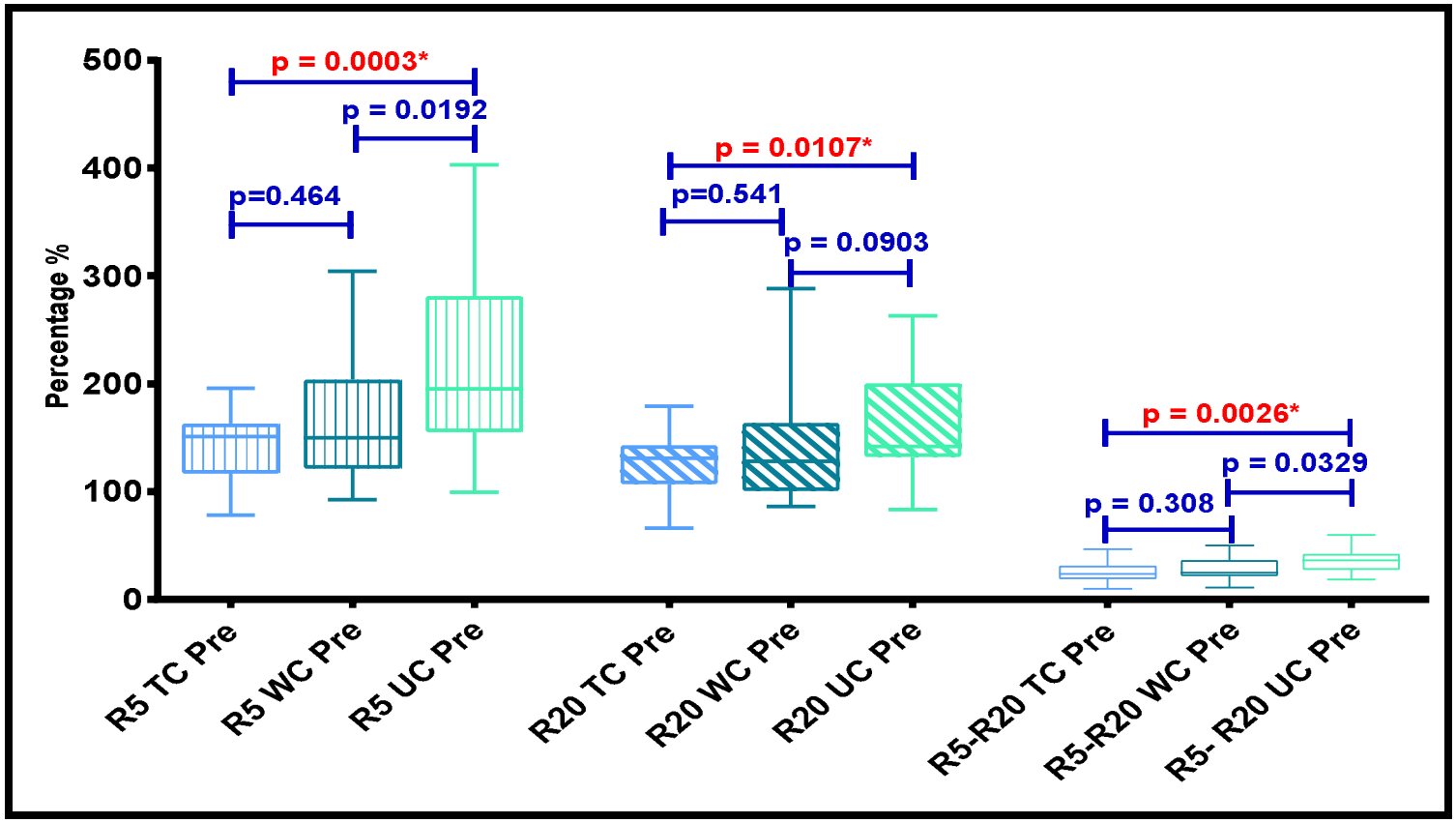

Figure 6.15: Pre-bronchodilator parameters for IOS resistance (R5, R20 and R5-R20), as percentage of predicted, comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

* p values reported as significant are depicted in **red**.

R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways,%: Percentage of predicted, Pre: Pre-bronchodilator, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

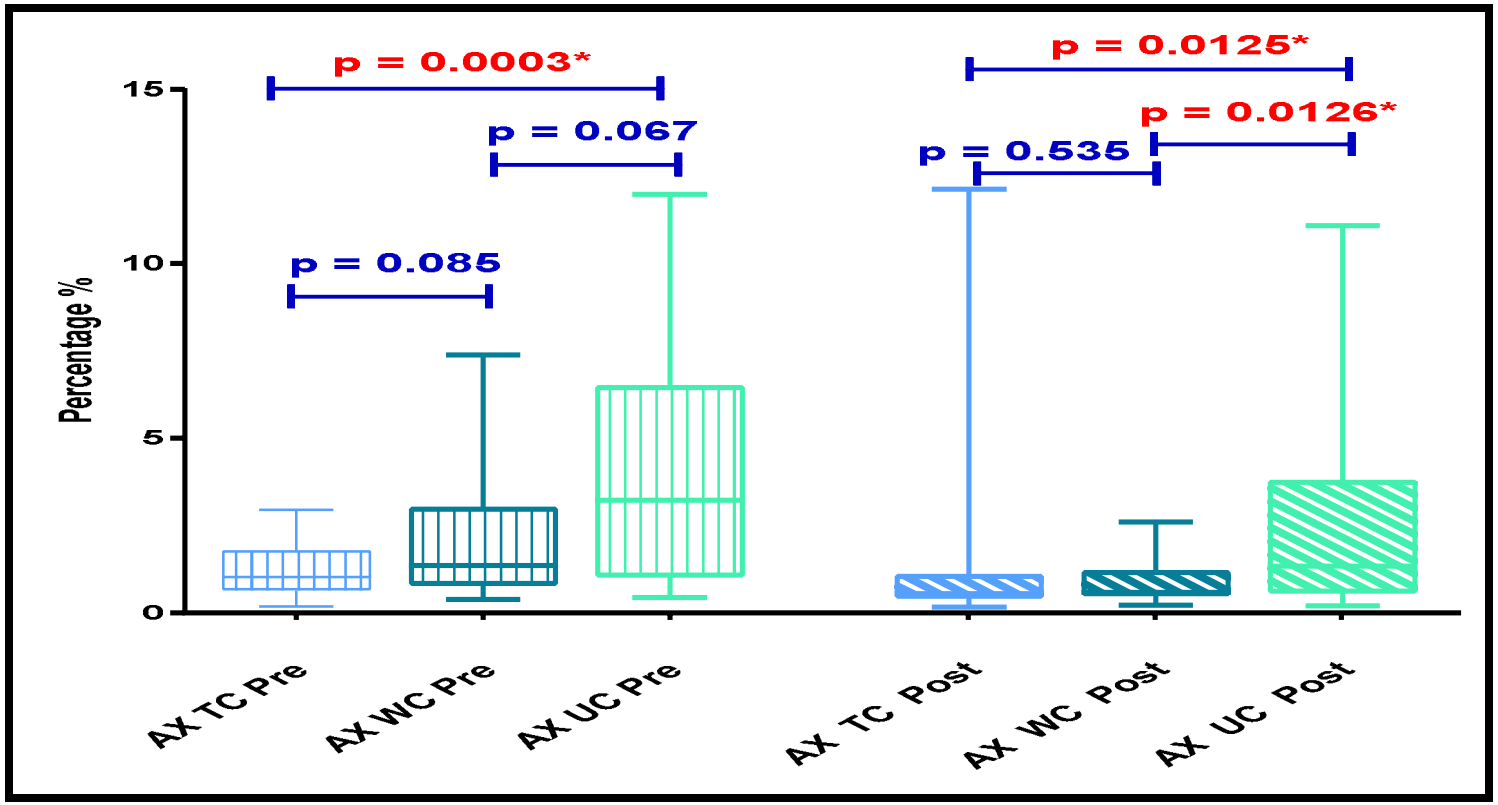

Figure 6.16: Pre- and post-bronchodilator parameters for IOS AX, as percentage of predicted, comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

* p values reported as significant are depicted in **red**.

AX: Reactance area, Pre: Pre-bronchodilator, Post: Post-bronchodilator, %: Percentage of predicted, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

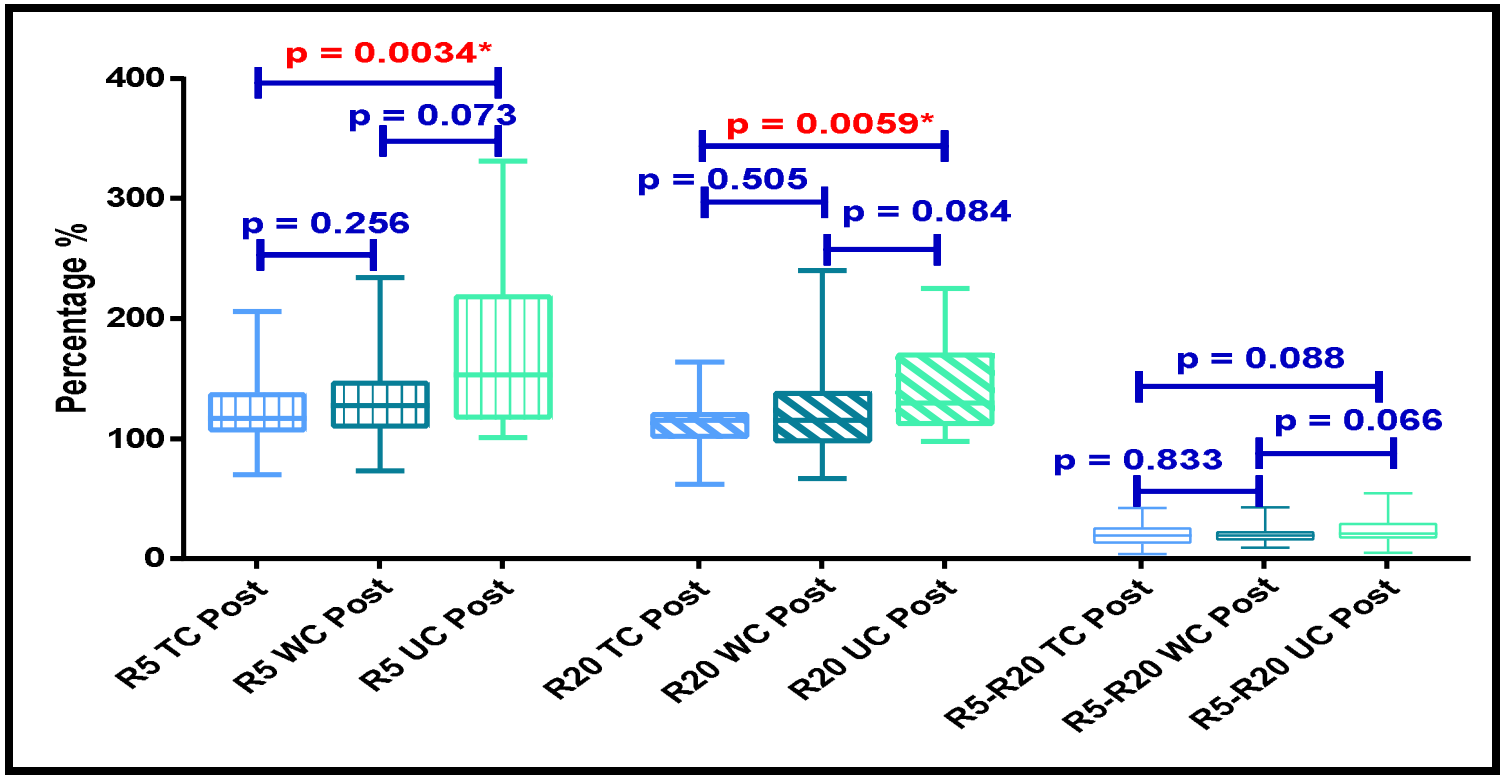

Figure 6.17: Post-bronchodilator parameters for IOS resistance (R5, R20 and R5-R20), as percentage of predicted, comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

* p values reported as significant are depicted in **red**.

R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, %: Percentage of predicted, Post: Post-bronchodilator, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

For the **group 2** analysis, changes in spirometric parameters post-bronchodilation (i.e. reversibility - %Δ) are summarised in Table 6.12 and Figure 6.18. Despite the differences in both the pre- and post-bronchodilator IOS parameters measured, there was no significant difference in the percentage change between pre- and post-bronchodilator values for any of the small airway IOS parameters, when the totally controlled group was compared to the well-controlled group and to the uncontrolled group (**group 2** analysis).

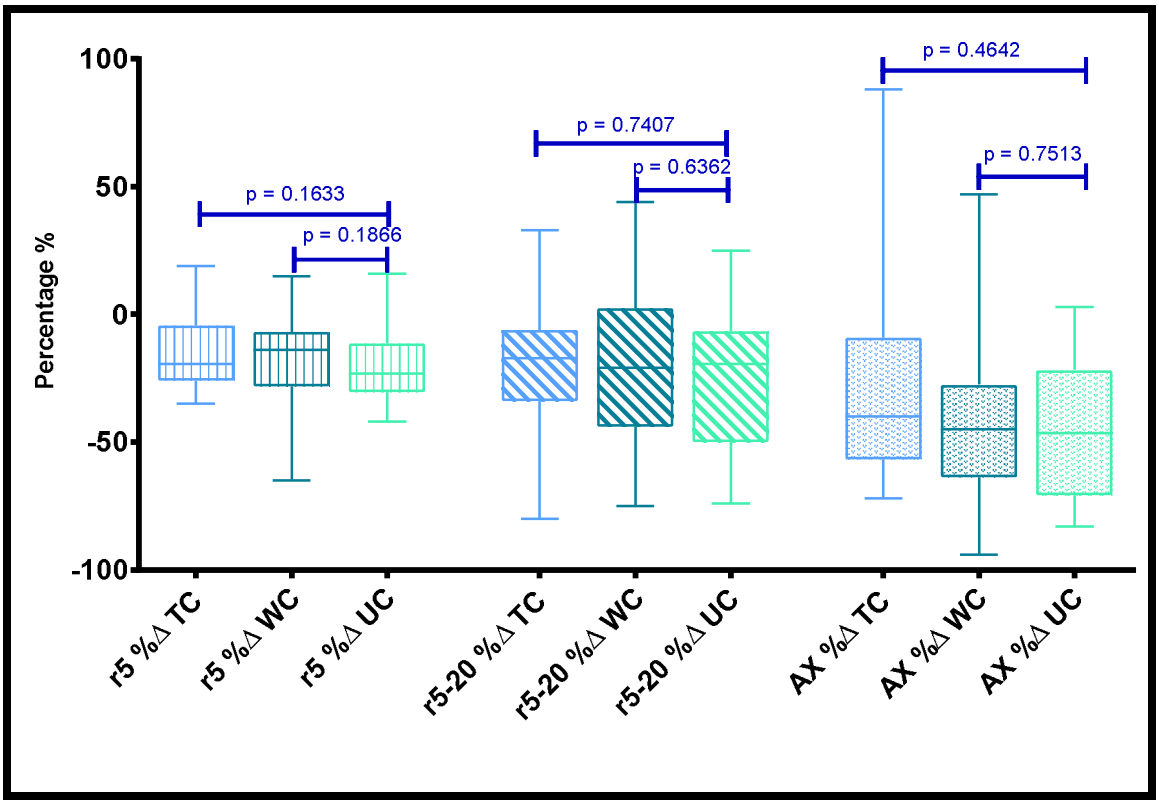

Figure 6.18: Percentage change post-bronchodilator for IOS parameters of the small airways (R5, R5-R20 and AX) comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)
R5: Total airway resistance, R5-R20: resistance of the small airways, AX: Reactance area,
TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled, %: Percentage of predicted, %Δ: % Change post-bronchodilator. Wilcoxon rank sum test.

Table 6.12: Percentage change post-bronchodilator IOS parameters (reported as percentage of predicted) comparing totally controlled, well-controlled and uncontrolled asthmatics (**group 2**)

IOS Parameters	Totally controlled n=30	Well-controlled n=22	Uncontrolled n=24	Totally controlled vs Well-controlled[1] (p values)	Totally controlled vs Uncontrolled[1] (p values)	Well-controlled vs Uncontrolled[1] (p values)
R5 %Δ	13.1 (9.1 - 18.8)	12.8 (8.0 - 20.4)	16.9 (11.2 - 25.6)	0.8384	0.1633	0.1866
R20 %Δ	11.6 (8.7 - 15.6)	6.0 (2.7 - 13.2)	6.9 (3.3 - 14.2)	0.2176	0.2996	0.6517
R5 - R20 %Δ	15.3 (8.3 - 27.9)	23.6 (16.6 - 33.5)	17.6 (10.6 - 29.2)	0.363	0.7407	0.6326
R35 %Δ	9.1 (6.9 - 12.1)	4.9 (1.8 - 13.0)	5.1 (2.5 - 10.3)	0.8167	0.2759	0.5227
Z5 %Δ	9.6 (4.6 - 20.1)	12.2 (5.7 - 26.3)	16.1 (7.9 - 32.7)	0.8675	0.0451	0.1559
X5 %Δ	12.0 (6.1 - 23.7)	16.9 (7.3 - 38.9)	28.1 (19.2 - 40.9)	0.1703	**0.0115***	0.4157
X20 %Δ	146.6 (79.1 - 271.4)	100.0 (69.0 - 145.1)	90.2 (51.5 - 157.8)	0.2432	0.0964	0.4221
X35 %Δ	40.3 (25.0 - 64.9)	93.2 (36.8 - 236.2)	63.0 (39.0 - 101.8)	0.2431	0.1017	0.9963
AX %Δ	21.5 (9.6 - 48.4)	40.2 (29.1 - 55.5)	18.7 (6.0 - 58.4)	0.3889	0.4642	0.7513
Res freq %Δ	20.1 (15.6 - 25.9)	17.9 (10.9 - 29.5)	7.1 (2.2 - 23.2)	0.7953	0.5901	0.3696

1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
Values highlighted ……. are IOS measurements of the small airways.
The results are reported as medians together with the 95% confidence intervals (CI).
R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, R35: Airway resistance at 35Hz, Z5: Respiratory impedance at 5 Hz, Hz: Hertz, X5: Peripheral capacitive reactance, X20: Reactance at 20Hz, X35: Reactance at 35Hz, V: Flow, AX: Reactance area, res freq: Resonant frequency, n: Number, %Δ: % Change post-bronchodilator, %: Percentage of predicted.

A further analysis of the correlation of the parameters measured by IOS and the corresponding parameters measured by spirometry was performed. There was a strong inverse correlation between AX (Goldman triangle = small airways) and FEF 25-75 for both the pre-bronchodilator measurements (r = -0.66; p = 0.0001) and the post-bronchodilator measurements (r = -0.63; p = 0.0001) (Table 6.13; Figure 6.18). However, there was poor correlation of the remainder of the pre- and post-bronchodilator spirometric measures of airway calibre and the IOS measurement of airways resistance (Table 6.13; Figure 6.19).

Table 6.13: Correlation between the spirometric parameters of the small and large airway calibre, and the IOS parameters of the small and large airway

Parameter measured	rho	p values
Pre FEV_1 vs Pre R20	-0.4	0.0003
Post FEV_1 vs Post R20	-0.45	0.0001
Post FEV_1 vs Post R20	-0.45	0.0001
Pre R5-R20 vs Pre FEF 25-75	-0.57	0.0001
Post R5-R20 vs Post FEF 25-75	-0.49	0.0001
Pre AX vs Pre FEF 25-75*	**-0.66****	0.0001
Post AX vs Pre FEF 25-75*	**-0.63****	0.0001
Post X5 vs Post FEV_1	-0.39	0.0001
Pre res freq vs Pre FEV_1	-0.53	0.0001
Post res freq vs Post FEV_1	-0.51	0.0001

Spearman rank correlation. ** = r ≥ +/- 0.6.
FEV1: Forced expiratory volume in one second, FEF25-75: Forced expiratory flow during the middle half of the FVC, R5: Total airway resistance, R20: Central airway resistance, R5-R20: Resistance of the small airways, X5: Peripheral capacitive reactance, AX: Reactance area, res freq: Resonant frequency, Pre: Pre-bronchodilator, Post: Post-bronchodilator.

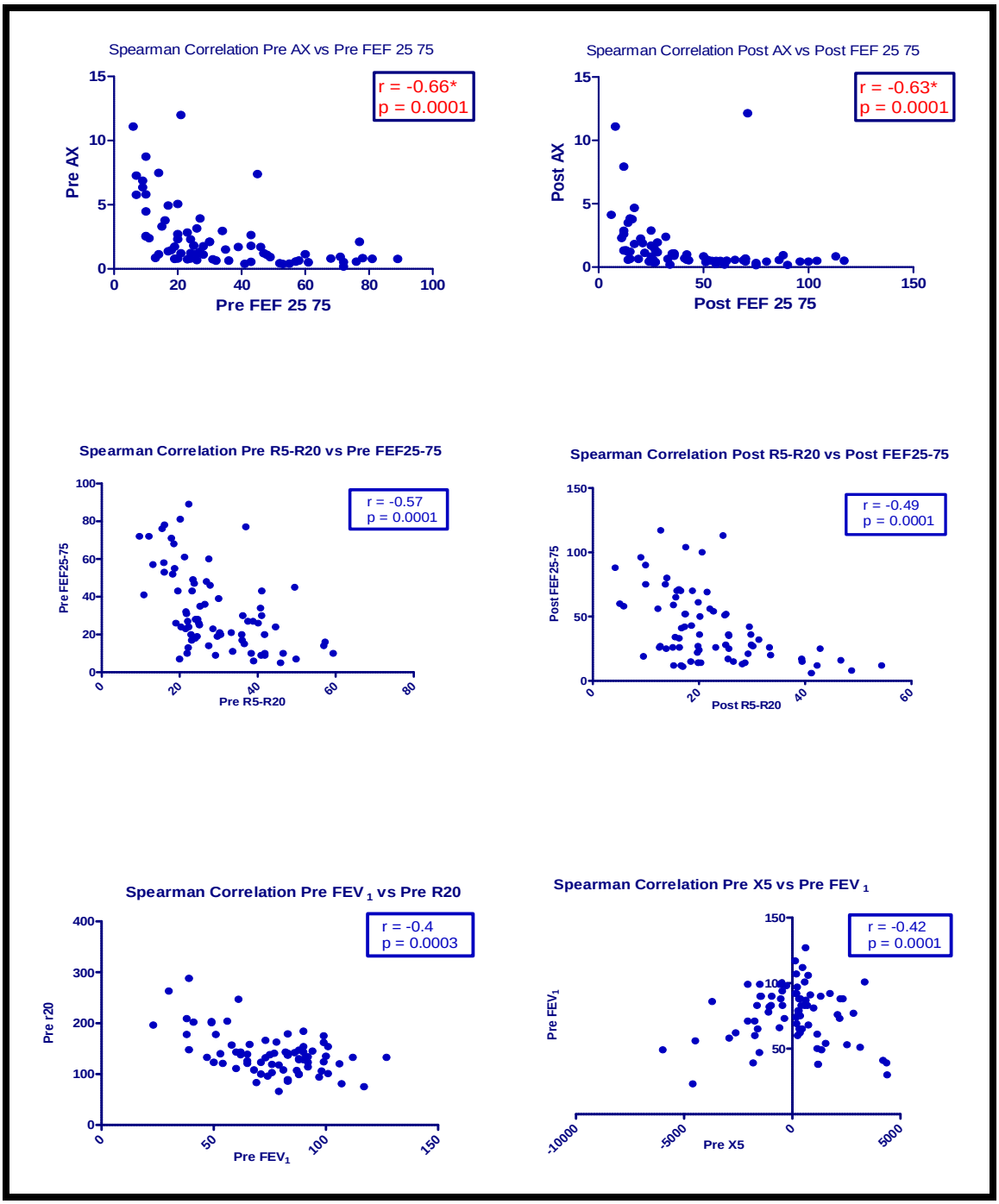

Figure 6.19: Correlation between the spirometric IOS parameters of the small and large airway calibre, and the IOS parameters of the small and large airway

* r values reported as a strong correlation are depicted in **red**.

FEV1: forced expiratory volume in one second, FEF25-75: forced expiratory flow during the middle half of the FVC, R20: central airway resistance, R5-R20: resistance of the small airways, X5: peripheral capacitive reactance, AX: reactance area, res freq: resonant frequency. Spearman rank correlation.

6.6 Discussion

One of the major contributors to the poor concordance between the patient's perception of their asthma control, and the objective assessment of that control, is the lack of a simple tool that can reliably assesses the state of the small airways (320, 351). This lack of an appropriate modality for appraisal of the small airways, is especially important in patients with apparently well controlled asthma, as these patients may have persistent small airways inflammation that is subclinical and might predispose them to recurrent exacerbations and airway remodelling (21). The assessment of the small airways in patients with asthma is often referred to as the assessment of the "silent zone" in asthma as is it is difficult to assess non-invasively (21, 22, 303). Therefore, the focus of this chapter was the non-invasive appraisal of the small airways in patients with controlled as well as uncontrolled asthma.

6.6.1 Assessment of the small airways with spirometry

There is a suggestion in the literature that methods of assessing the small airways using non-invasive modalities such as high resolution CT (HRCT) scan of the chest and IOS are important to validate since they will improve the evaluation of newer asthma therapies (353). Unfortunately, to date the most validated tool we have to assess airway calibre and airflow dynamics is still spirometry. However, spirometry is not very sensitive in detecting changes in the small airways (351). Thus, small airway inflammation with the resultant increase in airway resistance due to airway remodelling can progress undetected (23).

Supportive of this hypothesis, is that in the present study for the **group 1** and the **group 2** analyses, spirometric measurements of the small airways (FEF 25-75) was significantly reduced when reported as a percentage of predicted. The pre-bronchodilator percentage predicted FEF 25-75 was a median of 35.7% (95% CI 28.5 - 44.7) for the totally controlled group of patients and a median of 27.3% (95% CI 20.8 - 36.0) for the well-controlled group (Table 6.4; Figure 6.7). This was also true for the post-bronchodilator FEF25-75 values (Table 6.5; Figure 6.8).

There was no significant difference in percentage change in FEF 25-75 post-bronchodilation when the totally controlled group was compared with the uncontrolled group ($p = 0.91$), as well as when the well-controlled group was compared with the uncontrolled group ($p = 0.55$) (Table 6.6; Figure 6.8). In addition, and equally importantly, FEF 25-75 values in the totally controlled, well-controlled and uncontrolled asthmatic patients demonstrated an absolute post-

bronchodilator reversibility of 32%, 26.5% and 31% respectively, confirming the presence of bronchial airway hyper-reactivity (BAH) in the small airways even in the controlled group of asthmatics studied (Table 6.6). The significance of this information is debatable, but it could possibly imply the presence of small airway inflammation and remodelling within this group of patients. Of note, this was evident despite patients within the totally controlled and well-controlled groups being asymptomatic and having normal FEV1 values.

Low FEF 25-75 values in the presence of a normal FEV1 (i.e. similar to patients within the totally controlled and well-controlled asthma groups as defined by the ACQ in this study) have been associated with increased asthma severity, systemic steroid use, and asthma exacerbations in children (354). This supports the theory of ongoing small airway inflammation within this group of patients. There is also literature to support the assumption that a reduced FEF 25-75 (< 65% of predicted) in the evaluation of patients with asthma and or allergic rhinitis, is indicative of a small airway asthma phenotype, and should be a target of therapy in patients with persistent symptoms even when conventional spirometry parameters (i.e. FEV1) are normal (71, 355). Thus, the above finding make it vitally important for clinicians to have a validated tool to assess the small airways, as they are the "silent zone" of the lungs (21, 22, 303).

6.6.2 Assessment of the small airways with IOS

In the present study R5-R20 (reported as a percentage of predicted), which is a measurement of small airway resistance, there was significantly lower pre-bronchodilator in the controlled group when compared with the uncontrolled group of asthmatic patients (**group 1** analysis). The median R5-R20 for the controlled group was 24.6% (95% CI 22.2 - 27.3) compared with 33.5% (95% CI 28.9 - 38.9) in the uncontrolled group ($p = 0.0026$) (Table 6.7; Figure 6.9).

This was also evident for the post-bronchodilator R5-R20 values for the controlled group with the median being 18.5% (95% CI 16.2 - 21.0) compared with the uncontrolled group with a medianof 23.1% (95% CI 18.7 - 28.6); $p = 0.04$ (Table 6.8; Figure 6.10). This finding mirrored the spirometry values for the small airways (i.e. FEF25-75), implying small airway hyper-reactivity within the entire cohort of patents in the present study, irrespective of the level of asthma control as defined by the ACQ. This finding probably alludes to the presence of bronchial airway hyper-reactivity (BAH) in the small airways, even in apparently controlled

asthmatics as defined by the ACQ, and confirmed by conventional spirometry (i.e. reduced FEF25-75) within this cohort of patients (Tables 6.4, 6.5 ; Figures 6.9, 6.10).

There was also a decrease in the median R5-R20 resistance, when comparing the pre- and post-bronchodilator values, for the controlled group (24.6 % vs 18.5%; p = 0.0001) and the uncontrolled group of asthmatic patients (33.5% vs 23.1%; p =0.0001) (Tables 6.4, 6.5; Figure 6.13). Importantly, this improvement in airway resistance in the small airways post-bronchodilator (commonly referred to as bronchial airway reversibility-BAH), for the controlled as well as the uncontrolled group measured by IOS, was only evident in the uncontrolled group of patients when assessed by spirometry (Tables 6.1,6.2; Figure 6.4). Thus, IOS may allow us to identify BAH in the controlled asthmatic patient that might not be evident if assessed with conventional spirometry only. This finding has not been reported in adult asthmatic patients, and if validated, IOS may prove to be an invaluable tool in identifying BAH in the controlled asthmatic patient.

The low-frequency reactance area AX (Goldman Triangle) (310, 312), was also significantly lower in the controlled group when compared with the uncontrolled group of asthmatic patients (**group 1** analysis), for both the pre- and post-bronchodilator values (p = 0.0013 and p = 0.0042 respectively). This appears to agree with the suggestion that small airways may be compromised in some "controlled" asthmatic patients, and which would not be evident if they are only assessed using spirometry and symptom questionnaires (i.e. ACT and ACQ). Thus, the measurement of these IOS parameters in the present study appear to confirm the discriminating value of IOS in differentiating the true controlled patient from the uncontrolled adult asthmatic patient. A systematic literature review of the prevalence of small airways disease in adult asthmatic patients by Usmani *et al.* (356) reported a prevalence of 50–60% of small airway disease in the absence of proximal airway obstruction. In that systematic literature review published in 2016, one of the modalities used to the small airways was IOS. Hence, IOS may provide a treating physician with a simple to perform, validated and objective tool to identify, monitor and appropriately treat those patients with "apparently" controlled asthma.

Studies by Qi *et al.* (357), and Batmaz *et al.* (358), have reported significant correlations between the FEV1 and PEFR, and the various IOS parameters analysed (i.e. R5, R20, R5-R20, X5 and AX). This present study could not confirm any of these findings. Reasons for this might

be that those studies were performed in a paediatric cohort of patients. In those studies, the actual correlation values were not reported; rather the receiver operating characteristic (ROC) curves with sensitivities and specificities were reported. Thus, their findings are difficult to extrapolate to the present study because we did not have a non-asthmatic patient arm and, therefore, ROC curves could not be calculated. Another study by Hafez *et al.* (359), in an adult asthmatic cohort, reported a negative correlation between R5 and the FEV1/FVC ratio ($r = -0.79$), as well as a negative correlation between R20 and FEV1% ($r = -0.7$). Theoretically, R5 is a measurement of large away resistance and thus the correlation with the FEV1/FVC ratio is difficult to interpret and probably relates to patients with obstructive airways diseases and not necessarily asthma (307). Furthermore, R20 is a measurement of total airway resistance, and therefore the correlation with FEV1 is again theoretically incorrect (309, 310).

Another reason for the discrepancy in the present study and the studies quoted above, might be that the IOS data analysed in our study were corrected for weight, height and age as per the prediction equation provided by the ERS/ATS task force, and pre-programmed into the Jaeger IOS equipment used. However, these reference values have not been validated in different patient populations or in different age groups, and thus cannot be adequately corrected for our South African patient population cohort (296). Finally, although most of the correlations reported in the above studies were of statistical significance ($p < 0.05$), they appear to be of little clinical significance as most of the rho (r) values reported were less than 0.6. This has been corroborated in a publication by Song *et al.* (360), who reported a significant, but weak, correlation between FEV1 and resistance (R) at 5, 10, 20 and 35 Hz. In that study FVC was similarly also reported as being significantly correlated with R at 5, 10, 20 and 35 Hz, but with only moderate r values, all being reported as $r \leq +/- 0.4$ (360).

One of the major limitations of IOS is the significant variability of the reference values applied in reporting the published results, with various arbitrary cut-off values applied as reference values for the IOS parameters measured. For example, Desiraju *et al.* (309) report resistance values to be considered normal when above 150% of predicted. However, Moratta *et al.* (361), reported a change in R5 by 30% to 35% to reflect a positive response to bronchodilators, whereas Komarow *et al.* (362), reported a 29.1% change in AX as being an optimal cut-off point (307). Bailly *et al.* (363) reported that X5 was the only parameter sensitive enough to detect bronchial hyper-reactivity and that a 20% decline in FEV1 correlated with a 50%

decrease in X5. Notably, all these reference ranges have only been validated in the paediatric population group. The lack of validated reference ranges as well as correction factors for age and height, highlight some of the major issues impeding IOS implementation in assessing adult patients with asthma (364).

One of the more important drawbacks in the interpretation of IOS measurements is also the lack of a correction algorithm for any changes in the lung volume that might occur while airway resistance is measured. Successful bronchodilation of moderate to severe airway obstruction in both asthma and COPD reduces air trapping and hyperinflation, leading to a reduction of the functional residual capacity (FRC), and thus, total lung volume when airway resistance is measured. However, breathing at a lower FRC increases the airway resistance when compared with breathing at a previously higher lung volume. The reason for this is that the small airways are theoretically more crowded within the smaller lung volumes, and thus, the parameters of IOS measuring airway resistance may be misleading by showing no change or a worsening of the airway resistance (365, 366).

Nonetheless to date, none of the studies published have evaluated the usefulness of IOS as an adjunctive tool in assessing asthma control in adults patients categorised by the ACQ (59). In the present study, when the IOS data were correlated with the ACQ none of the IOS parameters measured had a rho value that demonstrated any clinically relevant correlation with the ACQ score. One of the major reasons for this may be that the cohort of patients analysed in this study was too small to perform appropriate correlation analyses that may have yield clinically significant outcomes.

Despite these limitations noted above IOS has been validated as a tool to assess and titrate asthma management in the paediatric population group (367), with good correlation between IOS and clinical symptoms, as well asthma control reported (317, 368). One of the major benefits of IOS documented in the paediatric population group is the ability of IOS to detect subtle changes in a patient's airway function earlier than with conventional spirometry (362). There are sufficient data to support using IOS in the paediatric population group to assess abnormal distal airway function, even in the setting of normal spirometry (358, 365). Importantly, in publications by Shi *et al.* (317, 318), neither FVC, FEF 25-75 nor FEV1 were

as effective as small airway IOS parameters in detecting poorly controlled asthma as well as in predicting loss of asthma control in children with normal spirometric parameters.

6.7 Conclusion

Assessment of the small airways is vitally important in the management of patients with asthma as they are the "silent zone" of the lungs (21, 22, 303). This is supported by the present study in that for the **group 1** and the **group 2** analyses, spirometric measurements of the small airways (FEF 25-75) was significantly reduced when reported as a percentage of predicted. FEF 25-75 values in the totally controlled, well-controlled and uncontrolled asthmatic patients also demonstrated an absolute post-bronchodilator reversibility of 32%, 26.5% and 31% respectively, possibly implying the presence of small airway inflammation and remodelling within this cohort of patients. IOS, once validated with appropriate reference ranges for adults, may provide the treating clinician with a valuable tool to assess these inflamed small airways in adult asthmatic patients. The major advantages of IOS is that it is independent of effort, reproducible and sensitive enough to allow titration of therapeutic interventions in the presence of normal spirometric parameters (317). The present study adds to the body of published literature on IOS in adult patients with asthma (357, 359). Furthermore, the present study confirms the utility of IOS in differentiating the controlled from the uncontrolled adult asthmatic patient as defined by the ACQ. However, more data is required from larger patient cohorts to determine appropriate cut-off values for these IOS parameters, that will have a sensitivity and specificity appropriate enough to detect or exclude small airway dysfunction and /or inflammation in the adult patient (369).

CHAPTER 7: EVALUATION OF THE UTILITY OF SERUM BIOMARKERS IN ASSESSING AIRWAY INFLAMMATION WHEN COMPARED TO CATEGORIES OF ASTHMA CONTROL AS DEFINED BY THE ACQ

7.1 Introduction

Asthma guidelines emphasize maintaining both current control and reducing future risk, but the relationship between these two targets are not well understood (343). The recurrent theme that is evident in studies on the management of many chronic diseases, especially diabetes mellitus and hypertension, is that once specified levels of control are achieved, future complications can be averted (370). There has been a significant shift in international guidelines, with a recognition of the need to treat patients to achieve disease control as a whole, as opposed to targeting individual aspects of disease manifestations such as normalisation of spirometric parameters in the case of asthma (7, 8). In the two examples cited above (i.e. hypertension and diabetes mellitus), treatment targets are clearly defined, simple to perform, reproducible and mostly involve the measurement of a single parameter, such as blood pressure or a glycosylated haemoglobin level, as a surrogate marker of disease control.

Unfortunately, asthma is a heterogeneous illness with several different mechanisms driving the underlying inflammation. (17). The identification of various different asthma phenotypes and endotypes has made the objective of using single parameters to assess airway inflammation far more challenging (77). Currently, the targets to assess the efficacy of therapy, recommended by the latest GINA guidelines (8), do not directly assess the inflammatory nature of the illness. One of the inherent characteristics of asthma is that uncontrolled patients have frequent acute episodic exacerbations. These exacerbations occur against a background of chronic persistent inflammation and structural changes within the airways, which leads to recurrent symptoms and reduced lung function (50). Thus, the assessment of airway inflammation in patients with asthma is vitally important to achieve asthma control as well as to reduce further risk in these patients.

The problem with our current assessment paradigm has been the assumption that by titrating therapeutic interventions to normalise symptoms and improve airflow dynamics, we will have

suppressed inflammation in the airways of asthmatic patients (7, 371). However, airway inflammation may persist in the apparently well-controlled asthmatic patient (66, 325). As a result, there has been an increasing focus on the actual assessment of airway inflammation in patients with asthma, 'Inflammometry' is now being increasingly used to titrate therapeutic interventions and to achieve better asthma control (73-76). Cellular, immunologic, and molecular biomarkers of airway inflammation can be studied by direct or indirect methods of sampling the airways.

Direct methods of assessing airway inflammation include bronchoalveolar lavage, biopsies of airways, bronchial brushing and induced sputum analysis. These methods have the advantage of being well validated, accurate and reproducible (372). The major drawback with bronchoscopy is that there is a theoretic potential of precipitating an acute bronchospastic response in a patient with inflamed airways during this invasive procedure. Furthermore, invasive procedures cannot be performed as a routine investigation at repeated visits (73, 75). Induced sputum analysis is a very attractive alternative as a direct assessment of airway inflammation, but the expertise and time needed to utilise this investigation on a routine basis, limits its practicality especially in non-academic facilities (324, 329).

Hence, the next objective of this study was to determine whether certain inflammatory serum biomarkers (i.e. CRP, SAAP and several cytokines derived from Th1 and Th2 T helper cells) demonstrated a significant difference between the groups of patients characterised by their asthma control as defined by the ACQ, as well as their correlation with ACQ the score. Moreover, the investigations were designed to determine whether the biomarkers could identify persistent airway inflammation in controlled asthmatic patients.

7.2 Design and Study Population

This study was designed as a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic with approval to conduct the study received from the University of the Witwatersrand Human Research Ethics committee (Protocol Number M060401- Appendix 3). The specifics of the design and population cohort are the same as the study population in Chapter 5 and have been described in detail in Chapter 4. In an attempt to reduce the statistical "noise to signal" from confounders that might dilute the study results, strict inclusion and exclusion criteria, as described in Chapter 4 were applied.

The reason for the application of strict inclusion and exclusion criteria is that this was a proof of concept study trying to identify a biomarker that might be affected by any concomitant co-existing illnesses. A total of 76 patients fulfilled the criteria for inclusion into the study.

7.3 Methods

A detailed description of the methodology employed in the present study has been described in Chapter 4. For the purposes of standardisation within this thesis, all patients completed the Asthma Control Questionnaire (ACQ), which was used as the basis for the assessment of asthma control in the entire cohort of patients studied (Appendix 1) (58). As indicated in Chapter 4, the population of patients studied were categorised into two groups, based on their level of control. **Group 1** consisted of the patients categorised into controlled (CC) and uncontrolled (UC), based on an ACQ level of 1.5. **Group 2** consisted of the same cohort of patients categorised by the ACQ into totally controlled (TC) having an ACQ < 0.75, well controlled (WC) having an ACQ of between 0.75 and 1.5 and uncontrolled (UC) with an ACQ > 1.5 (59).

In all the studies, the evaluation was first for **group 1** (i.e. primary/initial analysis: controlled versus uncontrolled asthmatic patients) and thereafter, the **group 2** analysis (i.e. subsequent/secondary analysis: totally controlled versus well controlled and versus uncontrolled asthmatic patients).

Of importance in this chapter was the measurement of serum biomarkers, which were divided into 2 basic categories. Firstly, non-specific systemic markers of inflammation were measured, and included C-reactive protein (CRP) and serum amyloid associated protein (SAAP). Secondly, specific markers of inflammation that are inherent to the underlying inflammatory pathogenesis of airway inflammation i.e. the Th1 and Th2 cytokines were measured: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IFNγ and TNFα.

All the biomarkers were measured via microplate-based multiplex immunoassay technology. Multiplex technology uses microscopic microspheres (beads), each with a different colour code (spectral address) to permit discrimination of different proteins that are measured simultaneously. Each of the beads in the assay are coupled to specific antibodies targeted against different serum protein epitopes being measured. A bead reader (i.e. the BioPlex™

reader) then uses laser technology to classify the beads according to their spectral addresses assigned, as per the manufacturer's information sheet, thus identifying the specific protein bound to the bead. The bead reader quantifies the concentration of the protein by measuring the fluorescent intensity of the light emitted. In this study, the Millipore human cardiovascular disease panel 2 (HCVD2 – 62 BK) was used according to manufacturer's instructions to measure the serum CRP and SAAP, and the Bioplex pro™ cytokine, chemokine and growth factor assay (Bio-Rad M500AD1ZL6) was used according to manufacturer's instructions to measure the cytokine levels. A detail description of the exact methodology of the preparation and measurement of the analytes is documented in Appendix 6 and 7.

7.4 Statistical Analysis

Methodology detailing the basic principles of the statistical analyses applied, including population size calculations, has been discussed in Chapter 4. The population of patients studied were 76 in total. The Shapiro-Wilks normality test indicated that the data reported was not normally distributed; hence, non-parametric statistical tests were used for all the analyses performed.

The results were reported in both a table format, as well as graphically using a box-and-whisker plot where applicable. The results were represented in a dual format for ease of reference and interpretation. The results included in the tables were reported as medians together with the 95 percent (%) confidence intervals (CI). The box-and-whisker plot displayed the data using the standard five number summary: top and bottom boundary of the box indicating the 75th and 25th percentiles, the line within the box depicted the median and the whiskers represented the minimum and maximum values measured.

Results reported in the tables that were statistically significant are highlighted in bold black text with an asterisk *, whereas those results that had a clinically significant correlation are highlighted in bold black text with a double asterisk **, for easy recognition. The corresponding p or Rho (r) values are represented in the associated figures in **red**.

All the laboratory investigations, measured as continuous variables, were compared to the categories of asthma control, first for **group 1** and then for **group 2**. For the patients categorised into **group 1** (i.e. controlled versus uncontrolled asthmatic patients), the association between

the variable measured and the level of asthma control was analysed using the Wilcoxon rank sum test. For the patients categorised into **group 2** (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Kruskal-Wallis one-way ANOVA test. The Bonferroni correction was applied to the analysis of the **group 2** cohort to control for multiplicity.

In all testing a p-value < 0.05 was considered significant, except where the Bonferroni correction was applied.

Specific to this chapter, is that the levels of all biomarkers measured via the bioplex assays were reported as fluorescent intensity units (FU). When the level of a biomarker was below the lower limit of detection, it was assigned half the value of the lowest limit of detection. When the level of a biomarker was above the upper limit of detection, it was assigned double the value of the uppermost limit of detection. These adjustments for outlying data have been verified in the literature as an acceptable method for data correction (337, 338), and are recommended by the manufacturers of the analyte kits as well.

As outlined in Chapter 4, the reason for reporting in fluorescent intensity units (FU) as opposed to standard international units (SI) was that fluorescent intensity is the actual measurement (raw data) obtained from the microplate-based multiplex immunoassay system. By using the raw data in FU as opposed to diluting the data with several correction steps, using pre-defined manufacturer provided control samples to determine the equivalent SI units, the statistical analysis and results obtained were optimised (Appendix 6 and 7).

The relationship between asthma control as defined by the ACQ, and levels of biomarkers grouped into Th1 predominant cytokines or Th2 predominant cytokines, was determined using a logistic regression analysis.

A Spearman's rank correlation was used to assess the correlation between the ACQ and all other variables measured. For the interpretation of the Spearman's rank correlation, as outlined in Chapter 4, only $r > 0.6$ were reported as a having a strong correlation, even though a $r < 0.6$ may have been statistically significant with a $p < 0.05$.

Data analysis of the association between the medication used by the patient and the levels of the biomarker measured, was performed using the Wilcoxon rank sum test.

The statistical analyses and graphics were performed using the SPSS software, version 12 (**SPSS**, Chicago, Illinois), Excel software (Microsoft, Albuquerque, New Mexico) and GraphPad Software Inc. (La Jolla, California).

7.5 Results

The results of all the serum biomarkers measured are reported as FU with the corrected concentrations in ng/ml reported in the supplementary data table in Appendix 11.

7.5.1 Nonspecific markers of systemic inflammation i.e. CRP and SAAP

CRP levels were significantly higher in the uncontrolled group when compared with the controlled group (**group 1**). The median CRP in the controlled asthmatic group was 1604 FU (95% CI 1136 - 2267) compared with a median of 2852 FU (95% CI 1388 - 5861) in the uncontrolled group (p = 0.03) (Table 7.1; Figure 7.1). The median SAAP levels were not significantly different when comparing the two groups of patients (p = 0.296) (Table 7.1; Figure 7.1). There was also no significant correlations between the CRP or SAAP and the ACQ score (Table 7.1; Figure 7.2).

Table 7.1: CRP and SAAP levels comparing controlled and uncontrolled asthmatic patients (**group 1**), and the correlation between levels of CRP and SAAP and the ACQ score

Biomarker+	Controlled n=51[1]	Uncontrolled n=24	Controlled vs Uncontrolled[2] (p value)	Rho values (p values) n= 75[1,3]
CRP	1604 (1136 - 2267)	2852 (1388 - 5861)	**0.03***	0.2127 (0.067)
SAAP	657 (452 - 954)	1078 (458 - 2537)	0.2961	0.1041 (0.374)

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
1: Biomarker analysis was of 51 controlled patients as the result of one patient being aberrant.
2: Wilcoxon rank sum test. * = $p < 0.05$
3: Spearman rank correlation between ACQ score and other variables.
The results are reported as medians together with the 95% confidence intervals (CI).
CRP: C - reactive protein, SAAP: Serum amyloid associated protein, n: Number.

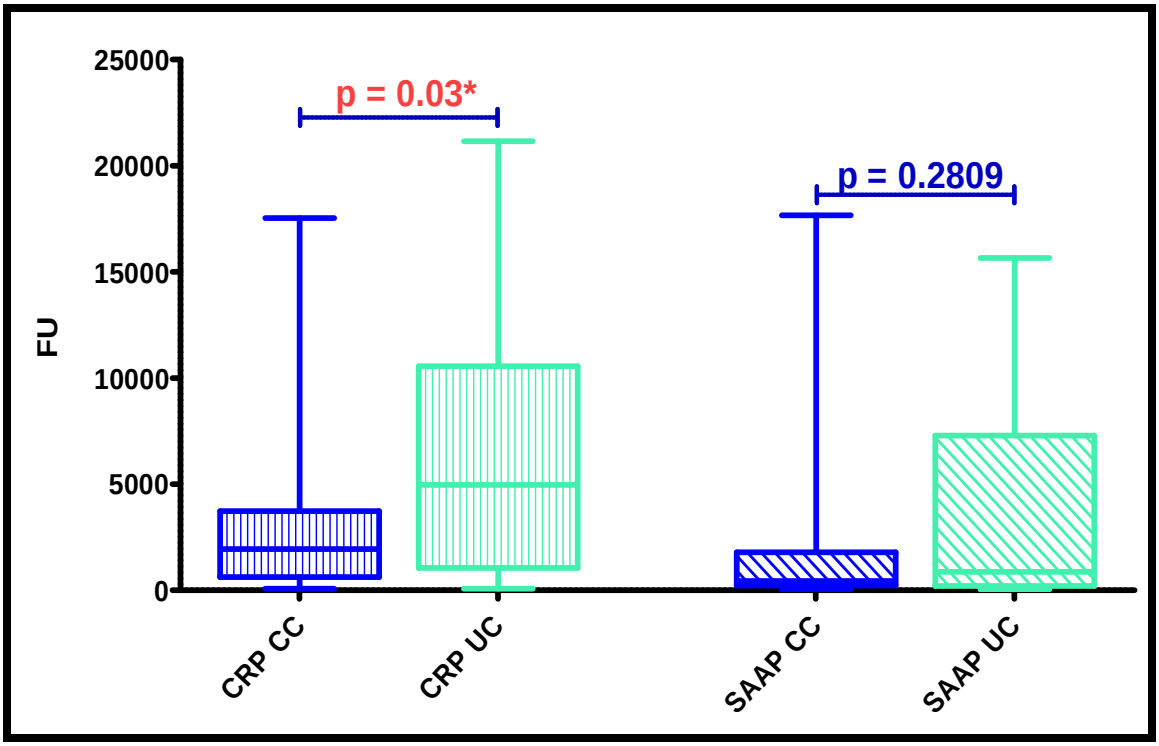

Figure 7.1: CRP and SAAP levels comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in **red**.
CRP: C - reactive protein, SAAP: Serum amyloid associated protein, FU: Fluorescent intensity units, CC: Controlled, UC: Uncontrolled. Wilcoxon rank sum test.

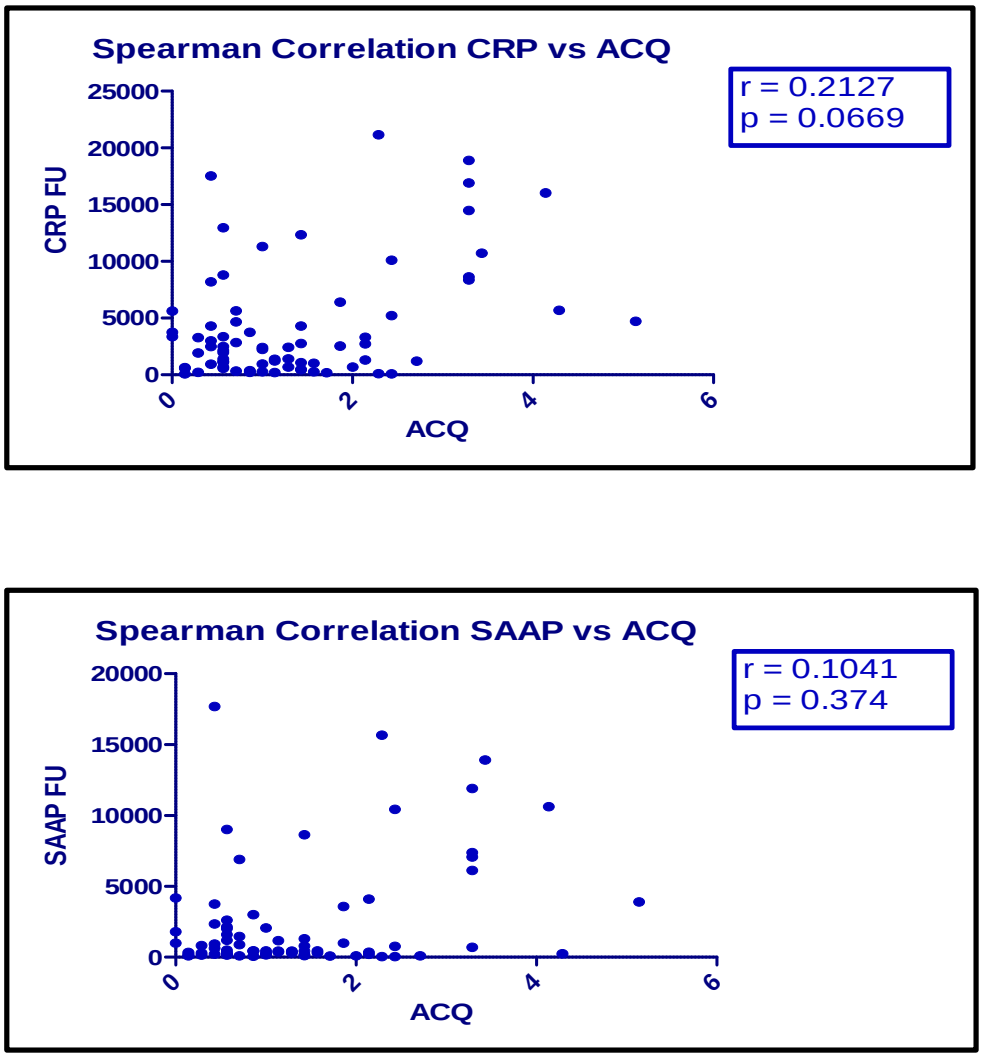

Figure 7.2: Correlation between CRP and SAAP levels and the ACQ score in the study population

CRP: C-reactive protein, SAAP: serum amyloid associated protein, FU: florescent intensity units, ACQ: Asthma Control Questionnaire. Spearman rank correlation.

In the comparison of the totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**), following the Bonferroni correction, there was no significant difference in the CRP or SAAP levels in the different asthma groups (Table 7.2; Figure 7.3).

Table 7.2: CRP and SAAP levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Biomarker[+]	Totally controlled n=30	Well-controlled n=21[1]	Uncontrolled n=24	Totally controlled vs Well-controlled[1,2] (p value)	Totally controlled vs Uncontrolled[2] (p value)	Well-controlled vs Uncontrolled[2] (p value)
CRP	1983 (1260 - 3120)	1185 (682 - 2059)	2852 (1388 - 5861)	0.09	0.13	0.03
SAAP	828 (490 - 1401)	471 (278 - 797)	1078 (458 - 2537)	0.19	0.57	0.17

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
1: Biomarker analysis was of 21 well-controlled patients as the result of one patient was aberrant.
2: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
The results are reported as medians together with the 95% confidence intervals (CI).
CRP: C-reactive protein, SAAP: Serum amyloid associated protein, n: Number.

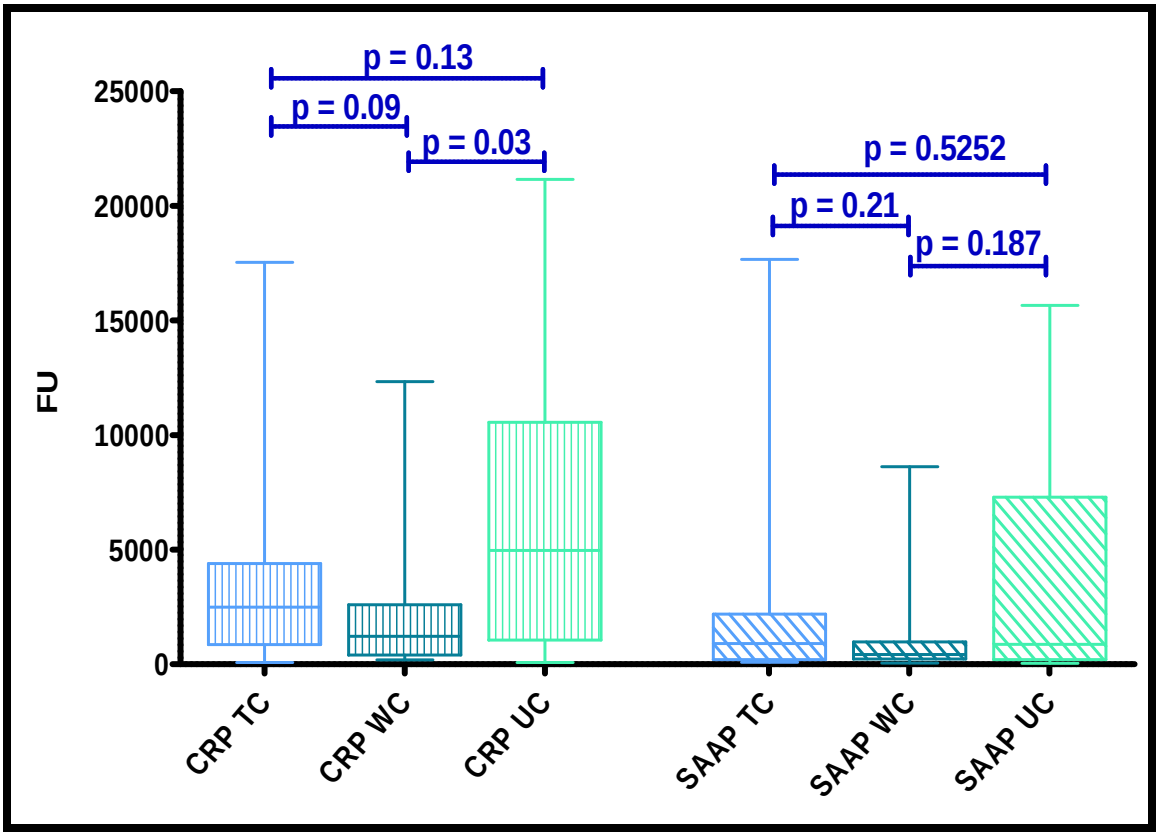

Figure 7.3: Comparison of CRP and SAAP levels in patients with totally controlled, well-controlled and uncontrolled asthma (**group 2**)
*p values reported as significant are depicted in **red**
CRP: C - reactive protein, SAAP: Serum amyloid associated protein, FU: Florescent intensity units, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

7.5.2 Th1 and Th2 cytokine profiles

The values of the major Th1 and Th2 derived cytokines in the serum of the patients are indicated in Table 7.3. They are reported as FU with the corrected concentration in ng/ml reported in the supplementary data table in Appendix 11.

Table 7.3: Th1 and Th2 cytokine levels comparing controlled and uncontrolled asthmatic patients (**group 1**), and the correlation between levels of these cytokines and the ACQ score

Biomarker[+]	Controlled n=51[1]	Uncontrolled n=24	Controlled vs Uncontrolled[2] (p values)	Rho values (p-values) n= 75[1,3,4,5]
IL-1β	10.87 (9.99 - 11.81)	11.04 (9.52 - 12.81)	0.93	0.0152 (0.8972)
IL-2	19.19 (16.57 - 22.23)	26.1 (20.06 - 33.95)	**0.02***	0.3514 (0.002)
IL-4	12.28 (11.42 - 13.20)	13.1 (10.43 - 16.46)	0.77	0.084 (0.4735)
IL-5	10.37 (8.76 - 12.28)	10.42 (7.78 - 13.95)	0.82	0.113 (0.3346)
IL-6[4]	17.69 (15.55 - 20.13)	22.66 (17.09 - 30.04)	0.07	0.2143 (0.0667)
IL-8 [5]	25.04 (22.84 - 27.44)	24.09 (21.21 - 27.36)[5]	0.75	0.0782 (0.5077)
IL-12	54.92(47.19 - 63.92)	59.78 (44.48 - 80.36)	0.48	0.1343 (0.2508)
IL-13	21.39 (18.47 - 24.79)	22.45 (15.36 - 32.81)	0.87	0.0645 (0.5823)
INFγ	16.62 (15.19 - 18.19)	17.88 (14.99 - 21.33)	0.21	0.1182 (0.3126)
TNFα	11.61 (10.37 - 12.99)	11.77 (9.79 - 14.16)	0.63	0.1489 (0.2022)

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
1: Cytokine analysis was of 51 controlled patients as one result was totally aberrant and discarded, n=75.
2: Wilcoxon rank sum test. * = $p < 0.05$
3: Spearman rank correlation between ACQ score and other variables.
4: IL-6 analysis of one patient in the controlled group (n=50) was not measurable by bioplex.
5: IL-8 analysis of one additional patient in the uncontrolled group (n=23) was totally not measurable by bioplex.
The results are reported as medians together with the 95% confidence intervals (CI).
IL: Interleukin, IFNγ: Interferon gamma, TNFα: Tumour necrosis factor alpha, n: Number.

With the exception of IL-2, there was no significant difference in the cytokine levels in the controlled group compared with the uncontrolled group of asthma patients (**group 1**) (Table 7.3; Figures 7.4, 7.5, 7.6). IL-2 was significantly higher in the uncontrolled group with a median of 26.1 FU (95% CI 20.06 - 33.95), compared with the controlled group median of 19.19 FU (95% CI 16.57- 22.23); $p = 0.02$ (Table 7.3; Figure 7.5). There was a trend towards a difference in IL-6 but this did not reach significance ($p = 0.07$).

More strikingly, for rest of the Th2 cytokines measured (i.e. IL-4, IL-5 and IL-13) there was no significant difference when comparing the controlled versus uncontrolled asthmatic patients (Table 7.3; Figure 7.6).

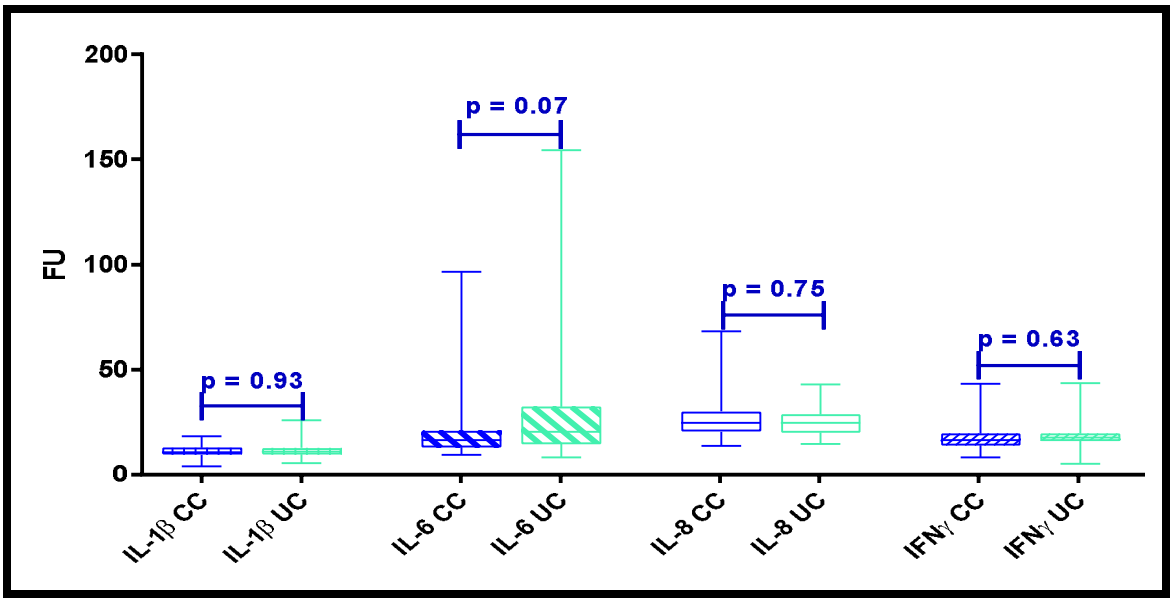

Figure 7.4: Th1 cytokines (**IL-1β, IL-6 and IFNγ**) comparing controlled and uncontrolled asthmatic patients (**group 1**)
FU: Florescent intensity unit, IL: Interleukin, IFNγ: Interferon gamma, CC: Controlled, UC: Uncontrolled.
Wilcoxon rank sum test.

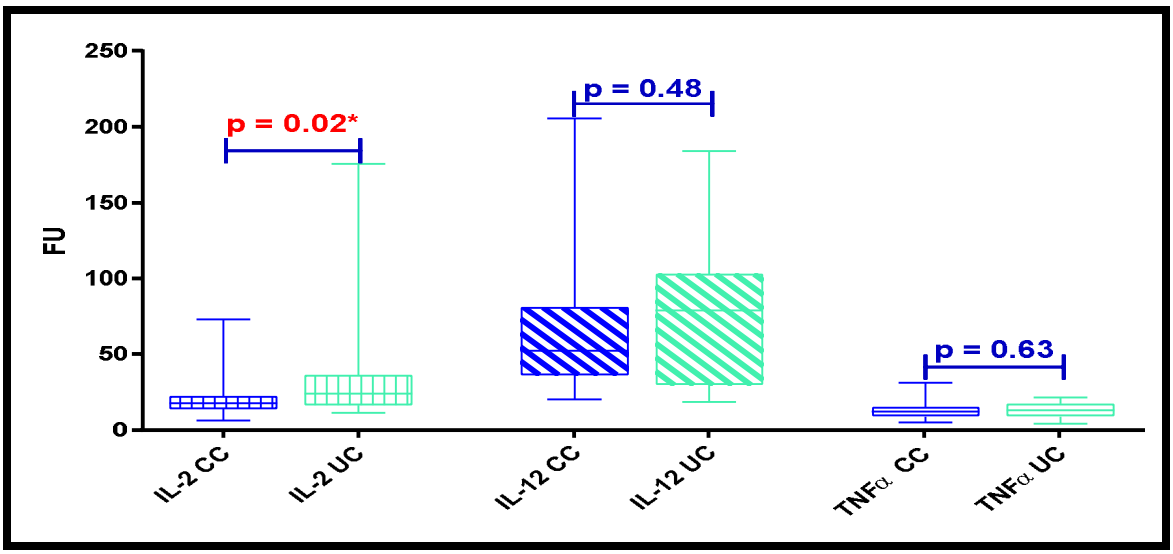

Figure 7.5: Th1 cytokines (**IL-2, IL-12 and TNFα**) comparing controlled and uncontrolled asthmatic patients (**group 1**)
*p values reported as significant are depicted in red.
FU: Florescent intensity unit, IL: Interleukin, TNFα: Tumour necrosis factor alpha, CC: Controlled, UC: Uncontrolled.
Wilcoxon rank sum test.

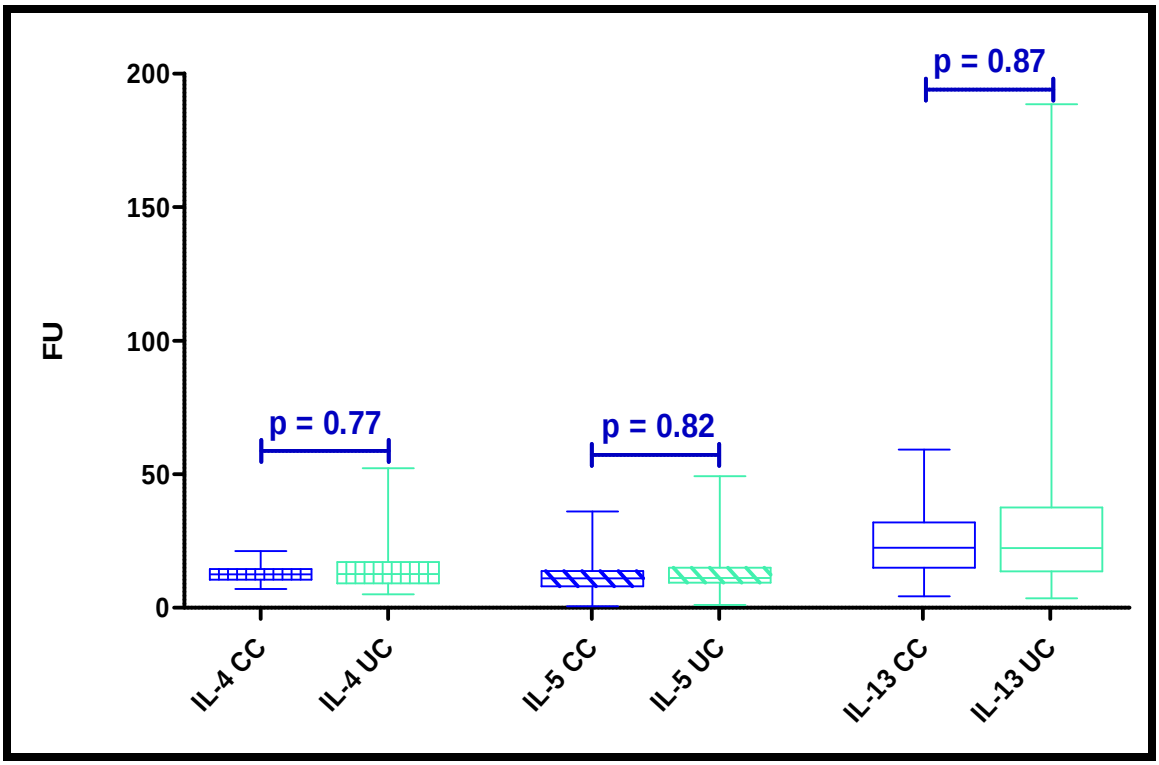

Figure 7.6: Th2 cytokines (**IL-4, IL-5 and IL-13**) comparing controlled and uncontrolled asthmatic patients (**group 1**)
FU: Florescent intensity unit, IL: Interleukin, CC: Controlled, UC: Uncontrolled. Wilcoxon rank sum test.

Furthermore, there was no clinically relevant correlation between any of the biomarkers measured and the ACQ score. Although the correlation between the IL-2 and the ACQ score was significant ($p = 0.02$), with $r = -0.4$, the result was clinically irrelevant (Figures 7.7a, 7.7b).

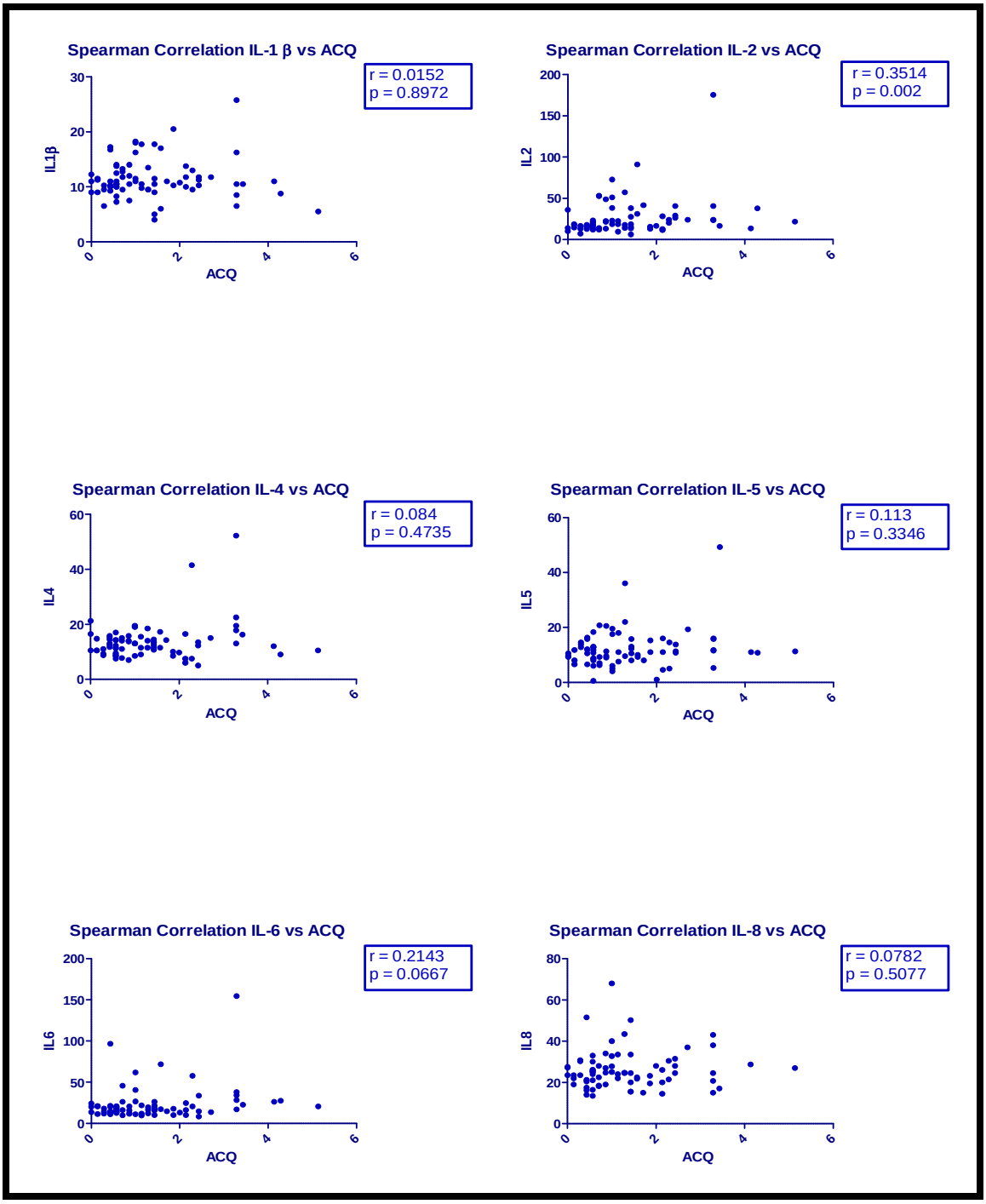

Figure 7.7a: Correlation between the Th1 cytokines levels and the ACQ score in the study population

IL: Interleukin, ACQ: Asthma Control Questionnaire. Spearman rank correlation.

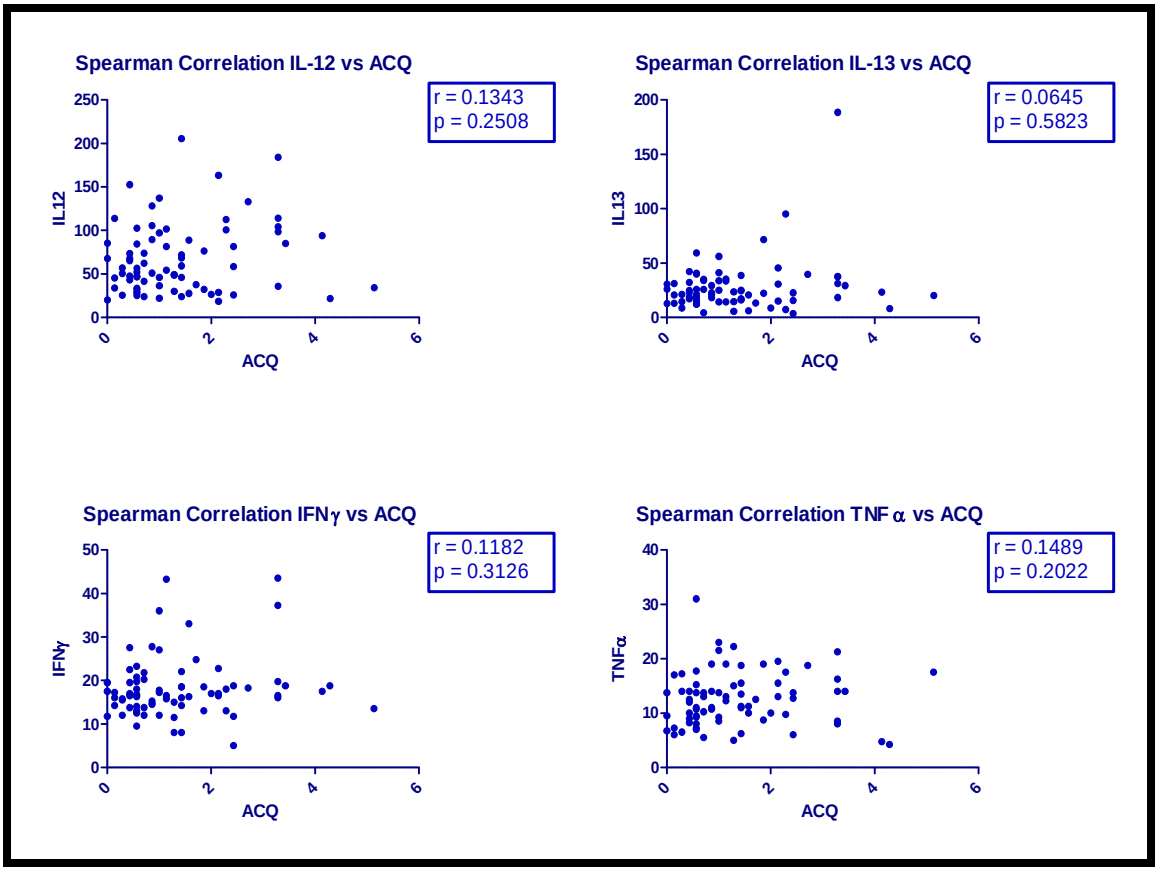

Figure 7.7b: Correlation between the Th2 cytokines levels and the ACQ score in the study population
IL: Interleukin, IFNγ: Interferon gamma: TNFα: Tumour necrosis factor alpha: ACQ: Asthma Control Questionnaire. Spearman rank correlation.

When comparing the totally controlled (TC), well-controlled (WC) and uncontrolled (UC) groups (**group 2**), the only significant finding was that IL-2 was significantly higher in the uncontrolled group with a median of 26.1 FU (95% CI 20.06 - 33.95), compared with the totally controlled group with a median of 17.13 FU (95% CI 14.65 - 20.04); p = 0.003 (Table 7.4; Figures 7.8, 7.9, 7.10). TNFα and IL-8 were higher in the uncontrolled group when compared with the totally controlled group, but this difference was not significant after the application of the Bonferroni correction (p = 0.05 and p = 0.07, respectively) (Table 7.4; Figures 7.8, 7.9).

Table 7.4: Th1 and Th2 cytokine levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Biomarker[+]	Totally controlled n=30	Well-controlled n=21[1]	Uncontrolled n=24	Totally controlled vs Well-controlled[2,3] (p values)	Totally controlled vs Uncontrolled[2,3] (p values)	Well-controlled vs Uncontrolled[2,4] (p values)
IL-1β	10.77 (9.95 - 11.66)	11.02 (9.20 - 13.18)	11.04 (9.52 - 12.81)	0.46	0.73	0.79
IL-2	17.13 (14.65 - 20.04)	22.56 (17.05 - 29.87)	26.1 (20.06 - 33.95)	0.047	**0.003***	0.45
IL-4	11.85 (10.76 - 13.04)	12.93 (11.51 - 14.53)	13.1 (10.43 - 16.46)	0.25	0.53	0.80
IL-5	9.56 (7.52 - 12.16)	11.65 (9.17 - 14.8)	10.42 (7.78 - 13.95)	0.34	0.55	0.73
IL-6[3]	17.74 (15.02 - 20.96)	17.62 (14.09 - 22.01)	22.66 (17.09 - 30.04)	0.73	0.11	0.12
IL-8[4]	23.15 (20.9 - 25.64)	28.01 (23.69 - 33.1)	24.09 (21.21 - 27.36)	0.07	0.58	0.18
IL-12	49.84 (41.41 - 59.98)	63.09 (48.46 - 82.14)	59.78 (44.48 - 80.36)	0.15	0.23	0.87
IL-13	20.71 (16.90 - 25.37)	22.41 (17.83 - 28.17)	22.45 (15.36 - 32.81)	0.60	0.75	0.93
IFNγ	16.36 (14.98 - 17.87)	17.0 (14.05 - 20.56)	17.88 (14.99 - 21.33)	0.94	0.26	0.30
TNFα	10.71 (9.27 - 12.37)	13.03 (10.85 - 15.63)	11.77 (9.79 - 14.16)	0.05	0.19	0.47

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
1: Cytokine analyses was of 21 well-controlled patients as one result was totally aberrant and discarded.
2: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
3: IL-6 analysis of one patient in the totally controlled group (n=29) was not measurable by bioplex.
4: IL-8 analysis of one additional patient in the uncontrolled group (n=23) was not measurable by bioplex.
The results are reported as medians together with the 95% confidence intervals (CI).
IL: Interleukin, IFNγ: Interferon gamma, TNFα: Tumour necrosis factor alpha, n: Number

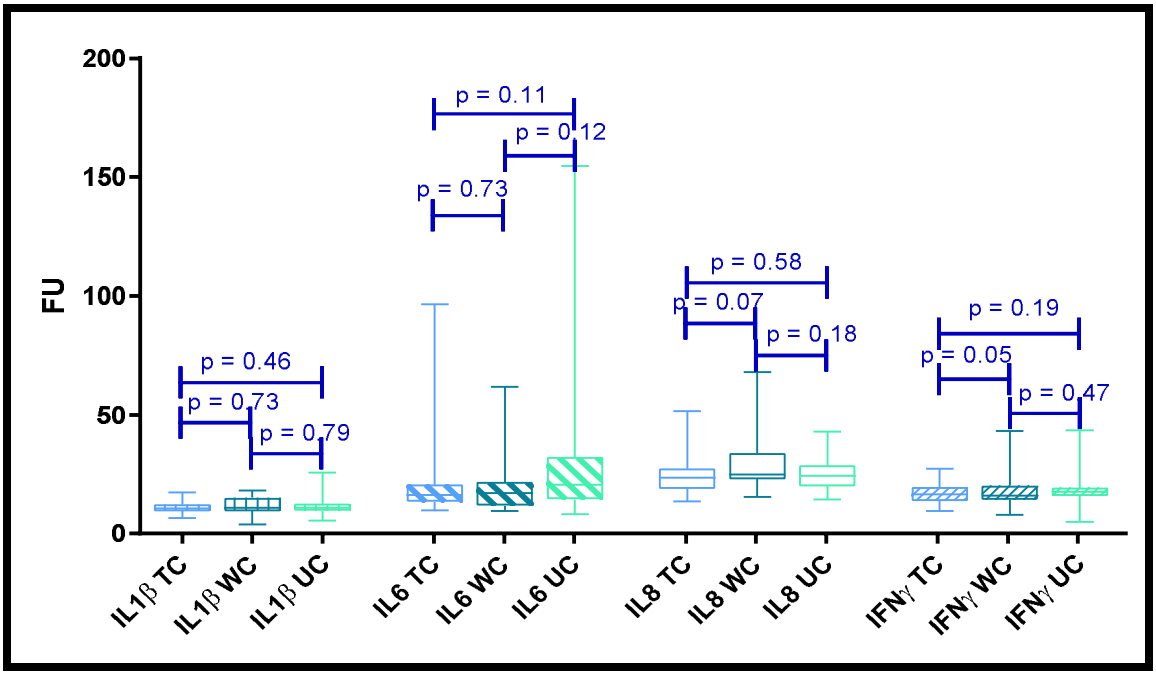

Figure 7.8: Th1 cytokines (**IL-1β, IL-6, IL-8 and IFNγ**) comparing totally controlled, well-controlled and uncontrolled patients (**group 2**)
FU: Florescent intensity unit, IL: Interleukin, IFNγ: Interferon gamma, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

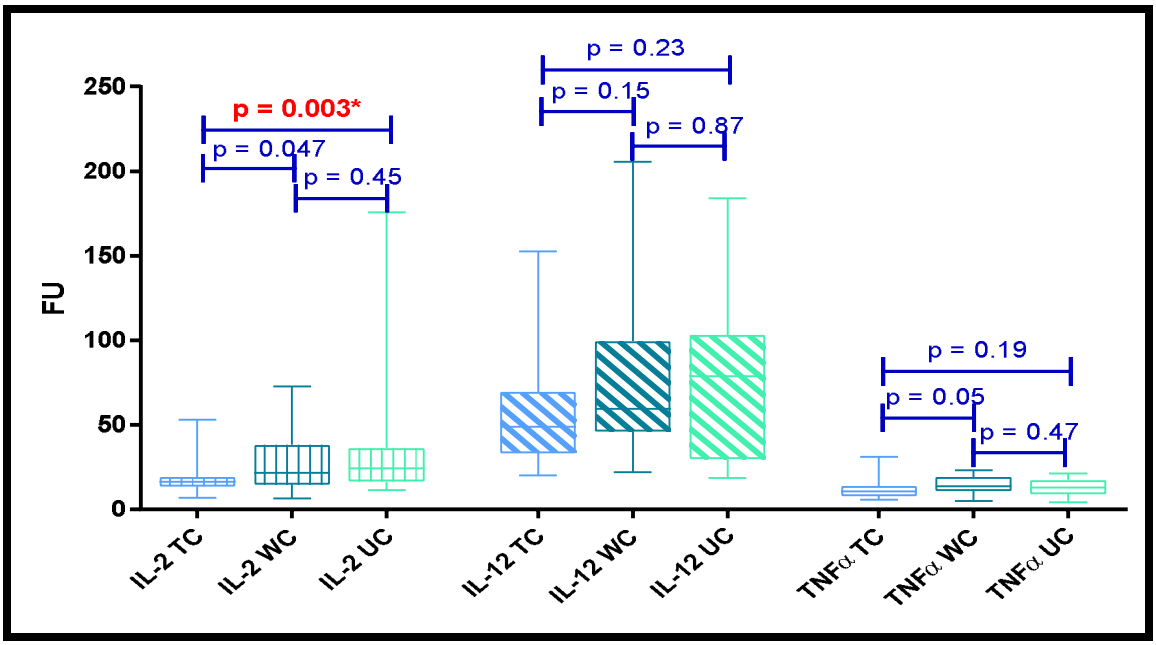

Figure 7.9: Th1 cytokines (**IL-2, IL-12 and TNFα**) comparing totally controlled, well-controlled and uncontrolled patients (**group 2**)
* p values reported as significant are depicted in **red**.
FU: Florescent intensity unit, IL: Interleukin, TNFα: Tumour necrosis factor alpha, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

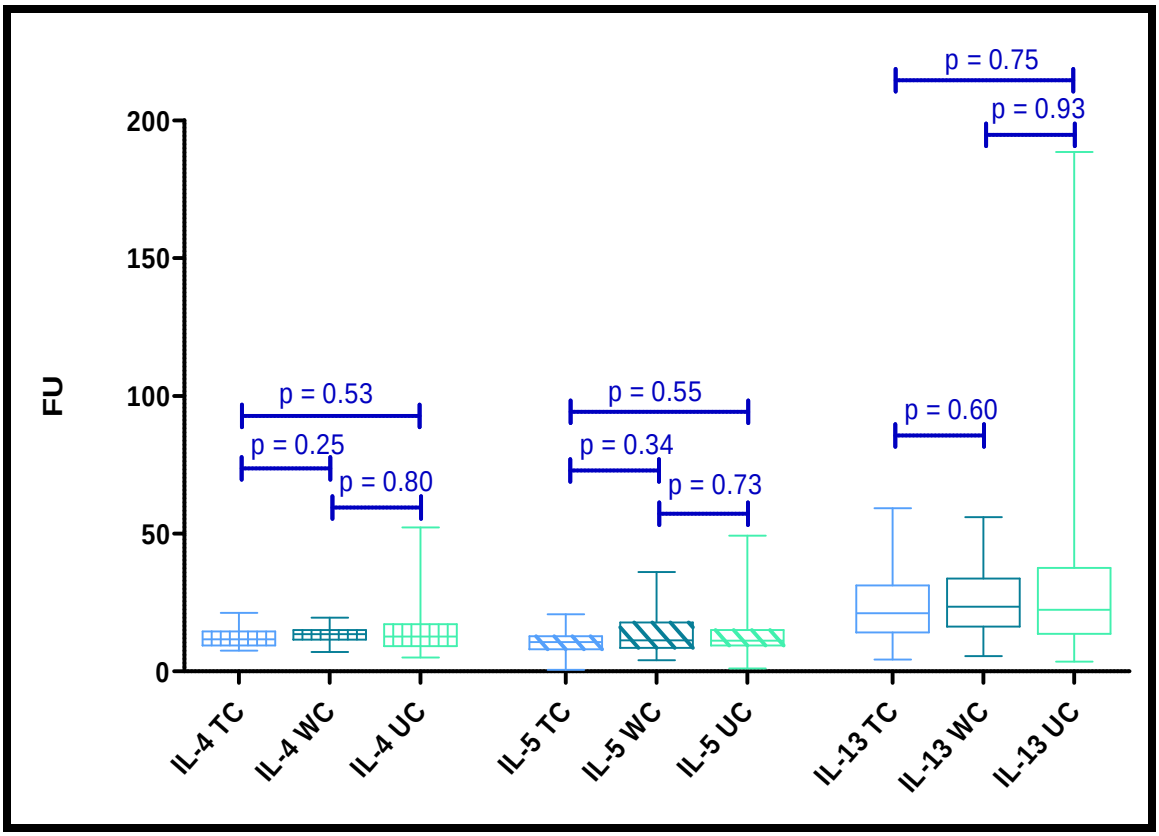

Figure 7.10: Th2 cytokines (**IL-4, IL-5 and IL-13**) comparing totally controlled, well-controlled and uncontrolled patients (**group 2**)
FU: Florescent intensity unit, IL: Interleukin, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

A scatter-dot plot of the Th1 and Th2 cytokines measured for the **group 2** analysis (totally controlled versus well-controlled versus uncontrolled asthmatic patients) is depicted in Figure 7.11.

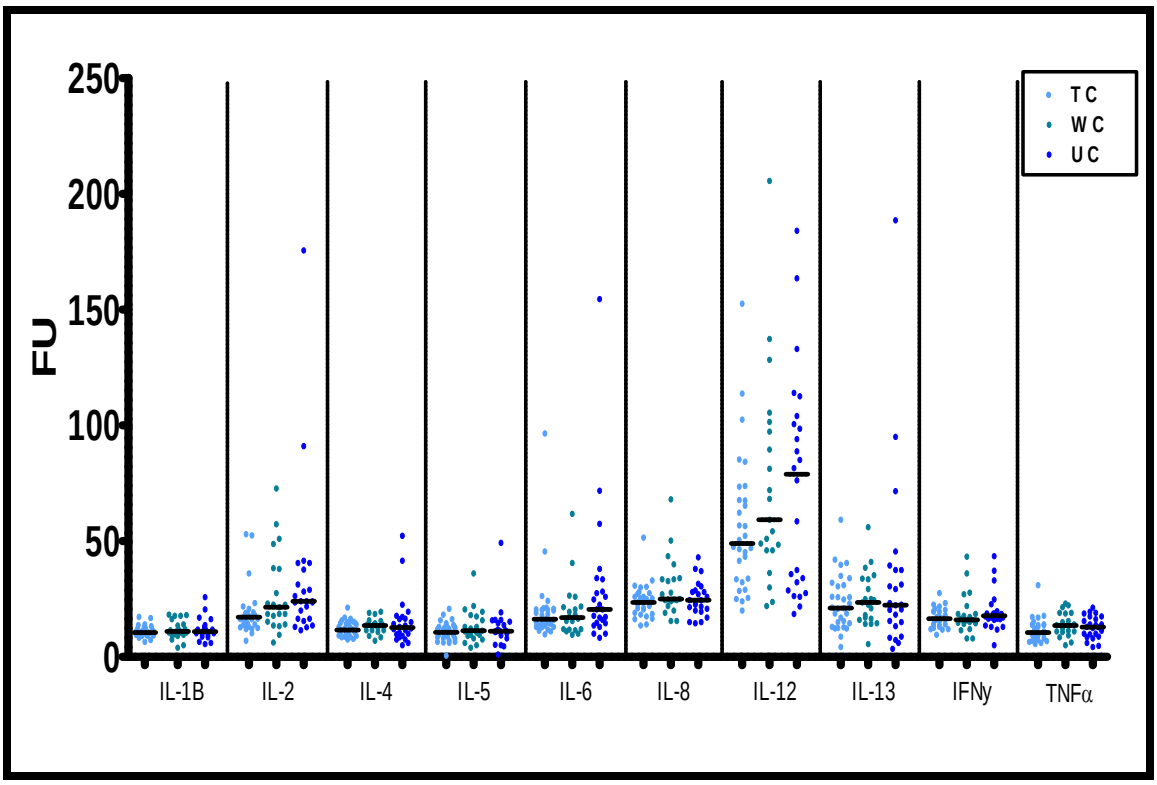

Figure 7.11: Scatter-dot plot of all the **Th1 and Th2** cytokines measured comparing totally controlled, well-controlled and uncontrolled patients (**group 2**)
Scatter-dot plot with bold line representing the median.
IL: Interleukin, IFNγ: Interferon gamma, TNFα: Tumour necrosis factor alpha, FU: Florescent intensity units, TC: Totally controlled, WC: Well-controlled, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

A logistic regression model was used to analyse the relationship between the biomarkers grouped into Th1 predominant cytokines (IL-2, IL-12, and IFNγ) or Th2 predominant cytokines (IL-4, IL-5, and IL-13), and the controlled or uncontrolled asthmatic patients (**group 1**). Among the patients with either controlled or uncontrolled asthma, the multivariate analysis of the Th1 cytokines as a group, or the Th2 cytokines as a group, did not prove to be a significant predictor of asthma control as defined by the ACQ (Table 7.5, 7.6).

Table 7.5: Logistic regression model analysing the relationship between the main serum Th1 cytokine (i.e. **IL-2, IL-12 and IFNγ**) levels and controlled or uncontrolled asthmatic patients (**group 1**)

ACQ CC / UC	Odds Ratio	Std. Err.	Z	P	[95% CI]	
IL-2	1.025	0.017	1.50	0.134	0.992	1.059
IL-12	1.006	0.007	0.84	0.403	0.993	1.019
IFNγ	0.993	0.440	-0.16	0.870	0.910	1.083

ACQ: Asthma Control Test, CC: Controlled, UC: Uncontrolled, IL: Interleukin, IFNγ: Interferon gamma, Std. Err.: Standard error, CI: Confidence interval, FU: Fluorescent intensity units.

Table 7.6: Logistic regression model analysing the relationship between the main serum Th2 cytokine (i.e. **IL-4, IL-5 and IL-13**) levels and controlled or uncontrolled asthmatic patients (**group 1**)

ACQ CC / UC	Odds Ratio	Std. Err.	Z	P	[95% CI]	
IL-4	1.061	0.044	1.43	0.152	0.978	1.152
IL-5	1.009	0.037	0.25	0.799	0.940	1.084
IL-13	1.018	0.014	1.30	0.192	0.991	1.046

ACQ: Asthma Control Test, CC: Controlled, UC: Uncontrolled, IL: Interleukin, Std. Err.: Standard error, CI: Confidence interval.

Finally, as most of the medications asthmatic patients use are inherently anti-inflammatory, a comparison was made between whether or not these anti-inflammatory medications were being used by the patients, and the serum concentration of the biomarkers. The reason for this analysis was to determine whether the medications the patients were prescribed had any effect on the levels of the inflammatory biomarkers measured. This comparison revealed no difference between whether or not anti-inflammatory medications were being used by the patients and the concentrations of the biomarker measured (Table 7.7).

The legend for table 7.7 has been included in a text box here as there is no space for it on page 141.

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
Wilcoxon rank sum test.* = $p < 0.05$.
Biomarker analysis was of 51 controlled patients as one result was totally aberrant and discarded.
1: IL-6 analysis of one patient in the totally controlled group (n=29) was not measurable by bioplex.
2: IL-8 analysis of one additional patient in the uncontrolled group (n=23) was not measurable by bioplex.
Budesonide hd: high-dose between 800 and 1600 µg/day; Budesonide md: moderate-dose ≤ 800 µg/day
Prednisone ld: low-dose ≤ 7.5 mg/day, µg/day: Microgram per day, mg/day: Milligram per day, CRP: C-reactive protein, SAAP: Serum amyloid associated protein, IL: Interleukin, IFNγ: Interferon gamma, TNFα: Tumour necrosis factor alpha.

Table 7.7: The concentrations of CRP, SAAP and the Th1 and Th2 cytokines comparing levels in patients using, versus not using anti-inflammatory medications

Biomarker[+]	Montelukast			Budesonide md: ≤ 800 µg/day			Budesonide hd: < 800 and ≤ 1600 µg/day			Prednisone ld: ≤ 7.5 mg/day		
	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value
CRP	2783.24 (1058.47-7318.57)	1785.90 (1260.22-2530.85)	0.27	1780.21 (1221.43-2594.63)	2734.16 (1444.12-5176.57)	0.39	2934.06 (1493.56-5763.92)	1766.25 (1219.04-2559.09)	0.29	2546.20 (601.55-10777.39)	1874.30 (1333.38-2634.65)	0.54
SAAP	1435.68 (438.20-4703.77)	675.53 (463.62-984.31)	0.20	704.86 (475.82-1044.14)	1130.50 (397.36-3216.28)	0.46	1081.36 (347.86-3361.51)	716.90 (486.39-1056.64)	0.59	842.05 (117.81-6018.86)	762.76 (525.21-1107.75)	0.99
IL-1	9.46 (7.97-11.22)	11.26 (10.40-12.19)	0.15	11.20 (10.39-12.06)	9.82 (7.84-12.30)	0.19	9.64 (7.57-12.27)	11.22 (10.42-12.07)	0.11	9.79 (7.59-12.63)	11.05 (10.24-11.93)	0.57
IL-2	25.72 (16.59-39.86)	20.33 (17.77-23.26)	0.37	20.30 (17.69-23.30)	25.43 (17.08-37.85)	0.11	25.74 (16.70-39.66)	20.33 (17.75-23.27)	0.13	33.90 (15.86-72.47)	20.17 (17.78-22.90)	0.06
IL-4	13.00 (10.69-15.81)	12.44 (11.30-13.70)	0.50	12.49 (11.29-13.81)	12.76 (10.98-14.84)	0.60	12.48 (10.68-14.59)	12.55 (11.36-13.86)	0.87	13.59 (10.10-18.30)	12.43 (11.35-13.62)	0.43
IL-5	12.10 (8.83-16.58)	10.06 (8.54-11.85)	0.72	9.77 (8.31-11.47)	13.60 (9.84-18.79)	0.07	13.85 (9.76-19.65)	9.78 (8.35-11.46)	0.05	15.36 (9.08-25.97)	9.98 (8.58-11.60)	0.14
IL-6 [1]	20.27 (13.26-31.00)	18.94 (16.64-21.56)	0.72	18.98 (16.45-21.91)	20.08 (15.24-26.45)	0.84	20.46 (15.16-27.61)	18.93 (16.44-21.80)	0.77	27.33 (13.13-56.89)	18.47 (16.35-20.88)	0.06
IL-8 [2]	24.33 (19.83-29.85)	24.82 (22.90-26.89)	0.81	23.98 (22.25-25.85)	28.62 (22.65-36.14)	0.17	28.28 (21.91-36.49)	24.11 (22.38-25.97)	0.29	28.59 (19.85-41.19)	24.42 (22.65-26.34)	0.22
IL-12	65.29 (44.78-95.20)	54.73 (47.17-63.51)	0.33	58.38 (50.06-68.08)	48.68 (35.40-66.94)	0.33	51.24 (37.01-70.94)	57.59 (49.39-67.15)	0.58	62.07 (30.75-125.30)	55.88 (48.58-64.28)	0.77
IL-13	27.14 (15.51-47.52)	20.74 (17.85-24.09)	0.20	22.01 (18.54-26.13)	20.52 (14.17-29.73)	0.77	21.35 (14.42-31.63)	21.81 (18.40-25.84)	0.92	28.03 (11.43-68.71)	21.17 (18.19-24.62)	0.70
INFγ	17.29 (13.89-21.52)	16.96 (15.50-18.55)	0.72	17.04 (15.54-18.69)	16.88 (13.91-20.49)	0.82	16.47 (13.45-20.18)	17.13 (15.64-18.77)	0.87	19.14 (13.42-27.32)	16.81 (15.44-18.30)	0.42
TNFα	13.45 (10.29-17.57)	11.32 (10.23-12.53)	0.06	11.58 (10.44-12.85)	12.01 (9.29-15.53)	0.56	11.65 (8.89-15.28)	11.66 (10.52-12.93)	0.82	11.95 (6.76-21.12)	11.63 (10.59-12.78)	0.43

7.6 Discussion

The current asthma guidelines recommend that treatment decisions in asthma should be made on the basis of symptoms, with or without using peak flow and spirometry (8, 373). The treatment algorithm advocates a stepwise approach to the management of patients with asthma and recommends adding therapy to the uncontrolled patient to achieve asthma control. More importantly, the guidelines also recommend a decrease in the usage or dosage of medication in the well-controlled asthmatic patient who has maintained symptom control for the last 3 months (8).

It has been well established that within a population of patients with asthma both symptoms and airway obstruction correlate poorly with the presence and severity of airway inflammation (66, 351). However, when doses of inhaled corticosteroids (ICS) medication, were reduced in stable asthmatic patients, without monitoring for changes in markers of airway inflammation, there was an increase in exacerbation events in these patients (324). Hence, a strategy where the patient with persistent airway inflammation can be correctly identified (inflammometry) would potentially prevent over treatment, but more significantly, under treatment (326, 327).

7.6.1 The association between CRP and asthma control

In the present study, there was a significant association between CRP, and the level of asthma control, as defined by the ACQ. CRP levels were higher in the uncontrolled group compared with the controlled group of asthmatic patients (**group 1** analysis); $p = 0.03$ (Table 7.1; Figure 7.1.). When this analysis was repeated comparing CRP and totally controlled, well-controlled and uncontrolled asthmatic patients, there was no significant difference in CRP values between these group of patients (**group 2** analysis). There was also poor correlation between the CRP levels and the ACQ; $r = 0.212$ (Table 7.1; Figure 7.2).

There have been several publications confirming the utility of CRP in assessing patients with asthma, but to date, none of the publications thus far has assessed the utility of CRP, in a group of asthmatic patients, in relationship to their level of asthma control as categorised by the ACQ. Takemura *et al*. (262), reported an association between CRP and decreased pulmonary function in steroid naive asthma patients. However, this association disappeared when the patients were treated with ICS (262). Qian *et al*. (374) reported a significantly higher hs-CRP in severe persistent asthmatics when compared with healthy controls ($p < 0.0001$). In that study there was a negative, but not a clinically significant correlation between serum hs-CRP and the FEV1

expressed as a percentage of predicted, in the mild persistent asthmatic (r = -0.23; p = 0.032) and the moderate persistent asthmatic (r = -0.33; p = 0.007) (374). Soferman *et al.* (375) reported a significant inverse correlation between hs-CRP and FEV1, with higher FEV1 values being significantly correlated with lower hs-CRP levels (p = 0.049). Sigari *et al.* (376) reported a significantly higher median hs-CRP levels of 11.5 mg/l in a totally controlled asthma group when compared with a healthy control group with a median hs-CRP level of 4.32 mg/l ($p < 0.05$). However, there was no correlation between hs-CRP and the level of asthma control as defined by the ACT (p = 0.27). These findings by Sigari *et al.* (376) were confirmed by Kilic *et al.* (377) who documented significantly higher hs-CRP levels in an uncontrolled asthmatic group (ACT < 19) compared with a controlled asthmatic group (ACT ≥20); p = 0.02. In that study there was also a strong inverse correlation between the hs-CRP and ACT (r = -0.91; $p < 0.001$), but no correlation between hs-CRP and FEV1 (r = -0.11; p = 0.54), alluding to the possibility that hs-CRP was an independent marker of asthma control (377).

Studies by Kilic *et al.* (377) and Monadi *et al.* (378) have suggested cut-off values for hs-CRP levels below 3 ug/ml and below 2.15 ug/ml respectively, which could be used as a biomarker in asthmatic patients to assess asthma control. In the present study, the number of control subjects recruited was not large enough to determine a specific cut-off value of CRP using receiver operating characteristic curve (ROC) analyses, which could discriminate between the various asthma control groups defined by the ACQ.

Significant heterogeneity in the published literature regarding CRP and asthma control exists, mirroring the heterogeneity of asthma as a disease process. This has limited the widespread use of CRP as a biomarker in asthma. One of the possible reasons for the discrepancies in the published literature is that the inclusion criteria of the patients differed widely between the different publications (379, 380). In conclusion, when strict exclusion criteria are applied, as in the present study, CRP may prove to be a valuable biomarker of airway inflammation in the uncontrolled asthmatic patient.

7.6.2 The association between SAAP and asthma control

The value of SAAP in the assessment of airway inflammation has been reported before, but the literature supporting the utility of SAAP in asthma is very limited (381). In the present study there was no significant association between SAAP and the level of asthma control as defined by the ACQ for either the controlled versus the uncontrolled asthmatic patients (**group 1**) as

well as for the comparison between totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**). Furthermore, there was also a poor correlation between SAAP and the ACQ.

The initial study on SAAP in asthmatic patients, was a population based study by Jousilahti *et al.* (332) published in 2002, documenting a significant association between age-adjusted asthma prevalence and SAAP quartiles ($p < 0.001$). However, that study was unique in that it only included men, and most notably, smoking and obesity were not exclusion criteria (332). Although smoking did not weaken the observation that serum concentrations of SAAP correlated with the prevalence of asthma, central obesity did, making it difficult to draw any conclusions with regard to the current study where obesity was an exclusion criterion. In another study by Buyukozturk *et al.*(265), SAAP levels measured were significantly higher in an asthma patient group when compared with a healthy control group ($p = 0.02$), but similar to the current study, there was no correlation between the SAAP levels and the patient's symptoms (265). Ozseker *et al.* (382) also reported higher SAAP concentrations in sputum and serum in treatment naive asthmatics, when compared with heathy controls, which did not change after six months of treatment with inhaled corticosteroids (ICS) ($p = 0.02$). Notably, there was no significant difference between the SAAP values pre-treatment and 6 months post-treatment, limiting its utility to monitor response to therapy (382).

In conclusion, the findings from previous studies suggest that SAAP may be a good biomarker for the identification of inflammation in patients with asthma when comparted to healthy controls (381, 382). However, as documented in the present study, its utility as a tool for identifying airway inflammation along the continuum of asthma control remains unclear.

7.6.3 The association between serum cytokines and asthma control

While the role of cytokines in asthma is clear, there have been no publications describing the association of the Th1 and Th2 cytokine concentrations in serum with a pre-defined level of asthma control as defined by the ACQ. The results from this study showed that of all the Th1 and Th2 cytokines measured, the only cytokines that demonstrated a difference or trend towards a significant difference for the **group 1** (controlled versus uncontrolled asthmatic patients) and **group 2** (totally controlled versus well-controlled and versus uncontrolled asthmatic patients) analyses, were IL-2, IL-6, IL-8 and TNFα. They will be discussed further in the following paragraphs.

7.6.3.1 IL-2

IL-2 was significantly higher in the uncontrolled group with a median of 26.1 FU (95% CI 20.06 - 33.95), compared with the controlled group with a median of 19.19 FU (95% CI 16.57 - 22.23); p = 0.02 (Table 7.3; Figure 7.5). In the **group 2** analysis, IL-2 was significantly higher in the uncontrolled group with a median of 26.1 FU (95% CI 20.06 - 33.95), when compared with the totally controlled group with a median of 17.13 FU (95% CI 14.65 - 20.04); p = 0.003 (Table 7.4; Figure 7.9).

Elevated IL-2 levels in patients with severe asthma defined by GINA guidelines (8) compared with patients with mild asthma have been previously reported by Rogala *et al*. (383). Unfortunately, those results were confounded by the fact that 40.3% of the severe asthmatic patients were former smokers and 14.3% current smokers, while 30.5% of the mild asthmatics were former smokers and 24.8% current smokers. This raises the possibility that IL-2 levels may have been suppressed in the mild asthmatic group by smoking (384, 385), or that the severe asthmatic patients developed an element of Asthma-COPD overlap (ACQ) (386-388). As smoking was an exclusion criterion in the present study those finding are difficult to compare to the findings reported in the present study. Ceyhan *et al*. (389) measured serum IL-2 levels in a group of asthmatic patients characterised by levels of asthma severity and reported mean IL-2 levels of 42.7 ± 21.1 pg/ml in the asthmatic group and 30.3 ± 2.4 pg/ml in the control group, but this difference was not significant. However, the entire cohort of patients studied was very small and included 6 mild asthmatics, 5 moderate asthmatics, 6 severe asthmatic and 5 control subjects (i.e. 17 patients and 5 controls), and thus, may not have been appropriately powered to yield a significant difference.

In another study by Vizmanos-Lamotte *et al.* (338), on paediatric patients, there were higher sputum IFNγ, IL-2, and IL-8 concentrations in atopic and non-atopic asthmatics, when compared with healthy controls ($p < 0.05$), but no correlation existed between the cytokine levels and asthma control. The IL-2 level was higher in the sputum of the atopic asthmatic group compared with the non-atopic asthmatic group ($p < 0.05$), but again, in this study a surprisingly low percentage of the patients were on appropriate therapy, with only 38% of the atopic asthmatic group and 24% of the non-atopic asthmatic group on any ICS treatment (338). The only other study documenting any association between asthma control and IL-2 was a publication by Boonpiyathad *et al*. (390), in which the concentration of IL-2 was measured in exhaled breath condensate in patients with asthma. In that study patients were categorised to

three groups; namely healthy controls, allergic asthmatic patients and non-allergic asthmatic patients. There was a signification correlation between IL-2 level and the ACT score (r = -0.684; p = 0.007). However, all of the patients in that study were steroid naive making the results difficult to extrapolate to a real world scenario and to the present study, where the majority of patients with asthma were on some form of ICS therapy (390).

Unfortunately, the latter two studies measured sputum IL-2 levels in patients in whom less than 40% were using ICS therapy, and the first study was confounded by 54% of the patient cohort being smokers, making the finding of these studies difficult to compare with this present study. Nonetheless, measuring serum concentrations of IL-2 has the potential to be used as a surrogate biomarker for asthma control. However, this needs to be confirmed in a larger study.

7.6.3.2 IL-6

In the present study there was a trend towards a difference for IL-6 when the controlled group was compared with the uncontrolled group of asthmatic patients (**group 1** analysis) (p = 0.07). Values for the controlled group were a median of 17.69 FU (95% CI 15.55 - 20.13) which appeared to be lower than in the uncontrolled group with a median of 22.66 FU (95% CI 17.09 - 30.04), but this difference was not statistically significant with p = 0.07 (Table 7.3; Figure 7.4). For the **group 2** analysis, there was no significant difference in the IL-6 levels in any of the subgroup comparisons (TC vs UC; p = 0.11), (TC vs WC; p = 0.73); and (WC vs UC; p = 0.12) (Table 7.4, Figure 7.8).

IL-6 is a unique cytokine in that it is produced by leucocytes and lung epithelial cells, which are increasingly becoming more important in the pathogenesis of asthma (44, 159). IL-6 plays an important role in determining the type of adaptive immune response, primarily in the differentiation of effector CD4 T helper cells, as well as inducing CRP transcription (391, 392). A study by Neveu *et al.* (393), reported elevated IL-6 concentrations in sputum of asthmatic patients when compared with healthy controls (p < 0.01). While the results from the current study did not show any significant difference between serum IL-6 values and the categories of asthma control as defined by the ACQ, there was a trend for the IL-6 to be higher in the uncontrolled asthmatic patient group. Therefore, IL-6 as a biomarker for airway inflammation needs to be verified within a larger cohort of asthma patients, categorised by their level of asthma control, using an objective asthma scoring system.

7.6.3.3 IL-8 and TNFα

The only other cytokines measured, where there appeared to be a trend towards a difference in the categories of asthma control as defined by the ACQ, were IL-8 and TNFα. For the **group 1** analysis, there was no difference for IL-8 and TNFα ($p = 0.75$ and $p = 0.63$, respectively) (Table 7.3; Figures 7.4, 7.5). However, for the **group 2** analysis (totally controlled versus well-controlled and versus uncontrolled), the levels in the well-controlled group appeared to be higher than in the totally controlled group for IL-8 and TNFα ($p= 0.05$ and $p = 0.07$ respectively) (Table 7.4; Figures 7.8, 7.9). This trend was non-significant when the Bonferroni correction was applied.

IL-8 and TNFα are important cytokines in the pathogenesis of asthma but importantly, these two cytokines play a more significant role in the immunopathogenesis of patients with severe asthma (162, 163). IL-8 is a potent neutrophil chemotactic cytokine, whereas TNFα functions predominantly as a Th1 cytokine in promoting airway inflammation and fibrosis (111). In a study by Meyer *et al.* (394), 191 patients who were admitted and treated for asthma had higher serum IL-8 levels pre-treatment when compared to the post treatment levels ($p < 0.05$). Another study by Silvestri *et al.* (395), found that serum IL-8 and TNFα levels were higher in severe asthmatics as compared with mild and moderate asthmatics.

In conclusion, although the data from the present study do not confirm a definite role for the cytokines IL-8 and TNFα, just as IL-6 above, in the assessment of the different categories of asthma control, these cytokines need to be studied in a larger cohort to determine if they have any value in the assessment of inflammation in asthmatic patients.

7.6.3.4 Multivariate analysis of the predominant Th1 and Th2 cytokines as a group compared to asthma control as defined by the ACQ

As discussed above, when the cytokines were analysed individually as to their relationship to asthma control, only IL-2 showed a significant difference between the controlled control and uncontrolled asthmatic patients. One of the possible mechanisms that may explain the above finding is that IL-2 binds to the IL-2 receptor on regulatory T (Treg) cells and upregulates its transcription of effector cytokines, which include TGFβ (48, 103). This upregulation and competitive binding of IL-2 by Treg cells leads to an IL-2 deprivation-mediated apoptosis of other effector T cells, which include the Th1 and Th2 subpopulations, as IL-2 functions as a autocrine cytokine to activate and upregulate all T cell subpopulations (25, 396). Thus, we

hypothesise that the elevated IL-2 levels in the uncontrolled asthmatic patient upregulates Treg cell function by preferentially binding to them. These upregulated Treg cell, via the transcription of IL-10 and TGFβ, then downregulate the transcription of the other cytokines (i.e. IL-4, IL-5, IL-12, IL-13 and IFNγ) produced by both the Th1 and Th2 cells (100).

To explore this hypothesis further, the relationship of the cytokines as a group (i.e. either Th1 (IL-2, IL-12 and IFNγ) or Th2 (IL-4, IL-5 and IL-13)) were compared to categories of asthma control, as defined by the ACQ, using logistic regression. This multivariate analysis indicated that even when analysing the cytokines as a group, there was no relationship between either the group of Th1 or the group of Th2 cytokines and asthma control (Table 7.5; 7.6). Therefore, it is possible that the Th1-Th2 cytokine axis in asthma is suppressed by an upregulated Treg cell function, as a consequence of the elevated IL-2 levels so that measurement of the systemic levels of the predominant Th1 and Th2 cytokines could not be shown to be related to the categories of asthma control defined by the ACQ (396). This relationship between the Treg cells and the Th1 and Th2 cells has been summarised in Figure 2.1 previously.

7.6.4 The association between serum cytokines and medications used

Finally, to ascertain whether the concentration of any the serum biomarkers measured in this present study were affected by any of the anti-inflammatory therapies the patients were using, a comparison was made between whether or not these anti-inflammatory medications were being used by the patients, and the serum concentration of the biomarkers. The reason for this analysis was to determine whether the medications the patients were prescribed had any effect on the levels of the inflammatory biomarkers measured.

Medication influencing serum cytokine levels have been previously documented in studies by Koto *et al.* (397) and Liu *et al*. (398). In these studies quoted, the levels of cytokines were measured before and after an acute asthmatic exacerbation in patients who were treated with systemic steroids. The cytokine concentrations were found to be lower in the treated cohort when compared with the pre-admission values (397, 398). However, in the present study there were no differences in the concentrations of the serum cytokines measured and the anti-inflammatory medications that the patients were using (Table 7.7). This finding was corroborated in a study by Puklesheim *et al*. where no association between asthma therapy and serum cytokine levels was observed (399).

7.7 Conclusion

The results of the present study support the hypothesis that while asthma is primarily an inflammatory airway disease, the inflammation in the airways is not associated with significant changes in the measured values of serum biomarkers. With the exception of CRP and IL-2, none of the serum biomarkers measured were associated with asthma control as defined by the ACQ. The reasons for this may include the fact that asthma is a complex and heterogeneous disease, consisting of many endotypes probably regulated by Treg cells (100, 114). Furthermore, it might be that the measured levels of specific serum cytokines differ from those measured in bronchoalveolar lavage fluid, or that the measured concentrations of these cytokines in serum are too low to yield statistically significant results (400, 401). Nonetheless, when strict exclusion criteria (Chapter 4) are applied (e.g. excluding patients with a significant smoking history or environmental and biomass exposure) as in the present study, inflammatory biomarkers such as CRP and IL-2 may prove to be valuable biomarkers of airway inflammation in the asthmatic patient.

CHAPTER 8: EVALUATION OF THE UTILITY OF FeNO, CHEMOATTRACTANTS - RANTES AND EOTAXIN, SERUM LEUKOTRIENES AS WELL AS IgE IN ASSESSING AIRWAY INFLAMMATION WHEN COMPARED TO CATEGORIES OF ASTHMA CONTROL AS DEFINED BY THE ACQ

8.1 Introduction

Precision medicine has been the buzzword for the management of patients with chronic illness in the 21st century. This concept alludes to a process whereby a patient's therapeutic interventions are tailored to address the heterogeneity of the specific illness, with respect to clinical manifestations and responses to therapy. Asthma is an illness that displays significant heterogeneity within patient populations groups (402). With a greater understanding of the molecular mechanisms driving the inflammation in asthma, we are now able to redefine subgroups of asthma into endotype specific and phenotype specific (78).

Asthma endotypes refer to a subtype of the disease, defined by an intrinsically distinct pathogenic or pathophysiological mechanism. Endotyping in asthma focuses on the cellular and molecular mechanisms that drive the clinical manifestations of the illness, and these have been briefly described in the previous chapter (80). Phenotyping in asthma describes the clinically observable characteristics of the disease without a direct relationship to an underlying pathophysiological process. It refers specifically to clinical and morphologic characteristics as well as unique responses to treatment. Asthma phenotypes are clinically relevant but do not necessarily relate to, or provide insights into, the underlying molecular and pathological mechanism driving the disease (77, 80).

The importance of the eosinophil in the immunopathogenesis of asthma has been confirmed in several studies (87). Patients with eosinophil predominant airway inflammation demonstrate a more severe, and exacerbation prone, clinical disease (403). Thus, while there have been several different asthma phenotypes described, the most extensively investigated to date, pertains to the presence or absence of airway eosinophilia. These two asthma phenotypes behave differently with respect to the presence of early airway remodelling, and the response to inhaled corticosteroid (ICS) therapy (14, 87, 88, 404). Thus, these two distinct asthma phenotypes have subsequently been reclassified as being either "Th2-high or Th2-low" asthma phenotypes (93).

The gold standard for the identification of the Th2-high phenotype has been the documentation of persistent airway eosinophilia in induced sputum analysis (323, 324). Unfortunately, induced sputum analysis is not widely available outside research centres and tertiary respiratory units, who have the expertise and time needed to use this investigation on a routine basis (324, 329). In view of this limitation, several novel surrogate biomarkers have been developed to assess the presence of eosinophilic airway inflammation (405). These biomarkers include fractional exhaled nitric oxide (FeNO) in breath condensates, as well as serum IgE, cysteinyl leukotrienes (LT) (i.e. LTC4, LTD4 and LTE4) and the major chemokines associated with asthma pathogenesis (viz. eotaxin and Regulated on Activation, Normal T Expressed and Secreted (RANTES)).

The association between FeNO and eosinophilic airway inflammation, as well as blood and sputum eosinophilia, in patients with asthma has been reported in several publications (291, 373). This relationship has also been described in the American Thoracic Society (ATS) consensus document detailing the use of FeNO in patients with asthma (334). The use of total serum IgE as a biomarker in the management of asthma has also been validated in previous studies, and has been recommended by an expert group of the National Institutes of Health (NIH) as a biomarker that should be used in future clinical research studies in patients with asthma (406). Furthermore, documentation of a longitudinal increase in total IgE in adults with asthma has been noted to be associated with poor asthma control (407). More importantly, FeNO and total IgE have been documented to have comparable diagnostic accuracy with regards to the presence of sputum eosinophilia in patients with asthma, irrespective of the asthma phenotype (408). Thus, FeNO and serum IgE are validated surrogate investigations to identify the presence of airway eosinophilia, and hence, airway inflammation in patients with asthma.

The chemokines eotaxin and RANTES are molecules directly related to the recruitment and functionality of eosinophils within the airways (409). Hence, measurements of eotaxin and RANTES and their association with asthma control as defined by the ACQ may function as possible biomarkers for a Th2 high asthma phenotype.

Leukotrienes, and in particular the cysteinyl leukotrienes (LTB4, LTC4 and LTE4) are important products of eosinophil activation and eosinophil function within the airways (235). LTC4, LTD4 and LTE4 are the active constituents of the so-called “slow reacting substance of

anaphylaxis" and thus play a significant role in the immunopathogenesis of asthma. They are important in promoting airway eosinophilia and perpetuating airway damage in patients with asthma (111, 226). There have been several publications documenting the measurements of urinary LT levels, and in particular, LTE4 levels in patients with asthma. LTE4 is the final product in the leukotriene synthesis pathway (Figure 2.2) and thus LTE4 levels are a reflection of whole body leukotriene synthesis (410, 411). Hence, serum LTC4, LTD4 as well as LTE4 levels were measured in the present study, to attempt to identify a biomarker that might be more reflective of the state of inflammation in the airways.

Therefore, the next objective in this thesis, was to undertake a study to determine whether any of these surrogate markers of airway inflammation (i.e. FeNO, serum IgE, serum leukotrienes LTB4, LTC4 and LTE4, eotaxin and RANTES) demonstrated any significant association and/or correlation with asthma control as defined by the ACQ.

8.2 Design and Study Population

This study was designed as a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic with approval to conduct the study received from the University of the Witwatersrand Human Research Ethics committee (Protocol Number M060401- Appendix 3). The specifics of the design and population cohort are the same as the study population in Chapter 5 and have been described in detail in Chapter 4. In an attempt to reduce the statistical "noise to signal" from confounders that might dilute the study results, strict inclusion and exclusion criteria as described in Chapter 4 were applied. The reason for the application of strict inclusion and exclusion criteria is that this is a proof of concept study trying to identify a biomarker that might be affected by any concomitant co-existing illnesses. A total of 76 patients fulfilled the criteria for inclusion into the study.

8.3 Methods

A detailed description of the methodology employed in the present study has been described in Chapter 4. For the purposes of standardisation within this thesis, all patients completed the Asthma Control Questionnaire (ACQ), which was used as the basis for the assessment of asthma control in the entire cohort of patients studied (Appendix 1) (58). As indicated in Chapter 4, the population of patients studied were categorised into two groups, based on their level of control. **Group 1** consisted of the patients categorised into controlled (CC) and uncontrolled (UC), based on an ACQ level of 1.5. **Group 2** consisted of the same cohort of

patients categorised by the ACQ into totally controlled (TC) having an ACQ < 0.75, well controlled (WC) having an ACQ of between 0.75 and 1.5 and uncontrolled (UC) with an ACQ > 1.5 (59).

In all the studies, the evaluation was first for **group 1** (i.e. primary/initial analysis: controlled versus uncontrolled asthmatic patients) and thereafter, the **group 2** analysis (i.e. subsequent/secondary analysis: totally controlled versus well controlled and versus uncontrolled asthmatic patients).

Of importance in this chapter was that the investigations chosen to be measured, were considered to be potential surrogate markers of eosinophilic airway inflammation (93). Online FeNO was measured with a NIOX MINO air inflammation monitor manufactured by Aerocrine, Solana Sweden. Airflow was measured at 50ml/sec, and regulated by a built-in flow regulator, as per the ATS guidelines for online FeNO measurements(334). FeNO levels were reported as parts per million (ppm).

Multi-analyte analysis of eotaxin and RANTES was performed using the magnetic luminex screening assay (Bio-Rad,M500AD1ZL6) on a Bio-Plex 200 (Bio-Rad, CA, USA) according to the manufacturer's instructions, and reported as fluorescent intensity units (FU). The rationale for reporting as FU as well as a brief outline of the multiplex technology has been discussed in Chapter 4. A detailed description of the exact methodology of the preparation and measurement of the analytes is included in Appendix 7.

Serum total IgE levels were measured using the IMMULITE 2000 - K132801 Total IgE solid phase, chemiluminescent immunometric assay (Siemens Healthcare Diagnostics Inc., LA, USA) and reported using the International System of Units (SI Units - 1 kIU/L = 1 IU/ml). A detailed description of the exact methodology of the measurement of IgE is included in Appendix 8.

Serum leukotriene levels were measured using an enzyme-linked immunosorbent assay (ELISA) procured from Caymen Chemicals, Ann Arbor, Michigan. Currently there is minimal data published on the appropriate methodology of measuring serum leukotriene levels using enzymatic immuno-assays (EIA) kits. Most of the data published thus far focus on the measurement of urinary leukotriene levels (406, 412-415). Hence, a pilot study to determine

the optimal method of measuring serum leukotriene level was performed as part of this thesis (416-418). The full protocol of the optimisation studies is included in the supplementary data in Appendix 9, but a brief description of the theory behind the optimisation tests is included in the main body of this chapter below.

8.3.1 Optimisation experiments to determine the methodology for the measurements of serum leukotriene levels

The EIA assay (Caymen Chemicals, Ann Arbor, Michigan) used, is based on the principle of a competitive reaction between serum LT and a LT–acetylcholinesterase (AChE) conjugate called an LT Tracer(416). The product of this enzymatic reaction has a distinct yellow colour and the intensity of this colour, determined spectrophotometrically, is proportional to the amount of free LT tracer bound to the well. This value is inversely proportional to the amount of free LT present in the well, and hence a measurement of the concentration of serum leukotriene levels (419) (Figure 8.1).

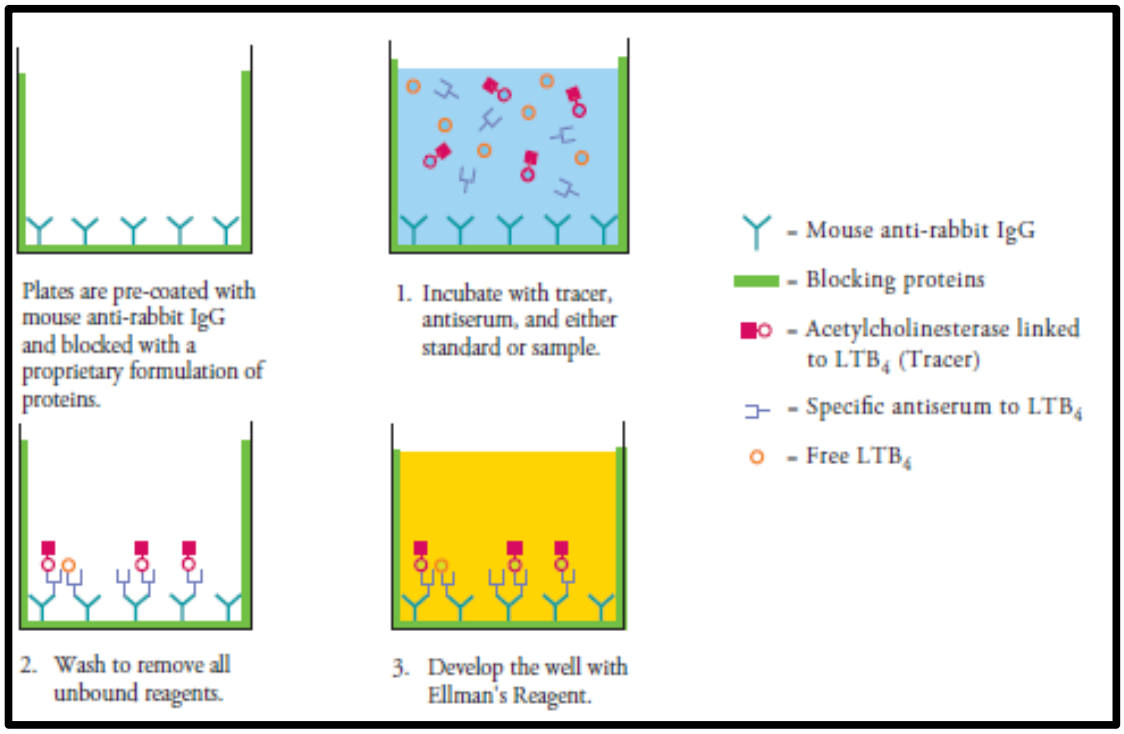

Figure 8.1: Schematic diagram of the technique of the ACE™ EIA kit. Figure courtesy of Caymen Chemicals

Step 1: Incubation of patient serum with manufacturer provided tracer and antiserum in wells pre-coated with IgG antibodies that are fixed to the wells, and are targeted against antiserum that binds leukotriene molecules and the free leukotriene molecules

Step 2: Washing of well to remove all unbound reagents

Step 3: Developing the well with Ellman's reagent and measuring the chemiluminescence emitted as a measurement of the serum leukotriene concentration.

LTB_4: leukotriene B4.

However, the technical problems associated with the measurement of serum LT concentration are twofold:

1) The concentration of LT levels in serum is miniscule. Thus, to improve detection of LT in serum, the manufacturer proposed that the specimens should be spiked with a known concentration of LT supplied by the manufacturer – a method called a cold spike. The spiking of patient serum is a process whereby the serum concentration of the analyte being measured is increased by the addition of a known concentration of same analyte. This improves the detection yield of the assay and after subtraction of the additional spiked concentration, the true concentration of the analyte is calculated.

2) The assay uses the intensity of a colour reaction determined spectrophotometrically to infer the concentration of the analyte. Thus, the serum/urine from which the analyte is being measured has to be purified of any substances that might interfere with this reading process. The reader determines the concentration of the analyte based on the colour the assay emits after the pre-preparatory steps. The major complication of sample purification is that while removing excess colour and impurities from the serum, purification might leach the analyte being measured as well, and thus falsely decrease the final concentration of the analyte.

Therefore, in view of the lack of published data to guide us, an optimisation study was performed. This was done with a set of serum from five of the final group of patients randomly selected according to the ACQ score as follows:

- two patients from the totally controlled group - ACQ < 0.75,
- one patient from the well-controlled group - ACQ between 0.75 and 1.5, and
- two patients from the uncontrolled group - ACQ > 1.5.

The purpose of the optimisations experiments was to answer the following questions:
1) Is it necessary to purify the sample or would the leaching process remove the analyte as well?
2) Is it necessary to spike the sample or could we derive the same results without spiking?
3) What is the optimal serum dilution to obtain results that will allow measurement of LT at both spectrums (high and low) of the kit?

The first step in the optimisation experiments was to determine if purification of the serum samples affected recovery of the analyte being measured (i.e. did purification influence the final concentration). This experiment was conducted by passing fluid with a known concentration of

leukotriene, provided by the manufacturers of the EIA kits (Caymen Chemicals), through carbon purification cartridges (HyperSep C8 SPE cartridges - Thermo Scientific). This was to assess if the carbon cartridges used for purification were leaching out the leukotriene as well. This part of the optimisation experiment was designed to determine the recovery factor of analyte after it has passed through the purification cartridge. Specimens with various different concentration of LT (called standards - Std) were prepared and passed through the cartridges and the concentration of the analytes recovered was measured. The results were compared with the specimens having the same concentrations of LT that were not passed through the purification cartridge. The difference between the two measured values (i.e. purified and not purified) gave an indication of how much leukotriene was being bound to the purification cartridge, and what the recovery factor was post-purification. Furthermore, if it was determined that there was no significant difference in measurements between purified and non-purified samples, then the purification step could be omitted.

The next step in the optimisation experiments was to determine the optimal range of analyte detection of the Caymen kits. Samples were spiked at the high end (Std 1 or Std 2), and low end (Std 7 or Std 8) of the range of the kits, and analysed. Spiking was the process of adding specific concentrations of leukotriene obtained from the manufacturers, to patient serum and measuring the levels of the leukotrienes via the EIA kits. The rationale for this was to determine if the analyte in the samples could be detected at the high and low ranges of concentrations, and to determine if dilution factor for the high ranges of analytes concentrations was required (i.e. LTC4, LTD4 and LTE4). This step also allowed the investigator to determine if samples with very low analyte concentrations would yield better results if the analyte concentration could be artificially increased by spiking the samples with a known concentration of the same analyte. The rationale was that the difference between the measured values minus the known spiked concentration would be the actual serum LT concentration.

The final step of the optimisation experiments was to assess the validity of the mathematical algorithm used by the computer in calculating the concentration of the analyte measured.

8.3.2 Results of optimisation study

A brief synopsis of the results, conclusion derived and recommendations made for the final testing protocol will be outlined for each of the leukotrienes measured. The full methodology and documentation of the results of the above experiments are included in Appendix 9.

8.3.2.1 LTB4

It was difficult to measure the concentration of the leukotriene lost due to the purification steps as poor results were obtained from the purified specimens, as well as the spiked specimens that were passed through the purification cartridges. This implied that the purification process was leaching / reducing the concentration of the analyte measured.

As the recovery concentration of the analyte measured was less than expected, the standard curve, which is a computerised program that mathematically converts the spectrophotometrically obtained value for LTB4 into the true serum concentration, had to be modified. This was because results obtained were out of range, and were a poor fit for the appropriate extrapolation of LTB4 values.

A rough estimate of the analyte lost due to the purification process was approximately 80 - 85% at higher concentrations (100-1000 pg/ml) and about 60-70% at the lower concentrations (3.9-62.5 pg/ml). When comparing the results from the purified and non-purified, as well as the spiked and un-spiked samples, an eight times dilution factor without spiking was determined as the most appropriate for this assay.

Thus, the final recommendation for the preparation for measurement of LTB4 was that non-purified serum samples with a factor of eight dilution should be used, and that Std 7 (15.6 pg/ml) and Std 8 (7.8 pg/ml) should be omitted from the standard curve for interpretation of results.

8.3.2.2 LTC4

The standard curve for LTC4 had very few results out of range and the standard curve was a good fit for the extrapolation of serum LTC4 concentration. There was no need for a mathematical modification of the computer algorithm. Loss due to purification was about 80% at higher concentrations (100-1000 pg/ml) and about 60-70% at lower concentrations (30-100 pg/ml). When comparing the purified and non-purified, as well as the spiked and un-spiked samples, a two times dilution factor without spiking was determined as the most appropriate for this assay.

Thus, the final recommendation for the preparation for measurement of LTC4 was that non-purified serum samples with a factor of two dilution should be used, while spiking was not

necessary and that Std 7 (15.6 pg/ml) and Std 8 (7.8 pg/ml) should be omitted from the standard curve for interpretation of the results.

8.3.2.3 LTE4

The standard curve for LTE4 had very few results out of range and the standard curve was a good fit for the extrapolation of serum LTE4 concentration. There was no need for a mathematical modification of the computer algorithm. Loss due to purification was about 80% at higher concentrations (100-1000 pg/ml) and 50-60% at lower concentrations (7.8–100 pg/ml). When comparing the purified and non-purified, as well as the spiked and un-spiked samples, a four times dilution factor without spiking was determined as the most appropriate for this assay.

Thus, the final recommendation for the preparation for measurement of LTE4 was that non-purified serum samples with a factor of four dilution should be used, while spiking was not necessary, and that Std 7 (15.6 pg/ml) and Std 8 (7.8 pg/ml) should be omitted from the standard curve for interpretation of results.

In summary, these optimisation experiments allowed us to develop the protocol and methodology necessary to carry out the tests on measuring serum leukotriene levels in asthmatic patients. Enzyme-linked immunosorbent assays (ELISA) were used to determine the serum concentrations of leukotriene B4, C4 and E4 (Cayman Chemicals, #520111; 520211; 520411 respectively). Sera were diluted 1 in 8, 1 in 2 and 1 in 4 for LTB4, LTC4 and LTE4, respectively, and assayed according to the manufacturer's recommendations (Appendix 9 - optimisation of LTB4, LTC4, LTE4). Optical density of the analytes was measured at 405 nm on a microplate reader (Elx800, BioTek, USA).

8.4 Statistical Analysis

Methodology detailing the basic principles of the statistical analyses applied, including population size calculations, has been discussed in Chapter 4. The population of patients studied were 76 in total. The Shapiro-Wilks normality test indicated that the data reported was not normally distributed; hence, non-parametric statistical tests were used for all the analyses performed.

The results were reported in both a table format, as well as graphically using a box-and-whisker plot where applicable. The results were represented in a dual format for ease of reference and interpretation. The results included in the tables were reported as medians together with the 95 percent (%) confidence intervals (CI). The box-and-whisker plot displayed the data using the standard five number summary: top and bottom boundary of the box indicating the 75th and 25th percentiles, the line within the box depicted the median and the whiskers represented the minimum and maximum values measured.

Results reported in the tables that were statistically significant are highlighted in bold black text with an asterisk *, whereas those results that had a clinically significant correlation are highlighted in bold black text with a double asterisk **, for easy recognition. The corresponding p or Rho (r) values are represented in the associated figures in **red**.

All the laboratory investigations measured, as continuous variables, were compared to the categories of asthma control, first for **group 1** and then for **group 2**. For the patients categorised into **group** 1 (i.e. controlled versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Wilcoxon rank sum test. For the patients categorised into **group 2** (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Kruskal-Wallis one-way ANOVA test. The Bonferroni correction was applied to the analysis of the **group 2** cohort to control for multiplicity.

In all testing a p-value < 0.05 was considered significant, except where the Bonferroni correction was applied.

Specific to this chapter, is that the levels of all biomarkers measured via the bioplex assays were reported as fluorescent intensity units (FU). When the level of a biomarker was below the lower limit of detection, it was assigned half the value of the lowest limit of detection. When the level of a biomarker was above the upper limit of detection, it was assigned double the value of the uppermost limit of detection. These adjustments for outlying data have been verified in the literature as an acceptable method for data correction (337, 338), and are recommended by the manufacturers of the analyte kits as well.

As outlined in Chapter 4, the reason for reporting in fluorescent intensity units (FU) as opposed to standard international units (SI) was that fluorescent intensity is the actual measurement (raw data) obtained from the microplate-based multiplex immunoassay system. By using the raw data in FU as opposed to diluting the data with several correction steps, using pre-defined manufacturer provided control samples to determine the equivalent SI units, the statistical analysis and results obtained were improved (Appendix 6 and 7).

A Spearman's rank correlation was used to assess the correlation between the ACQ and all other variables measured. For the interpretation of the Spearman's rank correlation, as outlined in Chapter 4, only $r > 0.6$ were reported as a having a strong correlation, even though a $r < 0.6$ may have been statistically significant with a $p < 0.05$.

For comparisons of FeNO values measured as a categorical variable (i.e. FeNO levels < or > 25ppm) and asthma control as defined by the ACQ, the chi-squared test was used and if any frequency was ≤ 5, a Fisher's exact (two-tailed) test was used. This analysis was performed using a FeNO cut-off level of 25 ppm as well as a higher cut-off value of 42 ppm (334, 420).

Data analysis of the association between the medication used by the patient and the levels of the biomarker measured, was performed using the Wilcoxon rank sum test.

The statistical analyses and graphics were performed using the SPSS software, version 12 (**SPSS**, Chicago, Illinois), Excel software (Microsoft, Albuquerque, New Mexico) and GraphPad Software Inc. (La Jolla, California).

8.5 Results

8.5.1 FeNO

For the **group 1,** analysis (controlled versus uncontrolled asthmatic patients), there was no significant difference in the FeNO levels when comparing the two groups of patients. The median FeNO level was 51.6 ppm (95% CI 41.3- 64.5) in the controlled group versus a median FeNO level of 50.2 ppm (95% CI 35.5– 70.9) in the uncontrolled group ; $p = 0.96$ (Table 8.1; Figure 8.2). There was also no clinically relevant correlation between the FeNO levels and the ACQ score.

Table 8.1: FeNO levels comparing controlled and uncontrolled asthmatic patients (**group 1**) and the correlation between levels of FeNO and the ACQ score

Biomarker	Controlled n=52	Uncontrolled n=24	Controlled vs Uncontrolled[1] (p values)	Rho values (p-values) n= 76[2]
FeNO ppm	51.6(41.3 - 64.5)	50.2 (35.5 – 70.9)	0.96	0.0895 (0.4421)

1: Wilcoxon rank sum test.
2: Spearman rank correlation between the ACQ score and spirometric variables.
The results are reported as medians together with the 95% confidence intervals (CI).
FeNO: Fractional exhaled nitric oxide, ppm: Parts per million, n: Number.

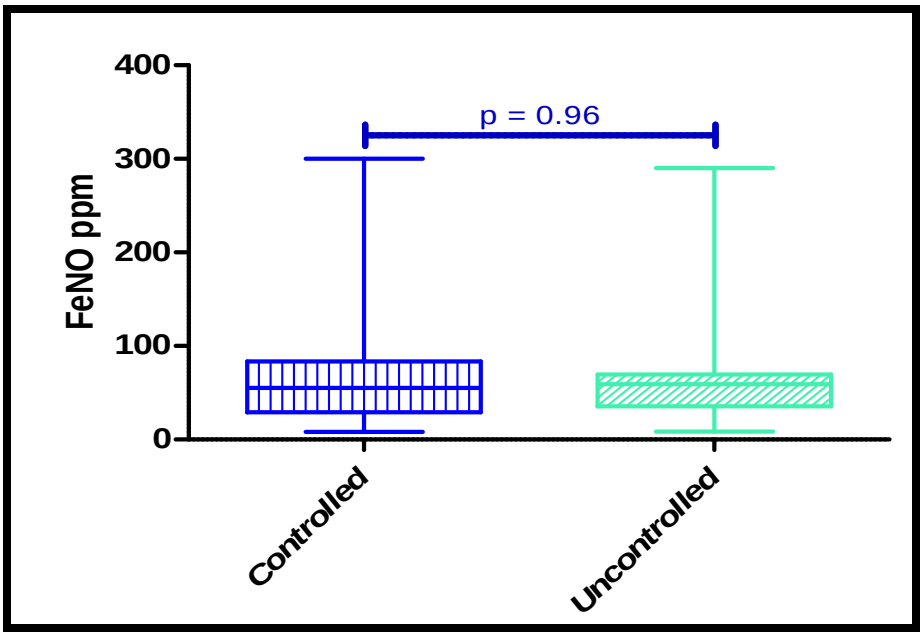

Figure 8.2: FeNO levels comparing controlled and uncontrolled asthmatic patients (**group 1**)
FeNO: Fractional exhaled nitric oxide, ppm: Parts per million. Wilcoxon rank sum test.

For the patients categorised into **group 2** (i.e. total controlled versus well-controlled and versus uncontrolled) there was still no significant difference between absolute FeNO levels and asthma control as defined by the ACQ, with levels in the totally controlled group being no different from that in either the well-controlled or uncontrolled groups (Table 8.2; Figure 8.3).

Table 8.2: FeNO levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Biomarker	Total control n=30	Well control n=22	Uncontrolled n=24	Total control vs Well controlled[1] (p value)	Total control vs Uncontrolled[1] (p value)	Well controlled vs Uncontrolled[1] (p value)
FeNO ppm	42.5 (32.4 – 55.6)	67.4 (46.5-97.8)	50.2 (35.5-70.9)	0.041	0.45	0.29

1: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
The results are reported as medians together with the 95% confidence intervals (CI).
FeNO: Fractional exhaled nitric oxide, ppm: Parts per million, n: Number.

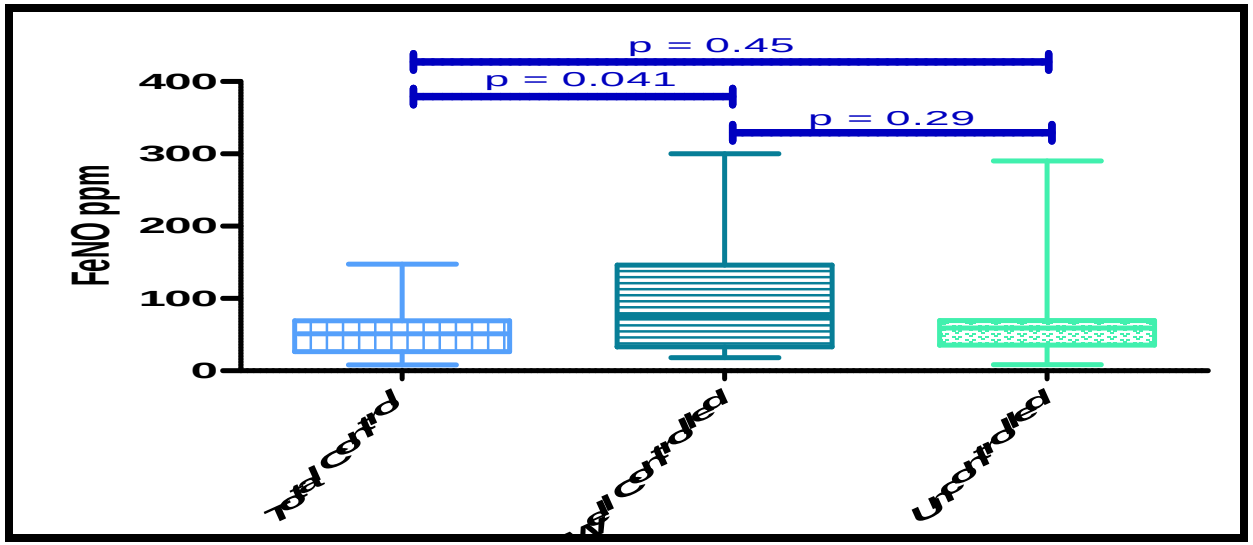

Figure 8.3: Comparison of FeNO levels in patients with totally controlled, well-controlled and uncontrolled asthma (**group 2**)
FeNO: Fractional exhaled nitric oxide, ppm: Parts per million. Kruskal-Wallis one-way ANOVA test.
p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).

Using a cut-off of 25 ppm, as suggested by the ATS guidelines, 15 patients (20%) were evaluated by FeNO as having controlled asthma whereas 61 (80%) had uncontrolled asthma (Figure 8.4).

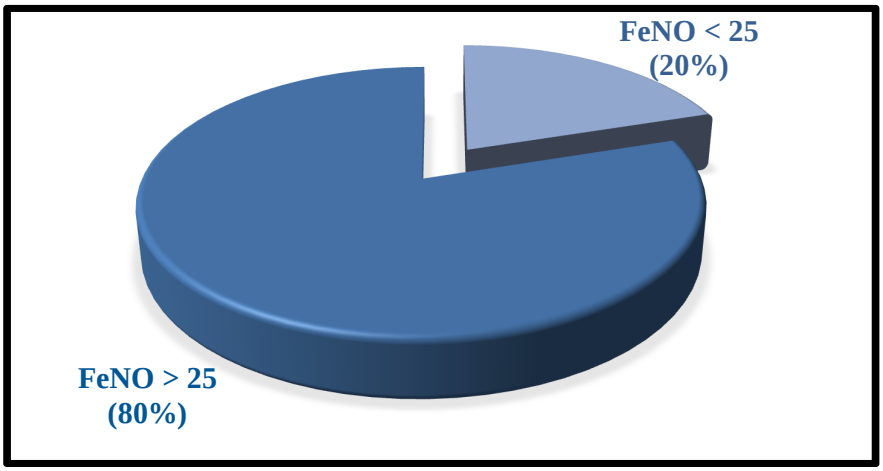

Figure 8.4: FeNO levels in the study population of 76 asthma patients
FeNO: fractional exhaled nitric oxide measured in parts per million

FeNO levels were also analysed as a categorical variable using the ATS cut-off value of 25 ppm, as well as a higher cut-off of 42 ppm, as suggested in a publication by Schleich *et al.* (420), and a cut-off of 50 ppm as suggested by Malinovschi *et al.* (421). There was no significant difference between a FeNO cut-off at different levels (i.e. above or below 25 ppm, 42 ppm and 50 ppm), compared to the categories of asthma control as defined by the ACQ for both the **group 1** analysis (i.e. controlled versus uncontrolled asthmatic patients), as well as for the **group 2** analysis (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients) (Table 8.3).

Table 8.3: Association between FeNO values reclassified categorically and asthma control as defined by ACQ

Biomarker	Group 1(ACQ) (Fischer exact) p values	Group 2(ACQ) (Pearson chi-squared) p values
FeNO > 25 ppm	0.76	0.81
FeNO > 42 ppm	0.77	0.80
FeNO > 50 ppm	0.99	0.51

Group 1: controlled = ACQ < 1.5; uncontrolled = ACQ ≥ 1.5
Group 2: totally controlled = ACQ < 0.75; well-controlled = 0.75 ≤ ACQ < 1.5; uncontrolled = ACQ ≥ 1.5
ACQ: asthma control test, FeNO: Fractional exhalation of nitric oxide, ppm: Parts per million.

8.5.2 Serum biomarkers of Th2 high or eosinophilic airway inflammation

8.5.2.1 RANTES

The **group 1** analysis revealed a significantly lower level for RANTES in the controlled group when compared with the uncontrolled group of patients as defined by the ACQ. The median level for RANTES in the controlled group was 5537.5 FU (95% CI 5296.6 - 5695.6) compared with 5928.6 FU (95% CI 5592.2 - 6285.1) in the uncontrolled group; p = 0.03 (Table 8.4; Figure 8.5).

Within the **group 2** analysis, RANTES was noted to be significantly lower in the totally controlled and well-controlled groups when compared with the uncontrolled group of asthmatic patients. The median RANTES level for the TC group was 5296.5 FU (95% CI 5094.2 - 5506.7) compared with 5785.19 FU (95% CI 5415.1 - 6180.6) for the WC group (p = 0.003), and compared with 5928.6 FU (95% CI 5592.2 - 6285.1) for the UC group (p = 0.014) (Table 8.5; Figure 8.6).

8.5.2.2 Eotaxin

Eotaxin levels demonstrated no significant difference and or correlation with asthma control in the **group 1** analysis, when comparing controlled versus uncontrolled asthmatic patients (Table 8.4; Figure 8.5), or in the **group 2** analysis, when comparing totally controlled patients to well-controlled and uncontrolled asthmatic patients (Table 8.5; Figure 8.6).

8.5.2.3 IgE

IgE levels demonstrated no significant difference and or correlation with asthma control in the **group 1** analysis, when comparing controlled versus uncontrolled asthmatic patients (Table 8.4; Figure 8.5), or in the **group 2** analysis, when comparing totally controlled to well-controlled and uncontrolled asthmatic patients (Table 8.5; Figure 8.6).

8.5.2.4 Leukotrienes

Of the leukotriene levels measured, for the **group 1** analysis, comparing controlled with uncontrolled asthmatic patients, there were no significant differences between serum leukotrienes and asthma control as defined by the ACQ (Table 8.4; Figure 8.7).

However, for the **group 2** analysis, LTE4 was significantly higher in the totally controlled (TC) group when compared with the uncontrolled group (UC), as well when the totally controlled group was compared with the well-controlled (WC) group. The median LTE4 level was 3547.3 pg/ml (95% CI 2592.2 - 4854.3) in the TC group, compared with 1728.2 pg/ml (95% CI 1167.4 - 2558.7) in the WC group (p = 0.007), and compared with 1692.6 pg/ml (95% CI 1055.6 - 2713.9) in the UC group (p = 0.006) (Table 8.5; Figure 8.8).

Table 8.4: Chemokine, IgE and leukotriene levels comparing controlled versus uncontrolled asthmatic patients (**group 1**) and the correlation between levels of chemokines, IgE and leukotriene to the ACQ score

Biomarker	Controlled n=52[1]	Uncontrolled n=24	Controlled vs Uncontrolled[2] p values	Rho values (p-values) n= 76[1,3,4]
Eotaxin FU [1]	77.12 (67.51 - 88.11)	75.84 (58.40 - 98.48)	0.65	-0.0657 (0.5753)
RANTES FU [1]	5537.5 (5296.6 - 5695.6)	5928.6 (5592.2 - 6285.1)	**0.03***	0.3409 (0.0028)
IgE IU/ml	407.4 (258.7 - 556.1)	332.3 (136.9 - 527.7)	0.41	-0.1518 (0.1906)
LTB4 pg/ml[4]	816.4 (581.6 - 1146.1)	1163.3 (881.9 - 1534.4)	0.29	0.1815 (0.1243)
LTC4 pg/ml	175.2 (152.3 - 201.5)	193.8 (156.8 - 239.6)	0.47	0.1593 (0.1692)
LTE4 pg/ml	2616.9 (2023.7 - 3383.8)	1692.6 (1055.6 - 2713.9)	0.08	-0.3321 (0.0034)

1: Chemokine analysis was of 51 controlled patients as one result was totally aberrant and discarded.
2: Wilcoxon rank sum test. * = $p < 0.05$
3: Spearman rank correlation between ACQ score and other variables.
4: LTB4 analysis was of 49 controlled patients as 3 patients results was not measured by EIA kit.
The results are reported as medians together with the 95% confidence intervals (CI).
FU: Fluorescent intensity units, IU/ml: International units per millilitre, pg/ml: picograms per millilitre, RANTES: Regulated on Activation, Normal T Expressed and Secreted, IgE: Immunoglobulin E, LT: Leukotriene, n: Number.

Table 8.5: Chemokine, IgE and leukotriene levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Biomarker	Totally control n=30	Well-control n=22[1,2]	Uncontrolled n=24	Totally controlled vs Well-controlled[1,2,3] (p values)	Totally controlled vs Uncontrolled[3] (p values)	Well-controlled vs Uncontrolled[1,2,3] (p values)
Eotaxin FU[1]	80.84 (65.87 - 99.21)	72.11 (61.74 - 84.21)	75.84 (58.40 - 98.48)	0.32	0.53	0.94
RANTES FU[1]	5296.5 (5094.2 - 5506.7)	5785.19 (5415.1 - 6180.6)	5928.6 (5592.2 - 6285.1)	**0.014***	**0.003***	0.70
IgE IU/ml	420.7 (236.4 - 605.0)	389.2 (124.6 - 653.7)	332.3 (136.9 - 527.7)	0.19	0.20	0.98
LTB4 pg/ml[2]	647.8 (409.9 - 1023.6)	1111.5 (662.4 - 1865.0)	1163.3 (881.9 - 1534.4)	0.18	0.14	0.81
LTC4 pg/ml	156.4 (131.7 - 185.7)	204.6 (162.1 - 258.4)	193.8 (156.8 - 239.6)	0.09	0.15	0.69
LTE4 pg/ml	3547.3 (2592.2 - 4854.3)	1728.2 (1167.4 - 2558.7)	1692.6 (1055.6 - 2713.9)	**0.006***	**0.007***	0.95

1: Chemokine analyses was of 21 well-controlled patients as one result was totally aberrant and discarded.
2: LTB4 analysis was of 19 well-controlled patients as 3 patients results was not measured by EIA kit.
3: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction).
The results are reported as medians together with the 95% confidence intervals (CI).
FU: Fluorescent intensity units, IU/ml: International units per millilitre, pg/ml: Picograms per millilitre, RANTES: Regulated on Activation, Normal T Expressed and Secreted, IgE: Immunoglobulin E, LT: Leukotriene, n: Number.

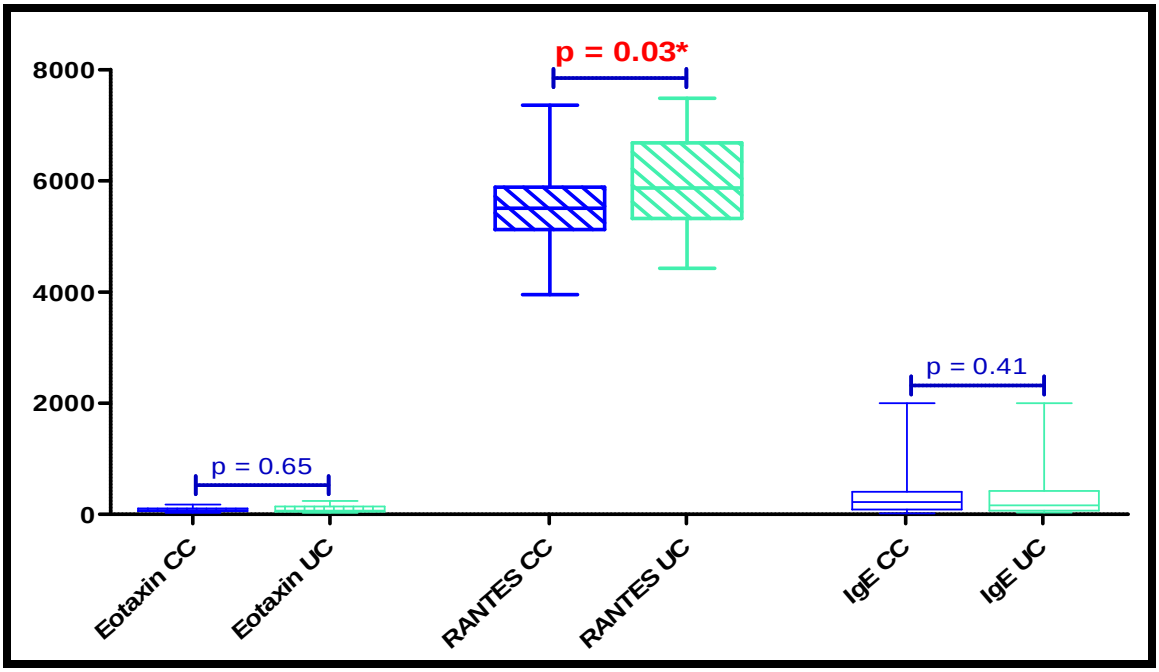

Figure 8.5: Eotaxin, RANTES and IgE levels comparing controlled and uncontrolled asthmatic patients (**group 1**)

* p values reported as significant are depicted in **red**.

RANTES: Regulated on Activation, Normal T Expressed and Secreted, IgE: Immunoglobulin E, CC: Controlled, UC: Uncontrolled. Wilcoxon rank sum test.

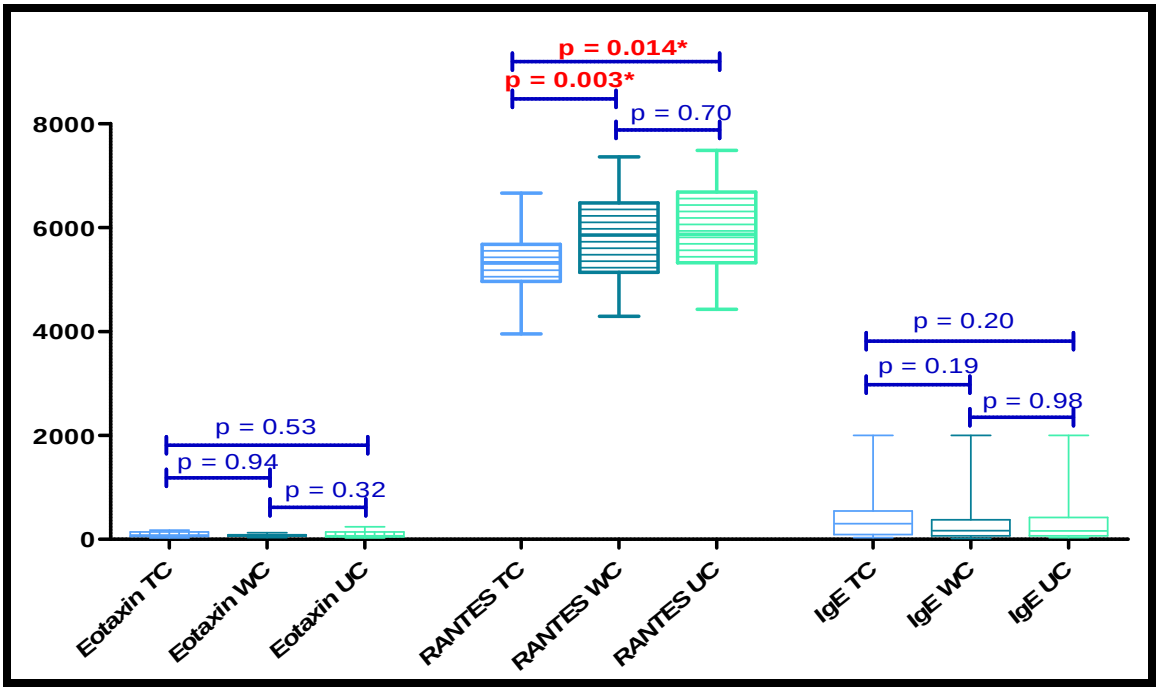

Figure 8.6: Eotaxin, RANTES and IgE levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

* p values reported as significant are depicted in **red**.

RANTES: Regulated on Activation, Normal T Expressed and Secreted, IgE: Immunoglobulin E, TC: Totally controlled, WC: Well control, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

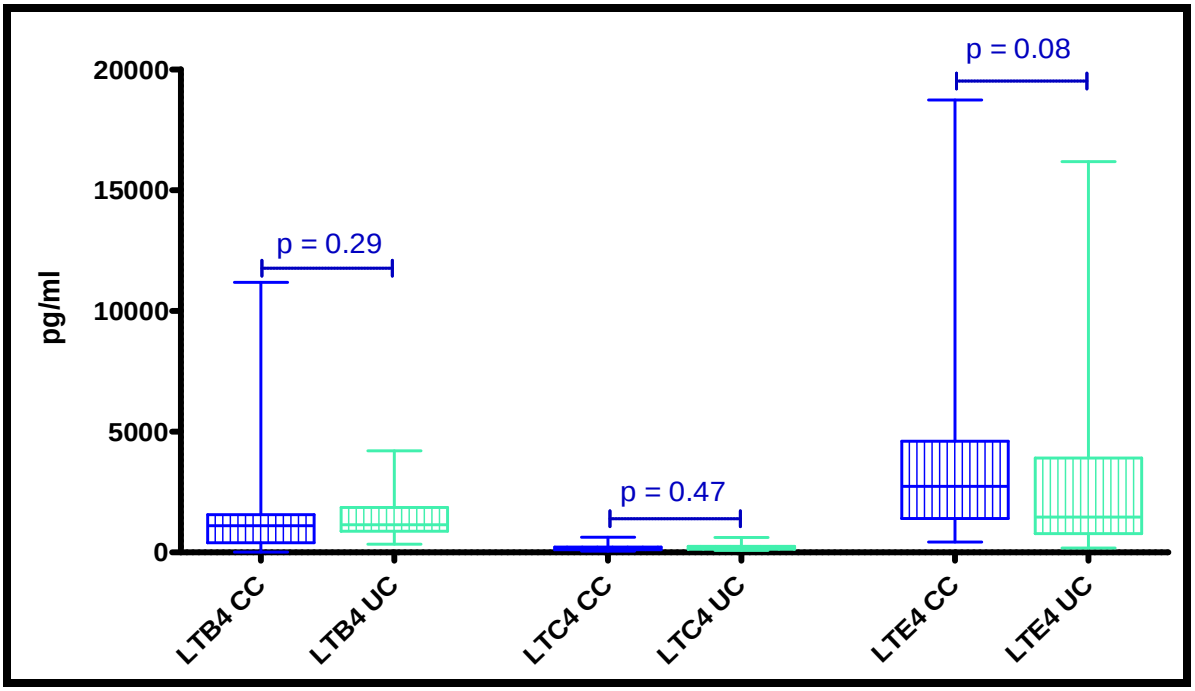

Figure 8.7: LTB4, LTC4 and LTE4 levels comparing controlled and uncontrolled asthmatic patients (**group 1**)
LT: Leukotriene, pg/ml: Picograms per millilitre, CC: Controlled, UC: Uncontrolled. Wilcoxon rank sum test.

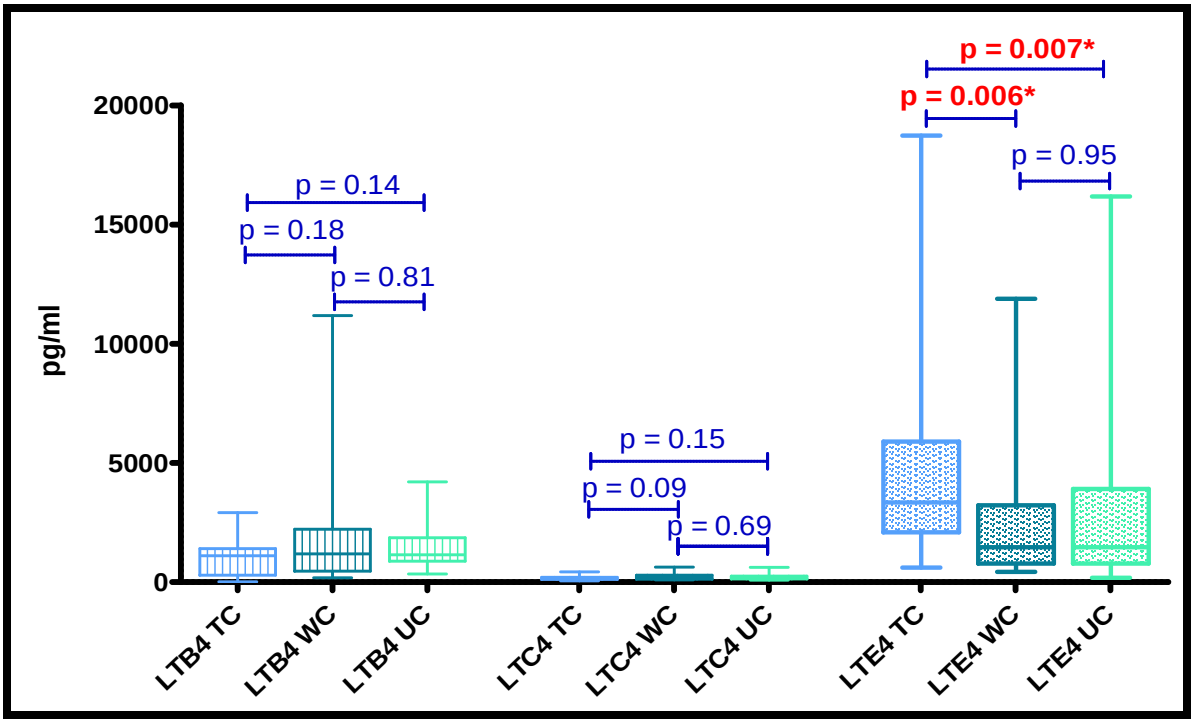

Figure 8.8: LTB4, LTC4 and LTE4 levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)
* p values reported as significant are depicted in **red**.
LT: Leukotriene, pg/ml: Picograms per millilitre, TC: Totally controlled, WC: Well control, UC: Uncontrolled. Kruskal-Wallis one-way ANOVA test.

There was no significant correlation between the FeNO levels and any of the chemokines, leukotrienes or the IgE levels with the ACQ (Figure 8.9).

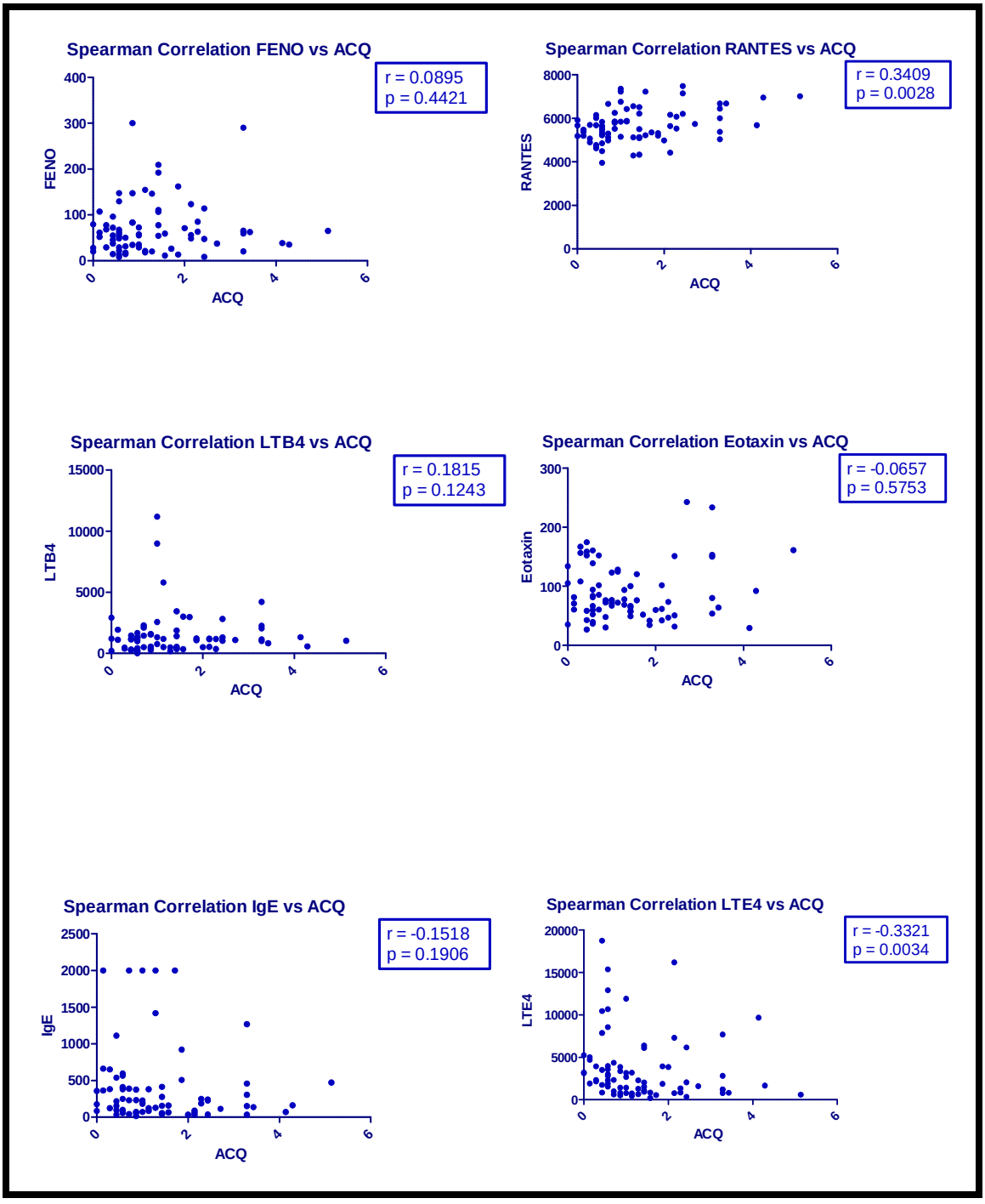

Figure 8.9: Correlation between FeNO, Chemokines, IgE and leukotrienes levels and ACQ score in the study population

FeNO: Fractional excretion nitric oxide, RANTES: Regulated on Activation, Normal T Expressed and Secreted, IgE: Immunoglobulin E, LT: Leukotriene, ACQ: Asthma Control Questionnaire. Spearman rank correlation.

A secondary analysis of the above measured surrogate mediators of eosinophilic inflammation (i.e. FeNO, IgE, chemokines and leukotrienes) was performed to determine if there was any correlation between these molecules and the Th2 cytokines analysed in Chapter 7 (Table 8.6). The correlation between RANTES and IgE ($p = 0.04$), and RANTES and IL-4 ($p = 0.01$) was statistically significant, but the result was clinically irrelevant ($r = -0.23$ and $r = 0.3$ respectively). The same was evident for the correlation between LTC4 and IgE as well LTB4 and FeNO (Table 8.6; Figures 8.10a, 8.10b).

Table 8.6: Correlation of eosinophilic mediators of airway inflammation with each other (i.e. FeNO, Chemokines, IgE and leukotrienes levels) as well as with the Th2 cytokines (i.e. IL-4 and IL-5)

Biomarker	rho	p values
IL-4 vs IgE	0.09	0.46
IL-5 vs IgE	0.13	0.26
FeNO vs IgE	0.07	0.56
RANTES vs IgE	-0.23	0.04
RANTES vs IL-4	0.3	0.01
RANTES vs IL-5	0.15	0.19
LTB4 vs IgE	0.14	0.22
LTC4 vs IgE	0.27	0.02
LTE4 vs IgE	-0.02	0.83
LTB4 vs FeNO	-0.26	0.03
LTC4 vs FeNO	-0.01	0.92
LTE4 vs FeNO	0.22	0.05

IL: Interleukin, IgE: Immunoglobulin E, RANTES: Regulated on Activation, Normal T Expressed and Secreted, LT: Leukotriene, FeNO: Fractional exhaled nitric oxide. Spearman rank correlation.

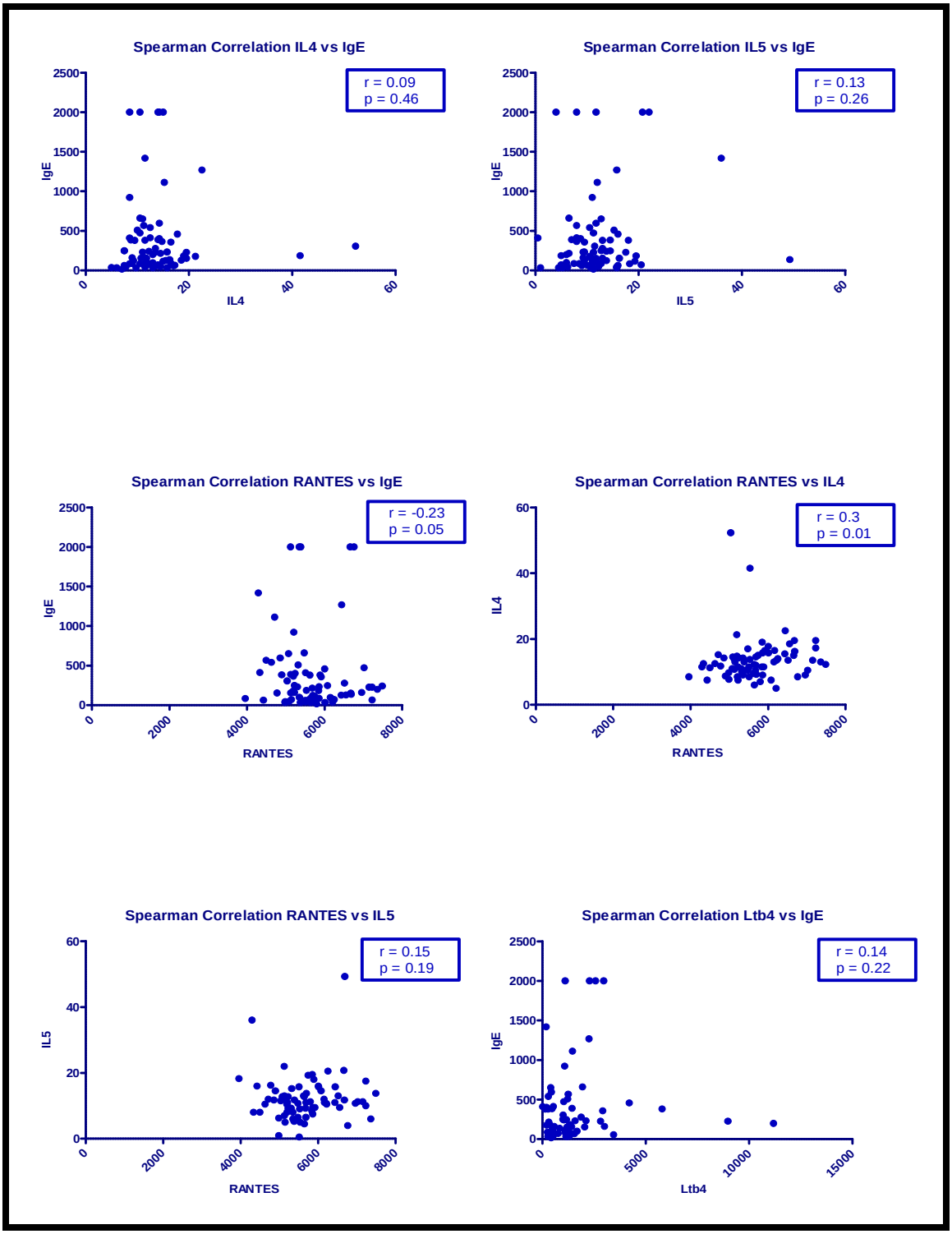

Figure 8.10a: Correlation between the various mediators of eosinophilic airway inflammation (i.e. FeNO, Chemokines, and IgE and leukotrienes levels) as well as with the Th2 cytokines (i.e. IL-4 and IL-5)

IL: Interleukin, IgE: Immunoglobulin E, RANTES: Regulated on Activation Normal T Expressed and Secreted, LT: Leukotriene. Spearman rank correlation.

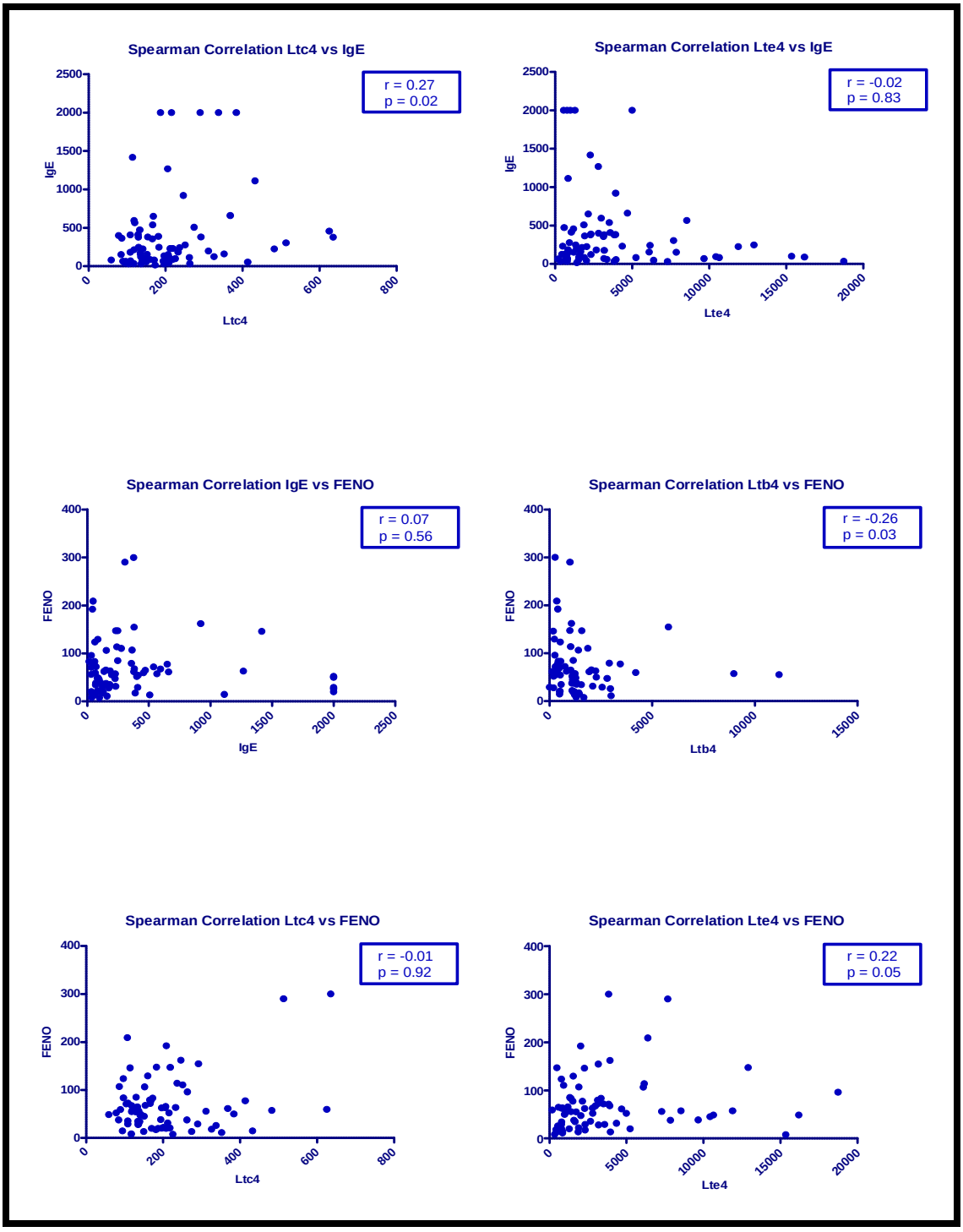

Figure 8.10b: Correlation between the various mediators of eosinophilic airway inflammation (i.e. FeNO, Chemokines, and IgE and leukotrienes levels) as well as with the Th2 cytokines (i.e. IL-4 and IL-5)

IgE: Immunoglobulin E, LT: Leukotriene, FeNO: Fractional exhaled nitric oxide. Spearman rank correlation.

Finally, as most of the medications asthmatic patients use inherently have anti-inflammatory activity, a comparison was made between the use or not of anti-inflammatory medications by the patients, and the serum concentrations of the biomarkers. This was done to ascertain whether the medication the patients were prescribed had any effect on the levels of the inflammatory biomarkers measured. The levels of RANTES were significantly higher in the patients using a low-dose of oral corticosteroids (≤ 7.5 mg/day) with a median RANTES level of 6269.21 FU (95% CI 5698.20 - 6897.44) versus a median RANTES level of 5566.31 FU (95% CI 5387.81-5750.73) in the group not on a maintenance dose of oral corticosteroids (p = 0.02) (Table 8.7).

The legend for table 8.7 has been included in a text box here as there is no space for it on page 174.

Wilcoxon rank sum test.* = $p < 0.05$.
1: LTB4 analysis was of 49 controlled patients as 3 patients results was not measured by EIA kit.
2: Chemokine analysis was of 51 controlled patients as one result was totally aberrant and discarded.
Budesonide hd: high-dose between 800 and 1600 µg/day; Budesonide md: moderate-dose ≤ 800 µg/day; Prednisone ld: low-dose ≤ 7.5 mg/day; IgE: Immunoglobulin E, RANTES: Regulated on Activation, Normal T Expressed and Secreted, LT: Leukotriene, FeNO: Fractional exhaled nitric oxide, pg/ml: Picograms per millilitre, IU/ml: International units per millilitre, ppm; Parts per million, FU: Fluorescent intensity units.

Table 8.7: Concentrations of the inflammatory biomarkers measured (i.e. FeNO, Chemokines, IgE and leukotrienes levels) comparing levels in those patients using, versus those patients not using anti-inflammatory medications.

Biomarker[+]	Montelukast			Budesonide md: ≤ 800 µg/day			Budesonide hd: < 800 and ≤ 1600 µg/day			Prednisone ld: ≤ 7.5 mg/day		
	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value
LTB4 pg/ml[1]	1029.27 (693.99-1526.52)	894.58 (670.45-1193.64)	0.76	890.93 (669.01-1186.47)	1036.69 (649.78-1654.00)	0.69	1017.98 (613.07-1690.31)	896.72 (676.47-1188.67)	0.81	1114.18 (742.80-1671.26)	898.47 (687.06-1174.93)	0.79
LTC4 pg/ml	161.02 (131.03-197.89)	185.29 (162.24-211.61)	0.46	174.26 (153.13-198.32)	210.57 (162.05-273.60)	0.20	215.76 (163.46-284.81)	173.83 (153.06-197.42)	0.16	189.96 (150.01-240.56)	180.00 (158.83-203.99)	0.55
LTE4 pg/ml	1570.47 (975.60-2528.05)	2462.92 (1900.40-3191.94)	0.13	2497.30 (1904.68-3274.29)	1576.17 (1108.06-2242.05)	0.10	1475.98 (1040.63-2093.46)	2515.86 (1926.70-3285.19)	0.06	1355.86 (618.05-2974.44)	2403.98 (1887.52-3061.76)	0.13
FeNO ppm	61.41 (41.85-90.10)	49.29 (39.99-60.74)	0.34	51.87 (42.40-63.47)	48.43 (29.82-78.65)	0.62	53.05 (32.82-85.75)	50.76 (41.43-62.19)	0.95	59.26 (38.25-91.81)	50.42 (41.33-61.50)	0.71
IgE IU/ml	202.31 (113.56-360.44)	198.61 (147.24-267.90)	0.98	180.97 (136.00-240.81)	294.61 (149.55-580.37)	0.23	331.00 (167.36-654.66)	177.66 (133.82-235.86)	0.11	353.79 (124.05-1008.97)	187.97 (142.96-247.14)	0.21
Eotaxin FU[2]	72.82 (48.77-108.73)	77.55 (68.46-87.85)	0.40	73.49 (64.64-83.55)	92.47 (66.20-129.15)	0.20	88.63 (62.45-125.77)	74.42 (65.43-84.64)	0.38	108.76 (59.45-198.98)	74.00 (65.62-83.45)	0.15
RANTES FU[2]	6035.01 (5586.16-6519.92)	5546.73 (5361.10-5738.78)	0.06	5547.79 (5364.97-5736.84)	5993.74 (5512.22-6517.32)	0.12	6035.54 (5517.43-6602.29)	5546.63 (5366.81-5732.46)	0.10	**6269.21 (5698.20-6897.44)**	**5566.31 (5387.81-5750.73)**	**0.02***

8.6 Discussion

With the evolution of our understanding of the heterogeneity within the asthmatic patient population, several distinct asthma phenotypes have been identified. The best-categorised asthma phenotype to date is the Th2 high phenotype and documentation of persistent airway eosinophilia in induced sputum has been its pathognomonic feature (323, 324). This is supported by a Cochrane review (422) stating that a treatment strategy based on the sputum eosinophil reduction, in contrast to a strategy titrating medication to asthma control as advocated by the current GINA guidelines (8), leads to a greater decrease in the overall risk of asthma exacerbations. However, induced sputum is not without its limitations. Although it a less invasive test and requires less expertise than bronchoscopy, sputum induction remains difficult to conduct in large population-based studies, is time consuming and requires specialised equipment and trained staff. Furthermore, the procedure itself may induce changes in inflammatory cell populations, particularly increases in sputum neutrophils within the airways (423). These limitations in the use of induced sputum analysis have led investigators to search for different biomarkers of eosinophilic inflammation. The surrogate markers of eosinophilic inflammation measured in the present study include FeNO, IgE, chemokines and leukotrienes and are discussed further.

8.6.1 The association between FeNO and asthma control

To date, FeNO has been the most extensively investigated surrogate maker of eosinophilic airway inflammation. The measurement and interpretation of FeNO levels have been standardised and published in the ATS/ERS consensus document of 2011 (334). There have been several publications confirming the close correlation between FeNO and sputum eosinophilia in patients with asthma (291, 373). However, in the present study, despite the fact that 80% of the patients tested had FeNO levels above 25ppm (a cut-off value defined as significant by the ATS guidelines), we could not demonstrate any association between FeNO levels above 25 ppm and asthma control as defined by the ACQ. The association between FeNO and asthma control, as defined by the ACQ, was further tested using a higher FeNO cut-off level of 42 ppm, as suggested by Schleich *et al.* (420), and a level of 50 ppm, as suggested by Malinovschi *et al*. (421), to assess the diagnostic yield of FeNO in this study. Even then, no association either within the **group 1** analysis or within the **group 2** analysis was demonstrated. Levels of FeNO could not differentiate between the uncontrolled, and both the totally and well-controlled asthmatic patients.

These results would be in line with the now significant body of literature published, that has cast doubt over the utility of a FeNO-based asthma management algorithm, particularly in adult patients. Malinovschi *et al.* (421) have reported that a higher FeNO cut-off of 50 ppm may identify patients with an adjusted odds ratio of 5.4 for asthma attacks, and 2.9 for asthma related emergency department visits compared with normal FeNO levels (< 25 ppb), and thus, this higher cut-off might have some utility in predicting a more exacerbation-prone asthma phenotype. However, this higher FeNO cut-off, though independently associated with asthma exacerbations, has not been validated as a surrogate marker for asthma control as defined by the ACQ. Rather, a FeNO above 50 ppm identifies a phenotype of uncontrolled asthmatic patients with eosinophilic airway inflammation.

A recent meta-analysis by Essat *et al.* (424), evaluating the effectiveness of FeNO in the management of adult asthmatic patients, reported no significant improvement in health related quality of life or asthma control in the FeNO treated group as opposed to the group treated with local best care practices. This finding was further corroborated by another meta-analysis by Guo *et al.* (425), evaluating the diagnostic accuracy of FeNO in asthma. The conclusion was that FeNO was accurate for the diagnosis of asthma in steroid naive or non-smoking patients, particularly in patients presenting with a chronic cough variant phenotype of asthma, but offered no utility in the management of patients with asthma (425). The most recent Cochrane Airways Review published in September 2016 (426), investigated whether using FeNO to guide asthma management is more effective than the established methods such as clinical guidelines. That meta-analysis reported that tailoring asthma medications based on FeNO levels, decreased the frequency of asthma exacerbations but did not affect day-to-day clinical symptoms, end-of-study FeNO levels, or inhaled corticosteroid dose. The recommendations from that Cochrane meta-analysis was that the universal use of FeNO, to help guide therapy in adults with asthma, could not be advocated as the main benefit was only a reduction in asthma exacerbations (426).

Reasons postulated for the discordant results with the use of FeNO, as opposed to sputum eosinophilia, included that FeNO levels are not unique to asthma and have been observed in eosinophilic bronchitis and allergic rhinitis (427, 428). The latest evidence explaining the discordance between FeNO and eosinophilic inflammation have come from the monoclonal antibody trials where anti-IL-5 (mepolizumab) (429) and anti-IL-13 (lebrikizumab) (430, 431) were used to treat patients with eosinophilic asthma. These Th2 interleukin targeted monoclonal antibody studies suggested that the cytokines that regulate the induction of iNOS and

production of NO in the airways are separate from those regulating eosinophil traffic through the airways, and this may result in a disassociation between FeNO levels and eosinophilic inflammation (432).

In conclusion, the present study demonstrated no significant differences in FeNO levels between the controlled versus the uncontrolled group of asthmatic patients (**group 1** analysis), or the totally controlled versus well-controlled, and versus uncontrolled asthmatic patients (**group 2** analysis**)** using FeNO at a cut-off level of 25 ppm, 42 ppm as well as 50 ppm. There was also no correlation between asthma control using the ACQ score and FeNO levels.

8.6.2 The association between IgE levels and asthma control

In the present study IgE levels were no different when compared to the categories of asthma control for either the **group 1** analysis (comparing controlled with uncontrolled asthmatic patients), or the **group 2** analysis (comparing totally controlled, well-controlled or uncontrolled asthmatic patients). Furthermore, there was no significant correlation between the ACQ and IgE levels.

Total serum IgE has been used as a biomarker for asthma severity in several studies but none of the studies compared an objective validated asthma control score (i.e. ACQ) with levels of IgE. One of these observational studies was the Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens (TENOR) study (91) with a cohort of over 4500 patients, conducted in patients with severe or difficult-to-treat asthma in which IgE was measured using the same method as the present study. In the TENOR study, IgE levels were stratified by asthma severity as evaluated by a physician. The overall median IgE values reported in that study were lower in patients with mild asthma than in patients with moderate asthma or severe asthma (91). In another study by de Marco *et al*. (433), patients with moderate and severe persistent asthma, characterised by early lung function deterioration, had higher IgE levels. However, two large global multicentre clinical trials namely the Severe Asthma Research Program - SARP (434) and the European Network For Understanding Mechanisms of Severe Asthma - ENFUMOSA (435) studies failed to demonstrate any correlation between asthma severity and levels of total and antigen specific IgE, findings similar to our present study.

Evidence from the literature suggests that it is useful to measure IgE levels in some types of asthma, and that it is still a good biomarker for atopy and uncontrolled allergic asthma (406,

436). An expert panel from the NIH (406) published a consensus document in 2012, recommending that total serum IgE be investigated as one of the biomarkers in the management of asthma. Elevated levels of IgE correlate with sputum eosinophilia (408), and a longitudinal increase in total IgE is associated with poor asthma control (407). More importantly, anti-IgE monoclonal therapy is now one of the biological therapies recommended by GINA for use in patients with severe and persistently uncontrolled asthma (8, 437). The dose of anti-IgE therapy prescribed is calculated based on the patient's weight and the serum IgE level. Furthermore, a recent publication by Syk *et al.* (438), reported that in patients optimised by ordinary asthma controller medications, there were significant decreases in total IgE levels. This decrease in total IgE levels corresponded to improvements in asthma control and quality of life. Thus, the role of total serum IgE levels may be for particular phenotypes of asthma and not every case.

In conclusion, while the data from the present study and other literature on the use of total serum IgE as a surrogate marker of airway inflammation in asthma is discordant, IgE is still recommended by the NIH as a valuable biomarker in the management of asthmatic patients (406). However, despite this recommendation that total serum IgE levels are useful in the management of asthmatic patients, the utility of total serum IgE when compared to categories of asthma control defined by a validated asthma control questionnaire (i.e. ACQ or ACT), requires further investigation.

8.6.3 The association between chemokine levels and asthma control

Chemokines are important molecules that regulate the trafficking of eosinophils and other inflammatory cells into the airways of patients with asthma (409). They are also important in eosinophil degranulation and are now increasingly being investigated as potential targets for modulating eosinophil trafficking and activation within the airways (439, 440). With this in mind, the level of two of the more important chemokines (i.e. eotaxin and RANTES) in the pathogenesis of airway inflammation, were measured as potential biomarkers in assessing airway inflammation, and more specifically eosinophilic airway inflammation.

In the present study, of the two chemokines measured, RANTES levels were significantly higher in the uncontrolled group when compared with the controlled group of asthmatic patients (**group 1**); p=0.03 (Table 8.4, Figure 8.5). The median level for RANTES in the controlled group was 5537.5 FU (95% CI 5296.6 - 5695.6) compared with 5928.6 FU (95% CI 5592.2 - 6285.1) in the uncontrolled group (Table 8.4; Figure 8.5).

This significant difference in the RANTES levels was further demonstrated in the **group 2** analysis. RANTES levels were significantly higher in the totally controlled (TC) group when compared with both the well-controlled (WC) and uncontrolled groups (UC), but could not discriminate between the well-controlled and the uncontrolled asthmatic patient groups (TC vs UC; $p = 0.014$, TC vs WC; $p = 0.003$ and WC vs UC; $p = 0.7$) (Table 8.5; Figure 8.6).

Chihara *et al*. (441), and Adachi *et al*. (442), reported elevated RANTES levels in patients diagnosed with an acute asthma attack compared with the levels in the same patient when in remission. In another study by Saad-El-Din Bessa *et al*. (443), serum levels of RANTES were significantly higher in all asthmatic patients when compared with healthy controls, and levels were significantly increased in patients with moderate and severe asthma as compared with those with mild asthma. The major difference in the publications cited above when compared with the present study, is that the RANTES levels were measured within a cohort of patients that were having an asthma exacerbation and not patients coming for routine check-ups, nor were the RANTES levels compared with the level of asthma control categorised by an objective asthma scoring questionnaire (i.e. ACQ).

In the present study there was no association between the **group 1** analysis and the eotaxin levels. Similar to the **group 1** analysis, there was no association between the **group 2** analysis and the eotaxin levels. Serum eotaxin levels have been measured as a surrogate marker for the diagnosis of atopy and asthma (444). Levels of eotaxin are also correlated with asthma severity defined by treatment intensity (445). However, serum eotaxin levels have not been compared with asthma control in any of the studies published to date.

In conclusion, elevated levels of RANTES in uncontrolled asthmatic patients might be a valuable biomarker in the assessment of airway inflammation that needs to be verified in larger trials. However, eotaxin in the present study demonstrated no utility as a biomarker in the assessment of asthma control as defined by the ACQ.

8.6.4 The association between leukotriene levels and asthma control

The final part of this study was the measurement of serum leukotriene levels and the determination of their association with asthma control as defined by the ACQ. To date, there have been no publications assessing this relationship and one of the reasons for this might be the difficulty in measuring serum leukotriene levels (416). There are several technical

difficulties in the measurement of serum LT concentrations. One of these difficulties include the minuscule levels of LT in serum (446). Thus, to improve detection of LT in serum, the manufacturers of the measurement kits (Caymen Chemicals) have advocated adding a known concentration of additional analyte, provided by the manufacturers, to the patient's serum. This process is known as spiking, and improves the analyte detection by the kits. For the final analysis, the spiked concentration is then subtracted from the measured concentration to yield the true value of the analyte measured.

The other major difficulty is that the assay uses the intensity of a colour reaction determined spectrophotometrically to infer the concentration of the analyte. Thus, the serum or urine from which the analyte is being measured has to be purified of any substances that might interfere with the reading process. The complication of the purification process is that purification might leach the analyte as well, and thus decrease the final concentration of the analyte.

To overcome these technical difficulties, optimisation experiments were performed as part of this present study to establish and validate a protocol for the measurement of serum leukotrienes in the asthmatic patient. The details of the findings of these optimisation experiments have been discussed briefly in the preceding paragraphs and are documented in more detail in Appendix 9. To the best of my knowledge, this is the first study documenting serum levels of the leukotrienes LTB4, LTC4 and LTE4 and their association with asthma control as defined by the ACQ.

In the present study, of the three leukotrienes measured, the **group 1** analysis did not demonstrate any significant differences between the controlled and uncontrolled groups of patients. For the **group 2** analysis, LTE4 was significantly higher in the totally controlled group when compared with the well-controlled and uncontrolled groups. The median LTE4 level was 3547.3 pg/ml (95% CI 2592.2 - 4854.3) in the TC group, compared with 1728.2 pg/ml (95% CI 1167.4 - 2558.7) in the WC group, and compared with 1692.6 pg/ml (95% CI 1055.6 - 2713.9) in the UC group (TC vs WC; $p = 0.006$ and TC vs UC; $p = 0.007$ (Table 8.5; Figure 8.8).

This is unexpected, as it is perceived that the serum levels of LTE4 would be lower in the patients using corticosteroids, even inhaled corticosteroids, as they suppress leukotriene synthesis by blocking the activity phospholipase A2 (235). Phospholipase A2 is the enzyme

that cleaves arachidonic acid from cell membrane phospholipids, which is the first step in leukotriene synthesis. Hence, patients with totally controlled asthma, who are on inhaled corticosteroid medication, should theoretically have the lowest levels of serum leukotriene levels (447). However, the findings in this present study with elevated levels of LTE4 in the totally controlled asthma patient group compared with the well-controlled and uncontrolled groups highlight the heterogeneity of asthma as a disease process. This unique finding of an elevated serum level of LTE4 in a group of totally controlled asthmatic patients, defined by the ACQ, has not been documented before. One of the reasons for the elevated serum LTE4 levels might be a reflection of ongoing eosinophilic airway inflammation within this group of patients. Another reason might be that the cysteinyl leukotriene pathway remains relatively unaffected by corticosteroid therapy in patients with asthma (i.e. corticosteroid therapy may selectively inhibit the cyclooxygenase pathway potentially leading to an increase in some of the leukotriene mediators produced via the lipoxygenase pathway) (411).

The finding of a significantly elevated serum LTE4 level in this present study is supported in published literature by findings of elevated urinary LT levels, especially LTE4 in patients with asthma. Abd El-Motaleb *et al.* (448), reported higher urinary LTE4 levels in children aged 3 to 14 years with acute asthma. Green *et al.* (449), reported higher urinary LTE4 levels in adults patients during an acute exacerbation when compared with levels obtained two weeks after the exacerbation. Kawagishi *et al.* (450), reported elevated urinary LTE4 levels in asthmatic patients without an acute asthma attack, that were positively correlated with the severity of the disease when compared with a cohort of healthy controls. Vachier *et al.* (411), reported higher urinary levels of LTE4 in patients with severe asthma, compared to patients with mild and moderate asthma as well as healthy controls. Notably, this difference persisted despite the patients with severe asthma being on oral corticosteroids. The initial two studied quoted above reported elevated urinary LT in patients with asthma exacerbations, while the latter two reported elevated levels in chronic stable asthmatic patients. In comparison, this present study is the first study to document elevated serum LTE4 levels in a group of totally controlled asthmatic patients defined by the ACQ, and therefore, may be a potential biomarker to assess asthma control and potentially airway inflammation in this cohort of patients.

However, when all the biomarkers (FeNO, IgE, eotaxin, RANTES, LTB4, LTC4 and LTE4) were correlated with the absolute ACQ score, as well as to the Th2 (IL-4, IL-5 and IL-13) cytokines, no significant association was demonstrated.

8.6.5 The association between FeNO, chemokines, IgE and leukotriene levels and the medications used

Finally, to ascertain whether the concentrations of any the serum biomarkers measured in this present study were affected by any of the anti-inflammatory therapies the patients were using, a comparison was made of levels of the biomarkers in patients using, versus patients not using anti-inflammatory medications. The levels of RANTES were significantly higher in the patients using a low-dose of oral corticosteroids ($\leq$ 7.5 mg/day) with median of 6269.21 FU (95% CI 5698.20 - 6897.44) versus a median of 5566.31 FU (95% CI 5387.81-5750.73) in the group not using a low-dose of oral corticosteroids; p = 0.02 (Table 8.7).

As it is unlikely that the low-dose oral corticosteroid (OCS) therapy was elevating the levels of RANTES, a more plausible explanation is that the patients who were using OCS, were those that had uncontrolled or difficult to control asthma and therefore had elevated RANTES levels. This is supported by the fact that only 4% (2/50) of patients with controlled asthma, as defined by the ACQ, were using low-dose OCS, whereas 21% (5/24) of the patients with uncontrolled asthma were using low-dose OCS because OCS are only recommended as add-on therapy in uncontrolled asthmatic patients with refractory symptoms. Notably, in this study population, patients using doses of OCS less than 7.5 mg per day (i.e. low-dose), were significantly more likely to be uncontrolled with an odds ratio of 6.58 (95% CI 1.08 - 39.91) (p=0.018) (Tables 5.6). Therefore, we hypothesise that the patients not using OCS are likely to be controlled asthmatic patients, and this group of controlled asthmatic patients have a lower RANTES level. (Tables 5.6, 5.7, 8.7). This alternative data analysis is supported by the finding in this present study, wherein we identified a significantly lower level of RANTES in the controlled group when compared with the uncontrolled group of asthmatic patients defined by the ACQ (**group 1**); p=0.03 (Table 8.4; Figure 8.5).

8.7 Conclusion

Of the biomarkers measured in this chapter, RANTES and serum LTE4 demonstrated significant difference between the controlled and uncontrolled asthmatic patients as defined by the ACQ. RANTES levels were significantly higher in the uncontrolled asthmatic patient when compared with the controlled asthmatic patient, highlighting its utility as possible biomarker for uncontrolled asthma. However, counterintuitively, LTE4 levels were elevated in the patients with totally controlled asthma when compared with the uncontrolled asthmatic patient. This finding might be a reflection of ongoing eosinophilic airway inflammation within this group of

totally controlled asthmatic patients, and/or the fact that the cysteinyl leukotriene pathway remains relatively unaffected by corticosteroid therapy in patients with asthma. Hence, we postulate that a high LTE4 coupled with a low RANTES level might be a surrogate marker for a yet undefined asthma phenotype, a finding that needs to be explored further in a larger cohort of asthmatic patients.

CHAPTER 9: EVALUATION OF THE UTILITY OF SERUM BIOMARKERS OF AIRWAY REMODELLING AND ANGIOGENESIS IN ASSESSING AIRWAY INFLAMMATION WHEN COMPARED TO CATEGORIES OF ASTHMA CONTROL AS DEFINED BY THE ACQ

9.1 Introduction

One of the major drivers of poor outcomes in patients with asthma is progression to airway remodelling and fibrosis by persistent and inadequately treated airway inflammation (451). The fallacy in our assessment of patients with asthma has been the assumption that by titrating therapeutic interventions to normalise symptoms and improve airflow dynamics, the inflammation present in the airways has been adequately suppressed (7, 371). Unfortunately, inflammation may persist despite normalisation of spirometric parameters and control of asthmatic symptoms (66). The most recent GINA guidelines have identified two major goals of asthma therapy (8). These goals are achieving and maintaining asthma control but, more importantly, also reducing the future risk to the patient. Unfortunately, the relationship between these two targets (i.e. achieving control and reducing future risk) is not well understood (343).

What is evident from previous studies is that persistent subclinical inflammation, even in the well-controlled asthmatic patient, leads to micro-architectural changes within the airways loosely termed “airway remodelling” (66, 68, 452). Amongst the most important factors determining future risk in asthmatic patients is airway remodelling. Airway remodelling predisposes patients to impaired lung development and accelerated loss of lung function (452). Currently, the best marker of airway remodelling is the measurement of the reticular basement membrane (RBM) thickness in histological specimens of the airways, obtained via endobronchial and transbronchial biopsies (49). Unfortunately, endobronchial and transbronchial biopsies are highly invasive procedures with a significant risk of complications to the patient. Hence, there is a need for a non-invasive biomarker that will detect airway remodelling and fibrosis, which might aid in defining the relationship between current control and future risk in asthma more clearly.

There are several cytokines known to promote fibrosis, including transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF). The most well documented of these cytokines is TGFβ (453). The importance of TGFβ in asthma has been demonstrated in

studies by Magnan *et al*. (454) and Redington *et al*. (256), which documented increased levels of TGFβ in bronchoalveolar lavage (BAL) fluid in asthmatic patients before and after an antigen challenge. The secreted TGFβ cytokine exists in three isoforms i.e. TGFβ1, TGFβ2 and TGFβ3 (455). TGFβ1 plays a critical role in controlling the immune system. It is secreted by most immune cells, most notably the Treg cells, and acts on various cells promoting cellular proliferation and differentiation (100, 253). In patients with asthma, TGFβ1 through signalling via the Smad pathway promotes transformation of airway epithelium into an epithelial-mesenchymal trophic unit that promotes airway remodelling. Remodelling occurs via differentiation of the airway myoepithelial cells into fibroblasts, which form collagen and secrete growth factors, perpetuating the remodelling process (49). TGFβ1 expression has also been correlated with RBM thickness and importantly, fibroblast number and asthma disease severity (456).

The other two forms of TGF have not been shown to play any significant role in airway remodelling. TGFβ2, also known as glioblastoma-derived T-cell suppressor factor (G-TSF), plays a role in embryonic development and has the ability to suppress the effects of interleukin-dependent T-cell tumours (254). It affects the development of the cardiac, lung, limb, eye and the urogenital systems. TGFβ3 regulates cellular adhesion molecules and extracellular matrix formation, as well as lung development (252). In asthma, there is greater expression of TGFβ1 mRNA that correlates with the severity of asthma and the degree of sub-epithelial fibrosis (252, 255). Release of TGFβ1 into BAL fluid has been documented after segmental airway allergen challenge and therefore, participates in modulating the T lymphocyte response in the airways of asthmatic patients (256).

Furthermore, instrumental to the airway remodelling process is neovascularisation of the airway basement membrane (453). This has been confirmed on histology specimens of patients with severe asthma who have airway remodelling, and it is driven primarily by the cytokine VEGF which is a potent angiogenic cytokine (457, 458).

The presence of airway remodelling was suggested in the present study by a decreased baseline FEF 25-75 in patients with totally and well-controlled asthma (i.e. in patients with normal FEV1 and appropriate symptom control - Chapter 6). With this in mind, the next objective of this thesis was to undertake a study to determine whether certain serum biomarkers of fibrosis and angiogenesis (i.e. VEGF and three subtypes of TGFβ (viz. TGFβ1, TGFβ2 and TGFβ3))

demonstrated a significant association and/or correlation with asthma control as defined by the ACQ (59). For a better appraisal of these pro-fibrotic molecules, they were also compared with the non-specific inflammatory biomarkers CRP and SAAP, as well as to cytokines known to promote a predominant neutrophilic inflammatory/Th2-low phenotype (i.e. IL-1, IL-2, IL-8, TNFα and IFNγ,) and those cytokines known to promote a more eosinophilic/Th2 high phenotype (i.e. IL-4, IL-5 and IL-13). These biomarkers, which have been discussed in Chapter 7, have also been included in this chapter to allow for their correlation with the biomarkers of angiogenesis and fibrosis.

9.2 Design and Study Population

This study was designed as a prospective, single centre, cross-sectional study of patients with confirmed asthma attending a dedicated specialist asthma clinic with approval to conduct the study received from the University of the Witwatersrand Human Research Ethics committee (Protocol Number M060401- Appendix 3). The specifics of the design and population cohort are the same as the study population in Chapter 5 and have been described in detail in Chapter 4. In an attempt to reduce the statistical "noise to signal" from confounders that might dilute the study results, strict inclusion and exclusion criteria as described in Chapter 4 were applied. The reason for the application of strict inclusion and exclusion criteria is that this is a proof of concept study trying to identify a biomarker that might be affected by any concomitant co-existing illnesses. A total of 76 patients fulfilled the criteria for inclusion into the study.

9.3 Methods

A detailed description of the methodology employed in the present study has been described in Chapter 4. For the purposes of standardisation within this thesis, all patients completed the Asthma Control Questionnaire (ACQ), which was used as the basis for the assessment of asthma control in the entire cohort of patients studied (Appendix 1) (58). As indicated in Chapter 4, the population of patients studied were categorised into two groups, based on their level of control. **Group 1** consisted of the patients categorised into controlled (CC) and uncontrolled (UC), based on an ACQ level of 1.5. **Group 2** consisted of the same cohort of patients categorised by the ACQ into totally controlled (TC) having an ACQ < 0.75, well controlled (WC) having an ACQ of between 0.75 and 1.5 and uncontrolled (UC) with an ACQ > 1.5 (59). In all the studies, the evaluation was first for **group 1** (i.e. primary/initial analysis: controlled versus uncontrolled asthmatic patients) and thereafter, the **group 2** analysis (i.e.

subsequent/secondary analysis: totally controlled versus well controlled and versus uncontrolled asthmatic patients).

Of importance to this chapter, is that multi-analyte analysis of TGFβ1, β2, β3, was performed using the Milliplex TGFβ1,2,3, kit (Millipore, TGFβ-64K-03) according to manufacturer's instructions. Similarly, interleukins, TNFα, IFNγ and VEGF were analysed using the Bio-Plex Pro™ Human Cytokine Standard Group (BioRad, 171D50001) according to manufacturer's recommendations. Levels of the multiplexed analytes were measured on a Bio-Plex 200 (Bio-Rad, CA, USA) and concentrations were determined using a 5-PL algorithm and reported as fluorescent intensity units (FU). The rationale for reporting as FU, as well as a brief outline of the multiplex technology has been discussed in Chapter 4. A detail description of the exact methodology of the preparation and measurement of the analytes is included in Appendix 10.

9.4 Statistical Analysis

Methodology detailing the basic principles of the statistical analyses applied, including population size calculations, has been discussed in Chapter 4. The population of patients studied were 76 in total. The Shapiro-Wilks normality test indicated that the data reported was not normally distributed; hence, non-parametric statistical tests were used for all the analyses performed.

The results were reported in both a table format, as well as graphically using a box-and-whisker plot where applicable. The results were represented in a dual format for ease of reference and interpretation. The results included in the tables were reported as medians together with the 95 percent (%) confidence intervals (CI). The box-and-whisker plot displayed the data using the standard five number summary: top and bottom boundary of the box indicating the 75th and 25th percentiles, the line within the box depicted the median and the whiskers represented the minimum and maximum values measured.

Results reported in the tables that were statistically significant are highlighted in bold black text with an asterisk *, whereas those results that had a clinically significant correlation are highlighted in bold black text with a double asterisk **, for easy recognition. The corresponding p or Rho (r) values are represented in the associated figures in **red**.

All the laboratory investigations, measured as continuous variables, were compared to the categories of asthma control, first for **group 1** and then for **group 2**. For the patients categorised into **group** 1 (i.e. controlled versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Wilcoxon rank sum test. For the patients categorised into **group 2** (i.e. totally controlled versus well-controlled and versus uncontrolled asthmatic patients), the association between the variable measured and the level of asthma control was analysed using the Kruskal-Wallis one-way ANOVA test. The Bonferroni correction was applied to the analysis of the **group 2** cohort to control for multiplicity.

In all testing a p-value < 0.05 was considered significant, except where the Bonferroni correction was applied.

Specific to this chapter, is that the levels of all biomarkers measured via the bioplex assays were reported as fluorescent intensity units (FU). When the level of a biomarker was below the lower limit of detection, it was assigned half the value of the lowest limit of detection. When the level of a biomarker was above the upper limit of detection, it was assigned double the value of the uppermost limit of detection. These adjustments for outlying data have been verified in the literature as an acceptable method for data correction (337, 338), and are recommended by the manufacturers of the analyte kits as well.

As outlined in Chapter 4, the reason for reporting in fluorescent intensity units (FU) as opposed to standard international units (SI) was that fluorescent intensity is the actual measurement (raw data) obtained from the microplate-based multiplex immunoassay system. By using the raw data in FU as opposed to diluting the data with several correction steps, using pre-defined manufacturer provided control samples to determine the equivalent SI units, the statistical analysis and results obtained were improved (Appendix 6 and 7).

A Spearman's rank correlation was used to assess the correlation between the ACQ and all other variables measured. For the interpretation of the Spearman's rank correlation, as outlined in Chapter 4, only $r > 0.6$ were reported as a having a strong correlation, even though a $r < 0.6$ may have been statistically significant with a $p < 0.05$.

Data analysis of the association between the medication used by the patient and the levels of the biomarker measured, was performed using the Wilcoxon rank sum test.

The statistical analyses and graphics were performed using the SPSS software, version 12 (**SPSS**, Chicago, Illinois), Excel software (Microsoft, Albuquerque, New Mexico) and GraphPad Software Inc. (La Jolla, California).

9.5 Results

There was no significant difference between the levels of any of the molecules of remodelling (i.e. TGFβ1, β2, β3) and angiogenesis (VEGF) when comparing the controlled with the uncontrolled asthmatic patients (**group 1** analysis) (Table 9.1; Figure 9.1). None of the TGFβ subclasses, nor the angiogenic molecule VEGF demonstrated any correlation with the ACQ score (Table 9.1; Figure 9.2).

The non-specific inflammatory biomarkers (i.e. CRP and SAAP), IL-2, the major cytokines responsible for perpetuating eosinophilic (IL-4, IL-5 and IL-13), as well as the major cytokines responsible for perpetuating neutrophilic airway inflammation (i.e. IL-8 and TNFα) are reported again in Tables 9.1 and 9.2. These biomarkers are repeated in this chapter to allow for a further correlation analyses with the markers of remodelling and angiogenesis. However, as reported in Chapter 7, with the exception of IL-2, there was no significant difference when comparing the controlled with the uncontrolled asthmatic patients (**group 1** analysis). There was also no clinically relevant correlations between these cytokine molecules measured and the ACQ score (Table 9.1; Figures 9.1, 9.2).

Table 9.1: CRP, SAAP, VEGF, TGFβ1, β2, β3 and cytokines levels comparing controlled and uncontrolled asthmatic patients (**group 1**) and the correlation between CRP, SAAP, VEGF, TGFβ1, β2, β3 and the ACQ score

Biomarker[+]	Controlled n=51[1]	Uncontrolled n=24	Controlled vs Uncontrolled[2] (p value)	Rho values (p-values) n= 75[1,3]
IL-1β	10.87 (9.99 - 11.81)	11.04 (9.52 - 12.81)	0.93	0.0152 (0.8972)
IL-2	19.19 (16.57 - 22.23)	26.1 (20.06 - 33.95)	**0.02***	0.3514 (0.002)
IL-4	12.28 (11.42 - 13.20)	13.1 (10.43 - 16.46)	0.77	0.084 (0.4735)
IL-5	10.37 (8.76 - 12.28)	10.42 (7.78 - 13.95)	0.82	0.113 (0.3346)
IL-8[4]	25.04 (22.84 - 27.44)	24.09 (21.21 - 27.36)	0.75	0.0782 (0.5077)
IL-13	21.39 (18.47 - 24.79)	22.45 (15.36 - 32.81)	0.87	0.0645 (0.5823)
IFNγ	16.62 (15.19 - 18.19)	17.88 (14.99 - 21.33)	0.21	0.1182 (0.3126)
TNFα	11.61 (10.37 - 12.99)	11.77 (9.79 - 14.16)	0.63	0.1489 (0.2022)
VEGF	138.86 (114.12 - 168.86)	178.33 (125.72 - 252.98)	0.24	0.1965 (0.091)
TGFβ1	4078.7 (3668.7 - 4534.5)	4193.6 (3586.5 - 4903.6)	0.60	0.1127 (0.3358)
TGFβ2	89.3 (79.4 - 100.5)	97.8 (81.6 - 117.3)	0.52	0.0807 (0.4915)
TGFβ3	7.6 (7.5 - 7.9)	7.2 (6.9 - 7.6)	0.13	-0.1727 (0.1385)
CRP	1604 (1136 - 2267)	2852 (1388 - 5861)	**0.03***	0.2127 (0.0669)
SAAP	657(452 - 954)	1078 (458 - 2537)	0.30	0.1041 (0.374)

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
1: Biomarker analysis was of 51 controlled patients as one result was totally aberrant and discarded.
2: Wilcoxon rank sum test. * = $p < 0.05$
3: Spearman rank correlation between ACQ score and other variables.
4: IL-8 analysis of one additional patient in the uncontrolled group (n=23) was not measurable by bioplex.
The results are reported as medians together with the 95% confidence intervals (CI).
CRP: C-reactive protein, IFNγ: Interferon gamma, IL: Interleukin, n: Number, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta, TNFα: Tumour necrosis factor alpha, VEGF: Vascular endothelial growth factor.

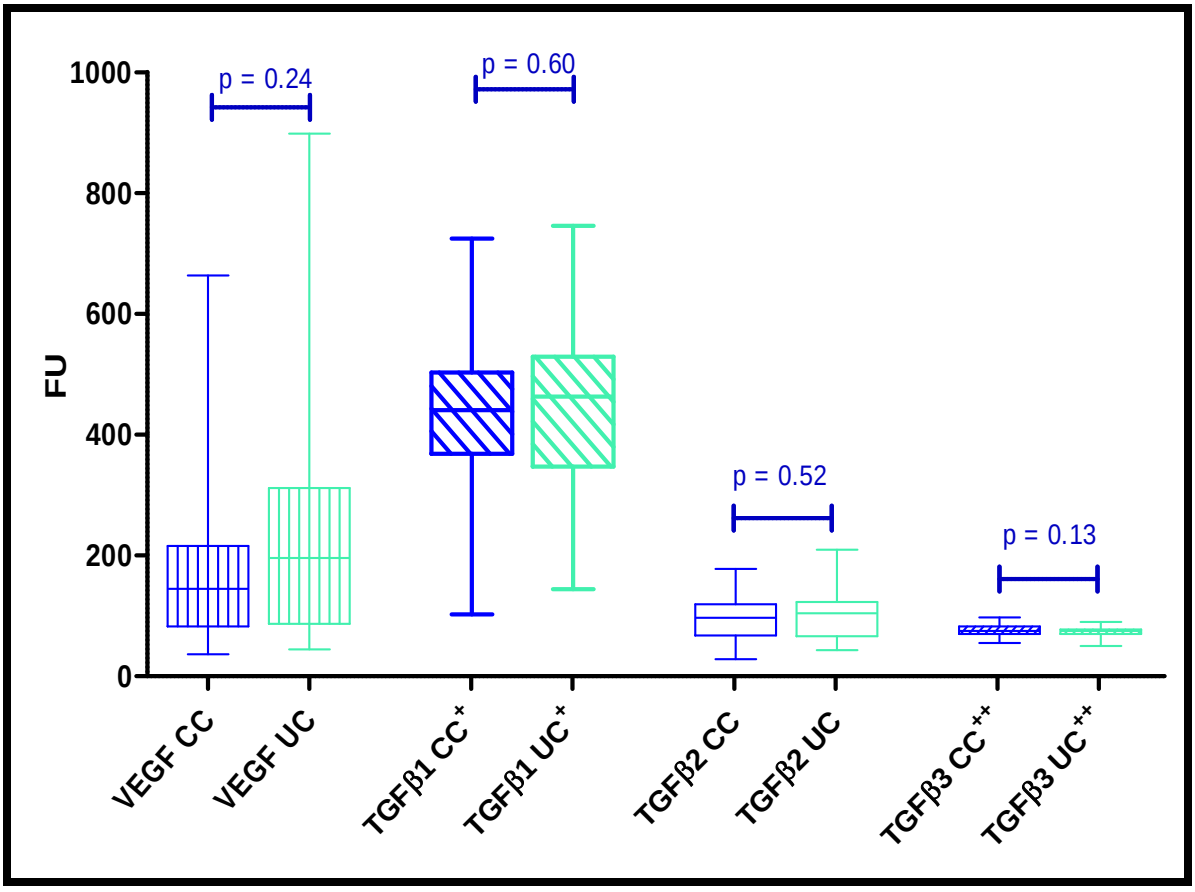

Figure 9.1: TGFβ1, β2, β3 and VEGF, levels comparing controlled and uncontrolled asthmatic patients (**group 1**)

FU: fluorescent intensity units, TGFβ: Transforming growth factor beta, VEGF: Vascular endothelial growth factor.

To improve the diagrammatic representation of the figure the following scales were applied:

$^{+}$TGFβ1 (FU x 10^{-1}), $^{++}$ TGFβ3 (FU x 10).

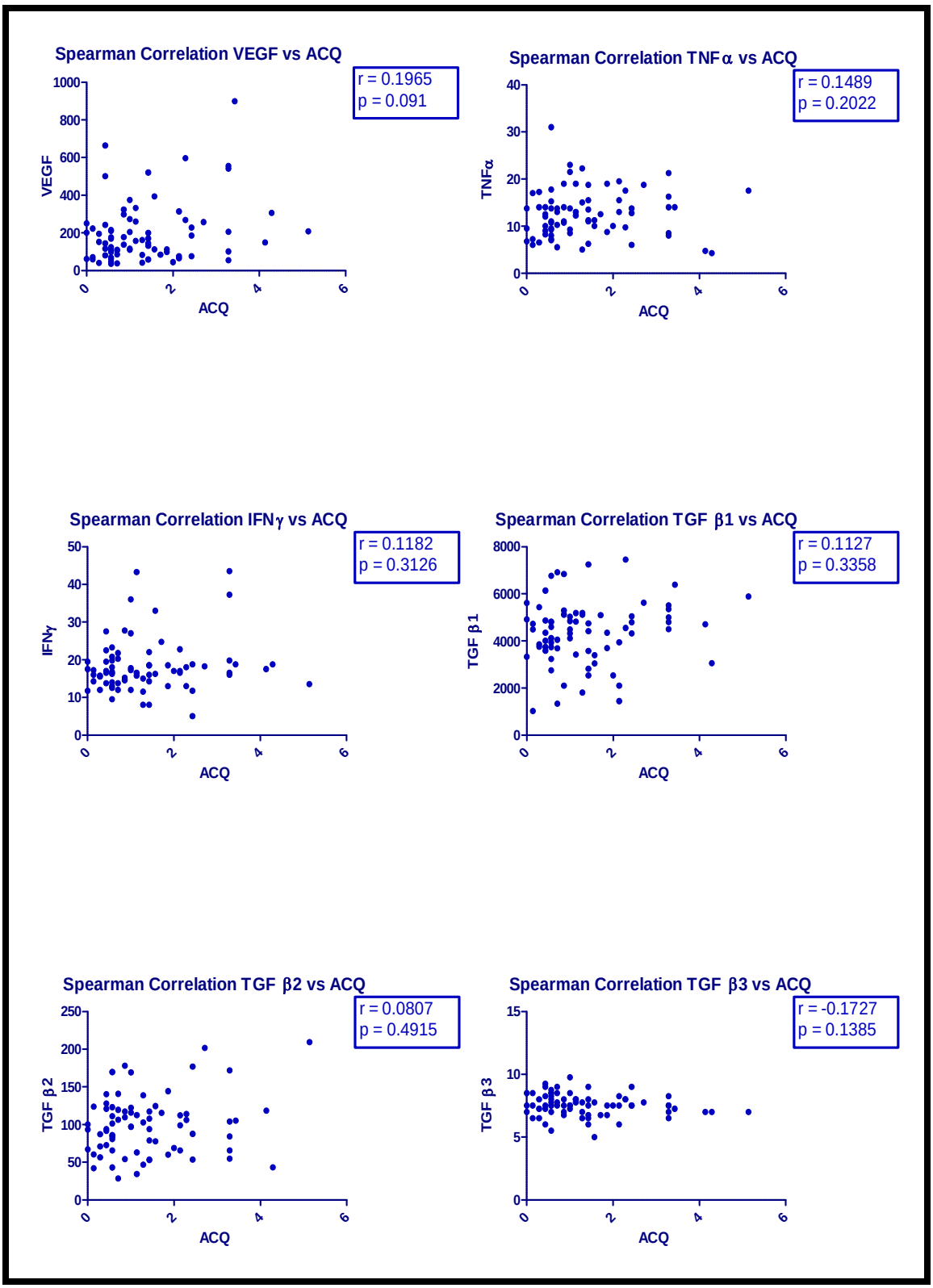

Figure 9.2: Correlation between the levels of CRP, SAAP, VEGF, TGFβ1, β2, β3 and cytokines levels and the ACQ score in the study population

ACQ: Asthma Control Questionnaire, IFNγ: Interferon gamma, TGFβ: Transforming growth factor beta, TNFα: Tumour necrosis factor alpha, VEGF: Vascular endothelial growth factor. Spearman rank correlation.

There was no significant difference between the levels of any of the molecules of remodelling (i.e. TGFβ1, β2, β3) and angiogenesis (VEGF), when comparing the totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2** analysis) (Table 9.2; Figure 9.3). There was a tendency for TGFβ3 to be higher in the totally controlled group when compared with the uncontrolled group. The median TGFβ3 level in the totally controlled group was 7.68 FU (95% CI 7.34 - 8.04), versus a median TGFβ3 level in the uncontrolled group of 7.28 FU (95% CI 6.92 - 7.65) ($p = 0.07$). Conversely, there was also a tendency for VEGF to be lower in the totally controlled group when compared with the well-controlled and uncontrolled groups. The median VEGF level in the totally controlled group was 119.24 FU (95% CI 91.11 - 156.04), versus a median VEGF level of 172.62 FU (95% CI 130.42 - 228.49) in the well-controlled group ($p = 0.04$) and versus a median VEGF level of 178.33 FU (95% CI 125.72 - 252.98) in the uncontrolled group ($p = 0.07$) (Table 9.2; Figure 9.3). However, when the Bonferroni correction was applied, none of the **group 2** analyses reported above yielded any significant results (Table 9.2; Figure 9.3).

Table 9.2: CRP, SAAP, VEGF, TGFβ1, β2, β3 and cytokines levels comparing totally controlled, well-controlled and uncontrolled asthmatic patients (**group 2**)

Biomarker[+]	Totally controlled n=30	Well-controlled n=21[1]	Uncontrolled n=24[2]	Totally controlled vs Well-controlled[1,3] p value	Totally controlled vs Uncontrolled[2,3] p value	Well-controlled vs Uncontrolled[1,2,3] p value
IL-1β	10.77 (9.95 - 11.66)	11.02 (9.20 - 13.18)	11.04 (9.52 - 12.81)	0.46	0.73	0.79
IL-2	17.13 (14.65 - 20.04)	22.56 (17.05 - 29.87)	26.1 (20.06 - 33.95)	0.047	**0.003***	0.45
IL-4	11.85 (10.76 - 13.04)	12.93 (11.51 - 14.53)	13.1 (10.43 - 16.46)	0.25	0.53	0.80
IL-5	9.56 (7.52 - 12.16)	11.65 (9.17 - 14.8)	10.42 (7.78 - 13.95)	0.34	0.55	0.73
IL-8[3]	23.15 (20.9 - 25.64)	28.01 (23.69 - 33.1)	24.09 (21.21 - 27.36)	0.07	0.58	0.18
IL-13	20.71 (16.90 - 25.37)	22.41 (17.83 - 28.17)	22.45 (15.36 - 32.81)	0.60	0.75	0.93
IFNγ	16.36 (14.98 - 17.87)	17.0 (14.05 - 20.56)	17.88 (14.99 - 21.33)	0.94	0.26	0.30
TNFα	10.71 (9.27 - 12.37)	13.03 (10.85 - 15.63)	11.77 (9.79 - 14.16)	0.05	0.19	0.47
VEGF	119.24 (91.11 - 156.04)	172.62 (130.42 - 228.49)	178.33 (125.72 - 252.98)	0.04	0.07	0.96
TGFβ1	3999.5 (3450.2 - 4636.2)	4194.7 (3568.1 - 4931.2)	4193.6 (3586.5 - 4903.6)	0.41	0.44	0.98
TGFβ2	88.6 (75.9 - 103.3)	90.3 (74.1 - 110.3)	97.8 (81.6 - 117.3)	0.82	0.53	0.64
TGFβ3	7.68 (7.34 - 8.04)	7.50 (7.12- 7.90)	7.28 (6.92 - 7.65)	0.32	0.07	0.55
CRP	3531 (2067 - 4994)	2404 (881 - 3927)	6694 (3915 - 9472)	0.09	0.13	0.03
SAAP	3382 (1932 - 5921)	1937 (1090 - 3443)	6018 (1875 - 19314)	0.21	0.53	0.19

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
1: Biomarker analyses was of 21 well-controlled patients as one result was totally aberrant and discarded.
2: IL-8 analysis of one additional patient in the uncontrolled group (n=23) was not measurable by bioplex.
3: Kruskal-Wallis one-way ANOVA test. * p values need to be interpreted as significant if < 0.05/3 = 0.017 (application of Bonferroni correction)
The results are reported as medians together with the 95% confidence intervals (CI).
CRP: C-reactive protein, IFNγ: Interferon gamma, IL: Interleukin, n: Number, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta, TNFα: Tumour necrosis factor alpha, VEGF: Vascular endothelial growth factor.

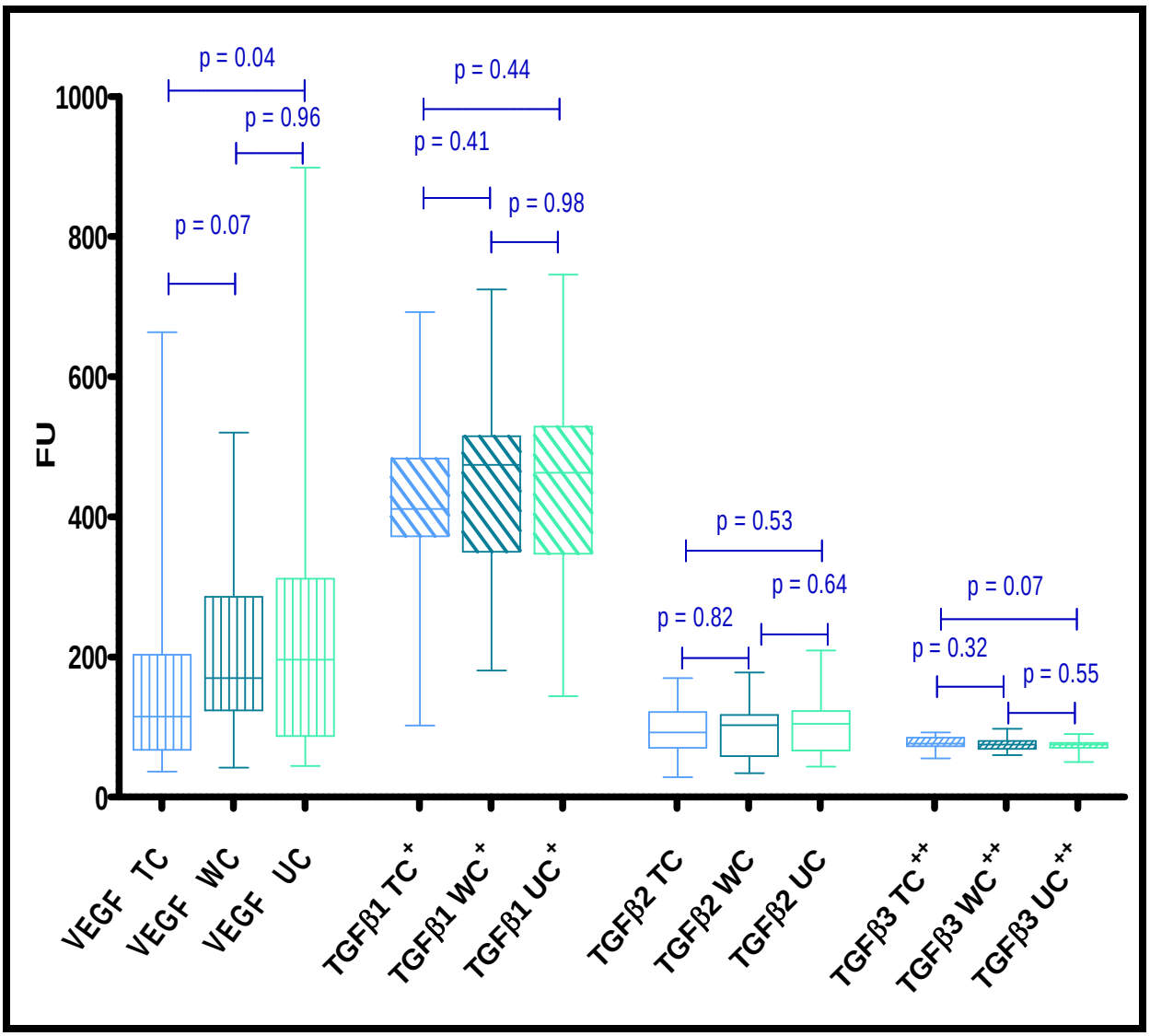

Figure 9.3: Comparison of TGFβ1, β2, β3 and VEGF levels in patients with totally controlled, well-controlled and uncontrolled asthma (**group 2**)
FU: fluorescent intensity units, TGFβ: Transforming growth factor beta, VEGF: Vascular endothelial growth factor.
To improve the diagrammatic representation of the figure the following scales were applied:
$^{+}$TGFβ1 (FU x 10^{-1}), $^{++}$ TGFβ3 (FU x 10).

A scatter-dot plot of CRP, SAAP, TGFβ1, β2, β3, VEGF and Th1 and Th2 cytokines measured for the **group 2** analysis (totally controlled versus well-controlled versus uncontrolled asthmatic patients) is depicted in Figure 9.4.

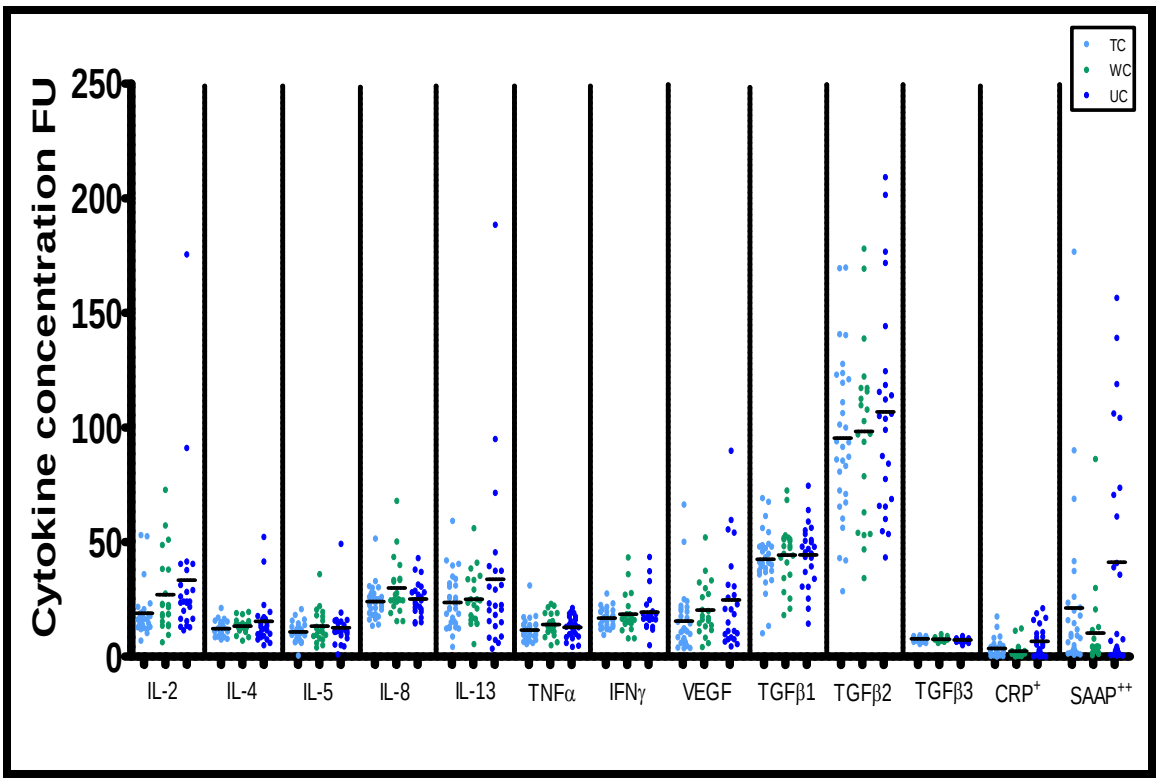

Figure 9.4: Scatter-dot plot of CRP, SAAP, TGFβ1, β2, β3, VEGF and cytokines measured comparing totally controlled, well-controlled and uncontrolled patients (**group 2**)

Scatter-dot plot with bold line representing the median.
CRP: C-reactive protein, FU: Fluorescent intensity units, IFNγ: Interferon gamma, IL: Interleukin, n: Number, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta, TNFα: Tumour necrosis factor alpha, VEGF: Vascular endothelial growth factor.
To improve the diagrammatic representation of the figure the following scales were applied:
$^{+}$ CRP (FU x 10^{-1}), $^{++}$ SAAP (FU x 10^{-2}).

For the completion of the analysis, the correlation between the pro-fibrotic mediators (VEGF and TGFβ1, TGFβ2 and TGFβ3) and nonspecific systemic biomarkers of inflammation (i.e. CRP & SAAP), as well as several serum cytokines (IL-1, IL-4, IL-5, IL-8 and IL-13) was done. (Table 9.3; Figures 9.5, 9.6). There was no clinically relevant correlation between any of the molecules measured and the ACQ score.

Table 9.3: Correlations between the markers of remodelling (**TGFβ1, β2, β3**) and angiogenesis (**VEGF**) and serum biomarkers including, nonspecific markers of inflammation (i.e. **CRP and SAAP**), as well as serum cytokines (**IL-1, IL-2 IL-4, IL-5, IL-8 and IL-13**)

Biomarker+	Rho (r)	p values
TGFβ1 vs CRP	0.16	0.18
TGFβ2 vs CRP	0.13	0.27
TGFβ3 vs CRP	0.01	0.96
TGFβ1 vs SAAP	0.15	0.21
TGFβ2 vs SAAP	0.24	0.04
TGFβ3 vs SAAP	0.07	0.57
TGFβ1 vs IL-2	0.02	0.85
TGFβ2 vs IL-2	0.17	0.15
CRP vs VEGF	0.23	0.05
SAAP vs VEGF	0.26	0.02
IL-4 vs TGFβ1	0.25	0.03
IL-8 vs TGFβ1	0.16	0.17
TNFα vs TGFβ1	0.04	0.71
TGFβ1 vs VEGF	0.32	0.01
TGFβ2 vs VEGF	0.19	0.1
TGFβ3 vs VEGF	0.13	0.27

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
Spearman rank correlation between biomarkers of remodelling (TGFβ1, β2, β3) and angiogenesis (VEGF) and serum biomarkers of inflammation.
CRP: C-reactive protein, IL: Interleukin, n: Number, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta, TNFα: Tumour necrosis factor alpha, VEGF: Vascular endothelial growth factor.

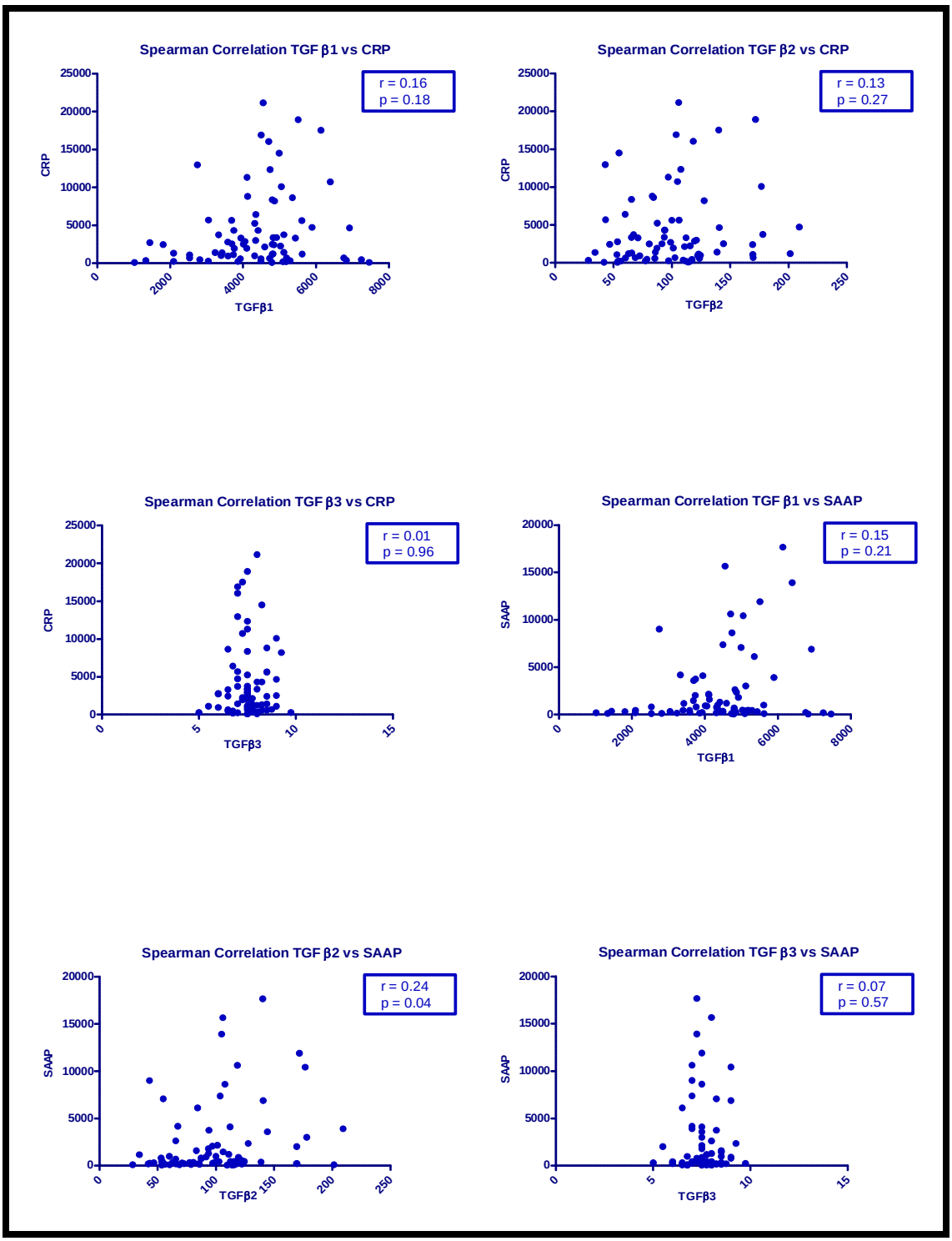

Figure 9.5: Correlation between markers of remodelling and non-specific biomarkers of inflammation

CRP: C-reactive protein, n: Number, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta. Spearman rank correlation.

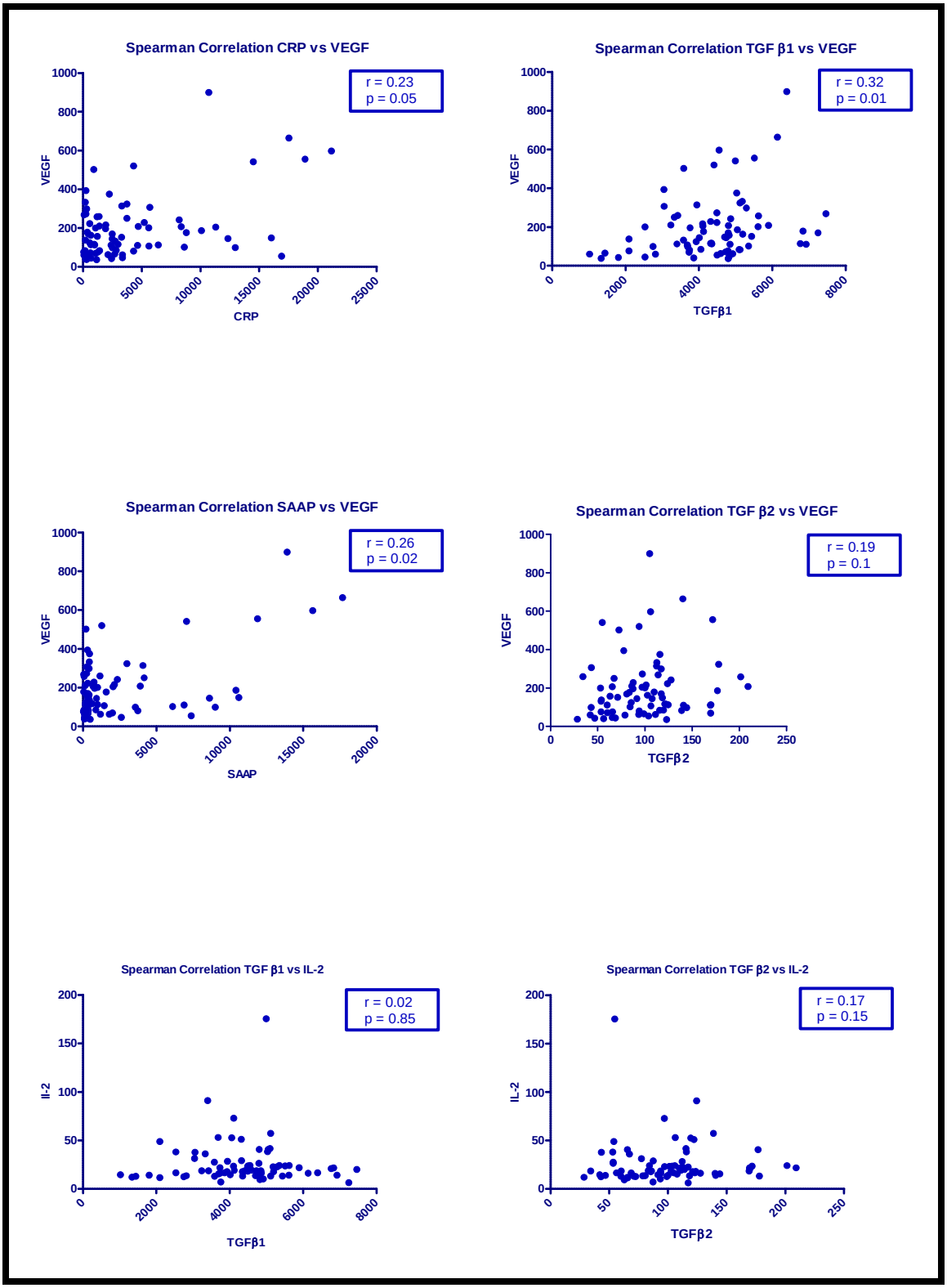

Figure 9.6: Correlation between markers of angiogenesis, remodelling, non-specific biomarkers of inflammation and serum cytokines

CRP: C-reactive protein, IL: Interleukin, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta, VEGF: Vascular endothelial growth factor. Spearman rank correlation.

Finally, to ascertain whether the concentration of any the fibrotic or angiogenic biomarkers measured in this present study were affected by any of the anti-inflammatory therapies the patients were using, a comparison was made between the serum concentrations of the biomarkers, in those patients using, versus those patient not using anti-inflammatory medication. The levels of TGFβ1 was significantly lower in the patients using the moderate doses of an inhaled corticosteroid (ICS) (i.e. budesonide md: ≤ 800 µg/day) with a median of 3940.5 FU (95% CI 3573.42 - 4345.275) versus a median of 4971.06 FU (95% CI 4270.319 - 5786.782) in the group not using moderate doses of budesonide; p = 0.02 (Table 9.4; Figure 9.7). By comparison, the levels of TGFβ1 were significantly higher in the group of patients using the higher dose ICS (i.e. budesonide hd: between 800 and 1600 µg/day) with a median of 5081.86 FU (95% CI 4342.73 - 5946.82), versus a median of 3937.05 FU (95% CI 3576.04 - 4334.50) in the group not using the higher dose ICS; p = 0.01 (Table 9.4; Figure 9.7) (ICS dose category as defined by GINA) (8).

The same difference with respect to the comparison between the use of moderate and high-dose budesonide, and TGFβ2 levels was observed. The levels of TGFβ2 were significantly lower in the patients using the moderate dose ICS with a median of 85.58 FU (95% CI 77.47 - 94.55), versus a median of 125.74 FU (95% CI 98.33 - 160.78) in the group not using the moderate dose ICS; p = 0.02 (Table 9.4; Figure 9.8) (ICS dose category as defined by GINA) (8). Similarly to TGFβ1, the level of TGFβ2 was significantly higher in the group of patients using the higher dose ICS with a median of 122.88 FU (95% CI 94.53-159.74), versus a median of 86.53 FU (95% CI 78.26-95.67) in the group not using higher dose ICS; p = 0.01 (Table 9.4; Figure 9.8).

Table 9.4: Concentrations of the biomarkers measured (i.e. CRP, SAAP, TGFβ1, β2, β3, VEGF and cytokine levels) comparing levels in those patients using, versus those patients not using anti-inflammatory medications

Biomarker+	Montelukast			Budesonide md: ≤ 800 µg/day			Budesonide hd: < 800 and ≤ 1600 µg/day			Prednisone ld: ≤ 7.5 mg/day		
	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value	Using Median (95% CI)	Not using Median (95% CI)	P-value
VEGF	240.33 (143.80-401.66)	136.36 (114.46-162.46)	0.02	142.93 (117.95-173.21)	188.00 (124.97-282.84)	0.28	203.07 (135.28-304.84)	141.26 (116.77-170.89)	0.15	258.95 (119.15-562.76)	142.25 (119.54-169.29)	0.07
TGFβ1	4067.82 (3085.48-5362.90)	4125.14 (3768.17-4515.92)	0.48	**3940.50 (3573.42-4345.28)**	**4971.06 (4270.32-5786.78)**	**0.02***	**5081.88 (4342.73-5946.82)**	**3937.05 (3576.04-4334.50)**	**0.01***	4400.11 (3021.74-6407.23)	4086.88 (3735.62-4471.17)	0.33
TGFβ2	96.71 (65.65-142.45)	90.99 (82.92-99.84)	0.42	**85.58 (77.47-94.55)**	**125.74 (98.33-160.78)**	**0.001***	**122.88 (94.53-159.74)**	**86.53 (78.26-95.67)**	**0.001***	85.76 (46.79-157.18)	92.62 (84.15-101.93)	0.54
TGFβ3	7.47 (7.04-7.92)	7.51 (7.27-7.74)	0.76	7.55 (7.33-7.78)	7.29 (6.77- 7.84)	0.22	7.44 (7.00-7.92)	7.51 (7.28-7.75)	0.45	7.23 (6.73-7.77)	7.53 (7.31-7.75)	0.2

+: All biomarkers are reported as fluorescent intensity units (FU), unless otherwise indicated.
Wilcoxon rank sum test. * = $p < 0.05$
Biomarker analysis was of 51 controlled patients as one result was totally aberrant and discarded.
Budesonide hd: high-dose between 800 and 1600 µg/day; Budesonide md: moderate-dose ≤ 800 µg/day; Prednisone ld: low-dose ≤ 7.5 mg/day
CRP: C-reactive protein, SAAP: Serum amyloid associated protein, TGFβ: Transforming growth factor beta, µg/day: Microgram per day, mg/day: Milligram per day.

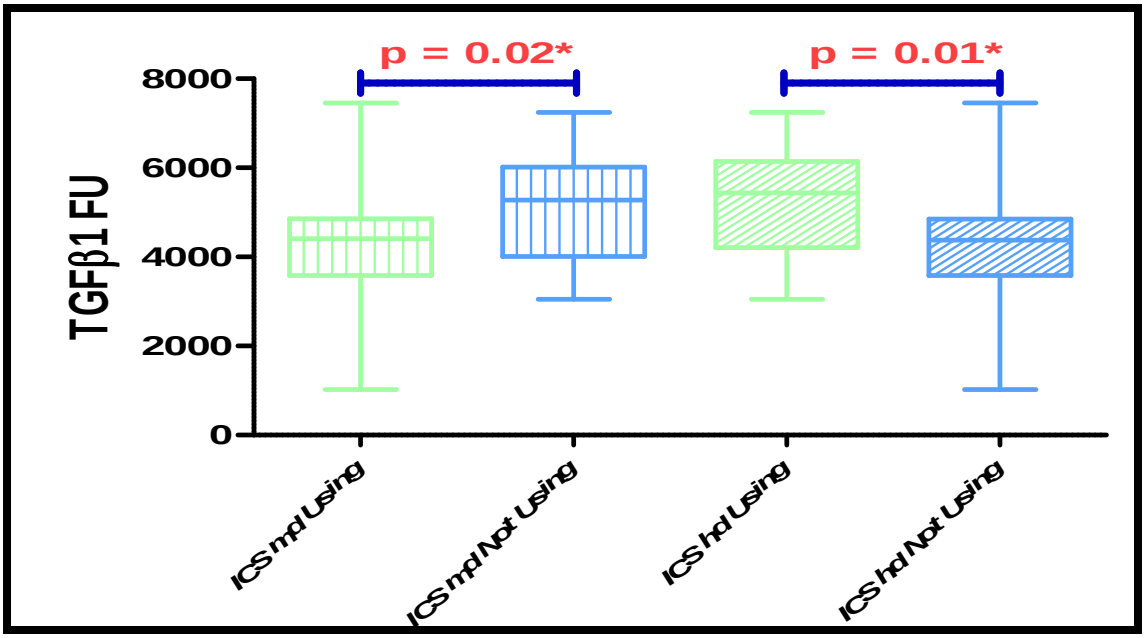

Figure 9.7: TGFβ1 levels comparing patients using and not using ICS therapy
* p values reported as significant are depicted in **red**.
FU: Fluorescent intensity units, ICS md: moderate-dose budesonide ≤ 800 µg/day, ICS hd: high-dose budesonide between 800 and 1600 µg/day, TGFβ: Transforming growth factor beta. Wilcoxon rank sum test.

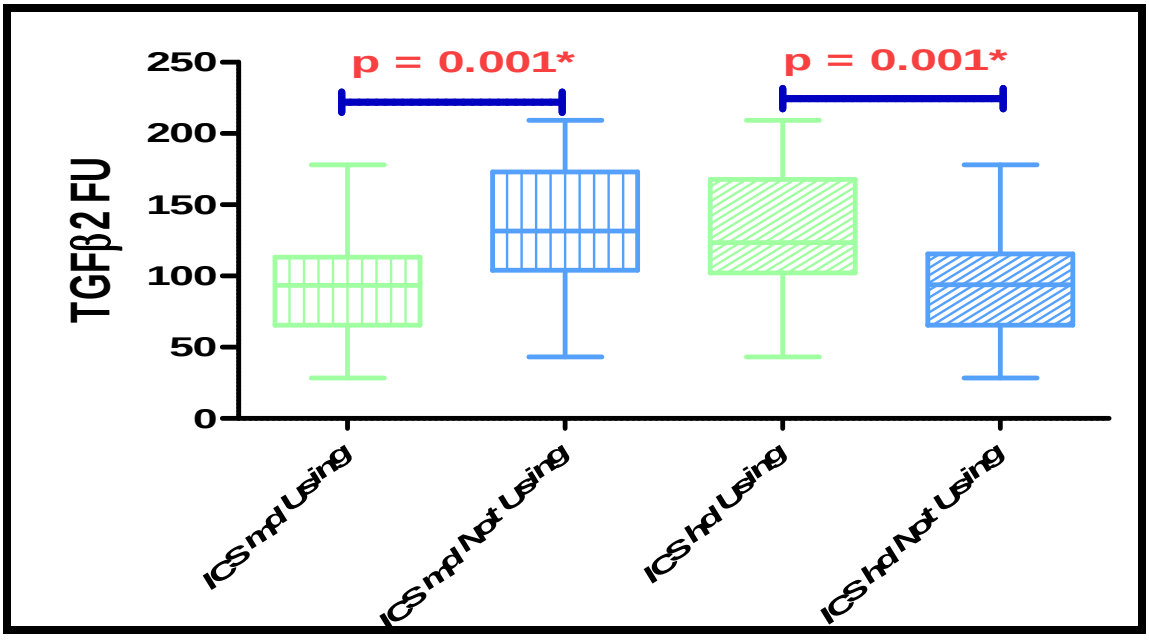

Figure 9.8: TGFβ2 levels comparing patients using and not using ICS therapy
* p values reported as significant are depicted in **red**.
FU: Fluorescent intensity units, ICS md: moderate-dose budesonide ≤ 800 µg/day, ICS hd: high-dose budesonide between 800 and 1600 µg/day, TGFβ: Transforming growth factor beta. Wilcoxon rank sum test.

9.6 Discussion

The latest GINA guidelines emphasize therapeutic strategies to maintain asthma control, but more importantly, focus on reducing future risk as well. However, the relationship between these two targets (i.e. asthma control and future risk) in patients with asthma is not well understood (8, 343). The reason for this dichotomy is that in some patients with apparently controlled asthma, airway inflammation may persist leading to structural changes within the airways - a process defined as airway remodelling (66). Airway remodelling develops when the airway repair process is dysregulated by inflammation, with excessive collagen deposition leading to irreversible structural airway damage. This subclinical airway inflammation is a significant risk factor for future asthma exacerbations, which predispose the airways to further damage and subsequent remodelling (343, 452).

The major driver of airway remodelling is repeated epithelial injury and subsequent epithelial cell activation (459). Airway epithelial cells, once activated, promote inflammatory cell recruitment and also mesenchymal signalling, which induces myofibroblast transformation (460). The major cytokine driving the remodelling process is TGFβ as it induces myofibroblast transformation and stimulates collagen synthesis (460). TGFβ also promotes angiogenesis in the basement membrane by inducing VEGF transcription (453). Airway remodelling develops when the repair process is dysregulated, with excessive collagen deposition leading to irreversible structural airway damage (461). With the above in mind, this study was performed to try to identify a non-invasive biomarker that might aid in identifying evidence of fibrosis and angiogenesis in patients categorised according to the categories of asthma control as defined by the ACQ.

9.6.1 The association between TGFβ levels and asthma control

In the **group 1** analysis (controlled versus uncontrolled asthmatic patients), there were no significant differences in the levels of any of the TGFβ subclasses (Table 9.1; Figure 9.1). There was also no correlation between the ACQ score and any of the TGFβ subclasses (Table 9.1; Figure 9.2). For the **group 2** analysis (totally controlled versus well-controlled versus uncontrolled asthmatics patients), there were no significant differences in the levels of any of the TGFβ subclasses when compared to the different categories of asthma control (Table 9.2; Figure 9.3).

However, equally important in the present study was that the levels of TGFβ1 and TGFβ2 were significantly higher in the patients using high-dose budesonide when compared with those patients not using high-dose budesonide ($p = 0.01$ and $p = 0.001$ respectively), and by comparison, significantly lower in the patients using moderate-dose budesonide when compared with those patients not using moderate-dose budesonide ($p = 0.02$ and $p = 0.001$ respectively) (Table 9.4; Figures 9.7, 9.8). A likely explanation for this finding is that the patients using high-dose inhaled corticosteroids (ICS) were more likely to be uncontrolled or difficult to control asthmatic patients, and therefore had elevated TGFβ levels. This explanation is supported by the documentation that only 15% (8/52) of the controlled group of asthmatic patients were using moderate-dose budesonide, whereas 29% (7/24) of the uncontrolled group of asthmatic patients were using high-dose budesonide (Tables 5.7, 9.4). Therefore, the interpretation of this data analysis is that TGFβ levels were elevated in a subgroup of uncontrolled asthmatic patients not defined by the ACQ, but rather categorised by their use or not of high-doses ICS.

A postulated pathogenesis for the significantly higher TGFβ levels in the above group of uncontrolled asthmatic patients, might be that in these patients, airway inflammation is modulated by the upregulation of Treg cells by TGFβ. Treg cells have a significant immunosuppressive potential within the airways in patients with asthma (103). Their function is regulated by the intracellular transcription factor forkhead box P3 (Foxp3) (101), which is the main control gene in the development of these cells (102), and Treg cells limit airway inflammation via the production of cytokines IL-10 and TGFβ (100). Furthermore, via a positive feedback loop, TGFβ plays an important role in increasing the expression of Foxp3, which promotes the transition of naïve T cells toward a Treg endotype. This Treg cell endotype has the potential to modulate other T cell activation, including Th1 and Th2 cells, by inhibiting the expression of effector cytokines including IL-4, TNFα, IFNγ, IL-17 and IL-21 (104).

Tsai *et al*. (462), reported significantly higher levels of TGFβ1 (40.73 ± 15.95 pg/mL) in a cohort of asthmatic children recovering from an acute exacerbation after treatment with oral corticosteroids for 7 days when compared with a non-asthmatic healthy control group of patients (28.77 ± 8.35 pg/mL); $p < 0.05$. These findings have been confirmed in another study by Liu *et al*. (463), which documented significantly lower levels of TGFβ1 in a group of paediatric asthmatic patients compared with a non-asthmatic healthy control group of patients. Yalcin *et al*. (464), reported a similar finding of elevated TGFβ levels in severe, persistent

asthmatic patients initiated on anti-IgE monoclonal antibody therapy. However, TGFβ levels remained elevated even after four months of therapy compared with their baseline level (p=0.013). More importantly, therapy with anti-IgE monoclonal antibodies was associated with a significant improvement (from a mean of 10 to a mean of 22) in the level of asthma control as defined by the ACT (464). These published data quoted above imply that severe persistent or uncontrolled asthmatic patients have higher levels of TGFβ, a finding similar to this present study.

In conclusion, despite the non-significant results in the primary analysis (**group 1**) and secondary analysis (**group 2**) reported in our study, data from the published literature, as well as analysis of TGFβ levels in comparison to medication used in the present study, suggests that serum TGFβ levels could be used as a biomarker for clinical asthma control (462-464). However, the exact immunopathogenesis and implications of this elevation in TGFβ levels in the asthmatic patient on high-dose ICS, and whether it is a biomarker for airway remodelling needs to be confirmed with further studies.

9.6.2 The association between VEGF levels and asthma control

The second molecule measured as a potential biomarker of airway remodelling was vascular endothelial growth factor (VEGF). VEGF is a potent angiogenic factor and histology specimens of patients with severe asthma who have airway remodelling demonstrate significant neovascularisation within the airways (457). In the **group 1** analysis, there was no significant difference in the levels of VEGF when the controlled group of patients was compared with the uncontrolled group of patients. There was also no significant correlation between the ACQ score and the VEGF levels measured. For the **group 2** analysis, there was a trend to lower levels of VEGF in the totally controlled group when compared with the well-controlled group (p = 0.05), as well as when the totally control group was compared with the uncontrolled group (p = 0.07), but this difference became insignificant following the Bonferroni correction (Table 9.2; Figure 9.3).

Koskal *et al.* (465), reported a significant positive correlation between VEGF levels and duration of asthma, a finding confirmed by Kanazawa *et al.*(466), and Lee *et al.* (467). A subsequent study by Lee *at al.* (468), assessing the potential value of VEGF as a biomarker for asthma, reported VEGF levels in serum to be significantly increased in stable asthmatic patients when compared with the levels in healthy control subjects. Furthermore, the VEGF levels were

even higher during an acute exacerbation than in the stable asthmatic patients. However, in that study there was no correlation between VEGF levels and the level of asthma control as defined by the asthma control test (AST), a finding which could have confirmed the results of the present study (468).

9.7 Conclusion

In the present study, while the serum biomarkers TGFβ and VEGF did not demonstrate any significant association with the level of asthma control as defined by the ACQ, they have nonetheless provided us with potential tools for a more specific and non-invasive assessment of airway inflammation. The analysis of the relationship between asthma medications and serum TGFβ levels suggested an alternative interpretation in that those patients on high-dose budesonide, who intuitively, were likely to be asthmatics that were uncontrolled or difficult to control, had higher levels of TGFβ than those asthmatics on moderate dose of budesonide, who intuitively, were likely to be better controlled. As such, in the present study when the data was interpreted differently, it suggested that controlled asthmatic patients when compared with uncontrolled asthmatic patients had lower levels of TGFβ1 and TGFβ2. Therefore, TGFβ will need to be validated as a potential biomarker for asthma control and VEGF will need to be validated as a potential biomarker for asthma exacerbations. The implications of the elevated TGFβ levels in this subgroup of uncontrolled asthmatic patients categorised by their use or not of high-doses of budesonide, as well as, whether TGFβ is a biomarker for airway inflammation within this group of uncontrolled asthmatic patients needs to be confirmed with further studies.

CHAPTER 10: CONCLUSIONS AND LIMITATIONS

There has been a paradigm shift in our understanding of asthma. It is becoming increasingly evident that asthma is an inflammatory lung disease, not only affecting the central airways, but also involving the small airways and the lung parenchyma (22, 72, 319). The inherent pathogenesis, causing calibre changes in the small airways, is inflammation (320). The fallacy in our current assessment of patients with asthma, with an increasing focus on asthma control, has been the assumption that by titrating therapeutic interventions to normalise symptoms and improve airflow dynamics (i.e. targeting asthma control) we will have suppressed the inflammation present in the airways (7). However, should this not be correct, the result of persistent airway inflammation might be the occurrence of structural damage within the airways, with airway remodelling, fibrosis and progressive loss of lung capacity over time (67, 68). Hence, a strategy where 'inflammometry' identifies the patient with persistent airway inflammation would potentially prevent over treatmentbut more importantly under treatment (326, 327).

Thus, this thesis was based on a series of studies with the aims to firstly identify relatively simple, non-invasive, and reproducible markers which might aid in the documentation of the presence or absence of airway inflammation, and in particular inflammation within the small airways, in patients with asthma. Secondly, the studies aimed to determine whether there was any association between these makers of inflammation and categories of asthma control as defined by the Asthma Control Questionnaire (ACQ) (331-333). A summary of the pertinent finding of each of the chapters is summarised below.

10.1 Chapter 5

In this study, we identified and described a group of asthmatic patients attending a tertiary referral asthma clinic at a University Hospital in Johannesburg, South Africa. Furthermore, we characterised their asthma control as defined primarily by the ACQ, and compared the agreement between the ACQ, the ACT and the physicians asthma rating (AST) in assessing asthma control. We documented that the two internationally validated asthma control questionnaires, namely the ACQ and the ACT, performed well and agreed not only with each other, but also with an independent physician's assessment of the level of asthma control. The ACQ performed robustly in the assessment of control, even when the spirometric measurements were omitted from the composite score (i.e. ACQ 6), enhancing its applicability as a tool in asthma management in resource - poor settings, where facilities for use of spirometry are

limited. Unfortunately, the study was not able to confirm the value of combination LABA-ICS therapy in attaining asthma control since this form of therapy was not routinely available to the patients in the study setting.

10.2 Chapter 6

In this study, using the same cohort of patients identified and described in Chapter 5, we measured small airway calibre via impulse oscillometry (IOS) and evaluated the association and correlation of IOS with conventional spirometry, as well as with asthma control, as defined by the ACQ. The purpose of this was for a better appraisal of the small airways in asthmatics, as the assessment of the small airways remains elusive for the practising clinician. We documented that even prior to bronchodilator use, there was a significant reduction in FEF 25-75 levels, expressed as percentage predicted, in the totally controlled, well-controlled and uncontrolled groups of asthmatic patients with medians of 35.7% , 27.3% and 17.3% respectively (Table 6.4; Figures 6.6, 6.7). This was also true for the post-bronchodilator FEF25-75 values (Table 6.5; Figures 6.6, 6.7). Furthermore, there was no significant difference in percentage change in FEF 25-75 post-bronchodilation when the totally controlled group was compared with the uncontrolled group ($p = 0.91$) or when the well-controlled group was compared with the uncontrolled group ($p = 0.55$) (Table 6.6; Figures 6.6, 6.7). This finding alludes to the possibility of an element of pre-existing small airway remodelling even in the groups of patients classified as being controlled by the ACQ.

However, equally importantly, the FEF 25-75 values in the totally controlled, well-controlled and uncontrolled asthmatic patients demonstrated an absolute post-bronchodilator reversibility of 32%, 27% and 31% respectively, confirming the presence of bronchial airway hyper-reactivity (BAH) in the small airways, even in apparently controlled asthmatic patients (Table 6.6).

We also documented a significant association between the main parameters of the small airways measured via IOS and asthma control as defined by the ACQ. The R5-R20 parameter, which is the measurement of small airway resistance, was significantly higher pre-bronchodilation in the uncontrolled group when compared with the controlled group. Furthermore, there was a significant decrease in the small airway resistance measured by the R5-R20 when comparing pre- and post-bronchodilator values for the controlled group (24.6 % vs 18.5%; $p = 0.0001$) and pre- and post-bronchodilator values for the uncontrolled group (33.5% vs 23.1%; $p = 0.0001$).

Of note, this improvement in airway resistance in the small airways post-bronchodilator (BAH), for the controlled as well as the uncontrolled group, as demonstrated by IOS, was only evident in the uncontrolled group of patients when assessed by spirometry (Tables 6.1, 6.2, 6.7, 6.8; Figures 6.1, 6.2, 6.9, 6.10). Thus, IOS when compared with spirometry was more sensitive in assessing changes in airway resistance in the small airways (BAH).

The low-frequency reactance area AX (Goldman Triangle) was also significantly lower in the controlled group when compared with the uncontrolled group of asthmatic patients (**group 1** analysis), for both the pre- and post-bronchodilator values ($p = 0.0013$ and $p = 0.0042$, respectively) (310, 312). R5, the measurement of total airway resistance, was also found to be significantly lower in the controlled group when compared with the uncontrolled group of asthmatic patients (**group 1** analysis) ($p = 0.004$ pre-bronchodilator and $p = 0.005$ post-bronchodilator, respectively).

However one of the major limitations in using IOS is a lack of standardised reference values to correct IOS parameters for age and height (364). Another limitation in the interpretation of IOS measurements is that they cannot be corrected for any changes in the lung volume that might occur while airway resistance is measured (365, 366). Nonetheless, the present study adds to published literature on IOS in adult patients with asthma (357, 359).

In summary, we documented that FEF25-75 provided valuable information of the state of the airways in patients with asthma, pre- and post-bronchodilation and that small airway inflammation and remodelling was evident within the totally controlled and well-controlled groups, despite these groups of asthmatic patients being asymptomatic and having normal FEV1 values. IOS has a greater discriminating value in differentiating the controlled from the uncontrolled adult asthmatic patient with respect to the evaluation of the small airways. However more data is required from larger patient cohorts to determine appropriate cut-off values for the IOS parameters, that will have a sensitivity and specificity appropriate enough to detect or exclude small airway dysfunction in the adult patient (369).

10.3 Chapter 7

In this study, using the same cohort of patients identified and described in Chapter 5, we measured non-specific systemic inflammatory biomarkers (CRP and SAAP) as well as specific systemic inflammatory cytokine biomarkers derived from Th1 and Th2 cells. The purpose was

to evaluate their utility in identifying airway inflammation when compared to the categories of asthma control as defined by the ACQ. In an attempt to reduce the statistical "noise to signal" from confounders that might dilute these study results, strict inclusion and exclusion criteria as described in Chapter 4 were applied. The reason for the application of these criteria is that this is a proof of concept study trying to identify a biomarker that might be affected by any concomitant co-existing illnesses.

We demonstrated that CRP was significantly higher in the uncontrolled group when compared with the controlled group of asthmatic patients, and may prove to be a valuable biomarker of airway inflammation in the uncontrolled asthmatic patient. However, the utility of SAAP as a tool for managing airway inflammation along the continuum of asthma control still remains elusive (381, 382). The cytokines that demonstrated a difference or trend towards a significant difference in the present study for the controlled versus uncontrolled asthmatic patients (**group 1** analysis) and totally controlled versus well-controlled and versus uncontrolled asthmatic patients (**group 2** analysis) analyses were IL-2, IL-6, IL-8 and TNFα.

IL-2 was significantly higher in the uncontrolled group with a median of 26.1 FU (95% CI 20.06 - 33.95), compared with the controlled group with a median of 19.19 FU (95% CI 16.57 - 22.23); p = 0.02 (Table 7.3; Figure 7.4). In the **group 2** analysis, IL-2 was significantly higher in the uncontrolled group with a median of 26.1 FU (95% CI 20.06 - 33.95), when compared with the totally controlled group with a median of 17.13 FU (95% CI 14.65 - 20.04); p = 0.003 (Table 7.4; Figures 7.9, 7.11).

IL-8 and TNFα demonstrated a trend towards an association with asthma control as defined by the ACQ, but predominantly for the **group 2** analysis (totally controlled (TC) versus well-controlled (WC) and versus uncontrolled (UC)). There appeared to be a trend towards a difference when comparing the TC versus the WC group of asthmatic patients for both IL-8 and TNFα (p= 0.05 and p = 0.07 respectively). However, this difference was not significant after correcting for multiplicity (Bonferroni correction).

The results of the present study support the hypothesis that while asthma is primarily an inflammatory airway disease, the inflammation in the airways is not associated with significant changes in the measured levels of serum cytokines in the blood. With the exception of IL-2, none of the cytokines measured, were associated with asthma control as defined by the ACQ.

To explore this unique finding further the relationship of the cytokines as a group (i.e. either Th1 (IL-2, IL-12 and IFNγ) or Th2 (IL-4, IL-5 and IL-13)) were compared to categories of asthma control, as defined by the ACQ, using logistic regression. This multivariate analysis indicated that even when analysing the cytokines as a group, there was no relationship between either the group of Th1 or the group of Th2 cytokines and asthma control. (Table 7.5; 7.6).

Therefore, it is possible that the Th1-Th2 cytokine axis in asthma is suppressed by the upregulated Treg cell function, as a consequence of the elevated IL-2 levels so that measurement of the systemic levels of the predominant cytokines produced by Th1 and Th2 cells could not be shown to be related to levels of asthma control (100, 396) (Figure 2.1). Another reason for the elevated IL-2 levels and suppression of all the rest of the cytokines in this thesis may include the fact that asthma is a complex and heterogeneous disease, consisting of many endotypes, some probably regulated by Treg cells (100, 114). Furthermore, it might be that the measured levels of specific serum cytokines in asthmatic patients differ from those measured in bronchoalveolar lavage fluid, or that the measured concentrations of these cytokines in serum are too low to yield statistically significant results (400, 401).

10.4 Chapter 8

In this study, using the same cohort of patients identified and described in Chapter 5, we measured FeNO, chemoattractants RANTES and eotaxin, serum leukotrienes and IgE in assessing airway inflammation and phenotyping patients with asthma. The data from the present study demonstrate no association or correlation between FeNO, at a cut-off level of 25 ppm, 42 ppm, as well as 50 ppm with asthma control, as defined by the ACQ (334, 420, 421). The findings of this study have been corroborated by a significant body of published literature, which has cast doubt over the utility of a FeNO-based asthma management algorithm, particularly in adult patients. A meta-analysis by Essat *et al.* (424), as well as the most recent Cochrane Airways Review published in September 2016 (426), documented that tailoring asthma medications based on FeNO levels decreased the frequency of asthma exacerbations but did not impact on day-to-day clinical symptoms, end-of-study FeNO levels, or inhaled corticosteroid dose.

Reasons postulated for the discordant results with the use of FeNO, as opposed to measurement of sputum eosinophilia, include the fact that FeNO levels are not unique to asthma and have been observed in eosinophilic bronchitis and allergic rhinitis (427, 428). The latest evidence

explaining the discordance between FeNO and eosinophilic inflammation have come from the monoclonal antibody trials where anti-IL-5 (mepolizumab) (429), and anti-IL-13 (lebrikizumab) (430, 431) were used to treat patients with eosinophilic asthma. These Th2 interleukin targeted monoclonal antibody studies suggested that the cytokines that regulate the induction of iNOS and production of NO in the airways, are separate from those regulating eosinophil traffic through the airways. This may result in a disassociation between FeNO levels and eosinophilic inflammation (432).

Total serum IgE levels were no different when compared to the categories of asthma control for either the **group 1** analysis (comparing controlled with uncontrolled asthmatic patients), or the **group 2** analysis (comparing totally controlled, well-controlled or uncontrolled asthmatic patients) (Tables 8.4, 8.5; Figures 8.5, 8.8). Furthermore, there was no correlation between the ACQ and IgE levels (Table 8.4; Figure 8.9). Evidence from the literature published about the utility of measuring IgE levels in asthma, advocates using IgE but rather as a good biomarker for atopy and uncontrolled allergic asthma (406, 436). Elevated levels of IgE correlate with sputum eosinophilia (408), and a longitudinal increase in total IgE is associated with poor asthma control (407).

Thus, while the data from the present study and reviewed literature supporting the use of total serum IgE as a surrogate marker of airway inflammation in asthma is discordant, the utility of IgE as a valuable biomarker is advocated by a recommendation from the National Institutes of Health (NIH) (406). Hence, the utility of total serum IgE in the management of asthmatic patients, categorised by asthma control using a validated asthma control questionnaire (i.e. ACQ or ACT), warrants further investigation.

Of the two chemokines measured, RANTES levels demonstrated a difference with respect to the **group 1** analysis, in that the levels were significantly higher in the uncontrolled group when compared with the controlled group of asthmatic patients ($p=0.03$) (Table 8.4; Figure 8.5). This difference in the RANTES levels was demonstrated in the **group 2** analysis as well. The levels of RANTES were significantly higher in the totally controlled (TC) group when compared with both the well-controlled (WC) and uncontrolled groups (UC), but the levels were not significantly different when comparing the well-controlled and the uncontrolled asthmatic patient groups (TC vs UC; $p = 0.014$, TC vs WC; $p = 0.003$ and WC vs UC; $p = 0.7$ (Table 8.5; Figure 8.7). In addition, the levels of RANTES were significantly higher in the patients using

a low-dose of oral corticosteroids (≤ 7.5 mg/day) with median of 6269.21 FU (95% CI 5698.20-6897.44) versus a median of 5566.31 FU (95% CI 5387.81-5750.73) in the group not using a low-dose of oral corticosteroids; p = 0.02 (Table 8.7). As it is unlikely that the low-dose oral corticosteroid (OCS) therapy was elevating the levels of RANTES, a more plausible explanation is that the patients who were using OCS were those that had uncontrolled or difficult to control asthma, and therefore had elevated RANTES levels. This is supported by the fact that in this study population, patients using doses of OCS less than 7.5 mg per day (i.e. low-dose), were significantly more likely to be uncontrolled with an odds ratio of 6.58 (95% CI 1.08 - 39.91) (p = 0.018) (Tables 5.6).

The final part of the studies in Chapter 8 was the measurement of serum leukotriene (LT) levels and the determination of their association with asthma control as defined by the ACQ. To date, there have been no publications assessing this relationship and one of the reasons for this might be the difficulty in measuring serum LT levels. There are several technical difficulties in the measurement of serum LT concentrations. To overcome these technical difficulties, optimisation experiments were performed as part of this present study to establish a protocol for the measurement of serum LT levels in the asthmatic patient. The details of the methodology and findings of these optimisation experiments are documented in Appendix 9. To the best of my knowledge, this is the first study documenting serum levels of the leukotrienes LTB4, LTC4 and LTE4 and their association with asthma control as defined by the ACQ.

Of the three leukotrienes measured, for the **group 2** analysis, LTE4 was significantly higher in the totally controlled group when compared with the uncontrolled group, as well when the totally controlled group was compared with the well-controlled group. The median LTE4 level was 3547.3 pg/ml (95% CI 2592.2 - 4854.3) for the TC group, compared with 1728.2 pg/ml (95% CI 1167.4 - 2558.7) for the WC group and compared with 1692.6 pg/ml (95% CI 1055.6 - 2713.9) for the UC group (TC vs UC; p = 0.007, and TC vs WC; p = 0.006 (Table 8.5; Figure 8.8). The reasons for the elevated LTE4 levels might be a reflection of ongoing eosinophilic airway inflammation within this group of patients, and/or the fact that the cysteinyl leukotriene pathway remains relatively unaffected by corticosteroid therapy in patients with asthma (i.e. corticosteroid therapy may selectively inhibit the cyclooxygenase pathway potentially leading to an increase in some of the leukotriene mediators in produced via the lipoxygenase pathway) (411).

In summary, from the biomarkers measured in this chapter, RANTES and serum LTE4 demonstrated significant difference between the controlled and uncontrolled asthmatic patients as defined by the ACQ. RANTES levels were significantly higher in the uncontrolled asthmatic patient when compared with the controlled asthmatic patient, highlighting its utility as possible biomarker for uncontrolled asthma. However, counterintuitively, LTE4 levels were elevated in the patients with totally controlled asthma when compared with the uncontrolled asthmatic patient. Hence, we postulate that a high LTE4 coupled with a low RANTES level might be a surrogate marker for a yet undefined asthma phenotype, a finding that needs to be explored further in a larger cohort of asthmatic patients.

10.5 Chapter 9

In the final study of this thesis, using the same cohort of patients identified and described in Chapter 5, we measured serum biomarkers of remodelling and angiogenesis (TGFβ and VEGF) compared them to the categories of asthma control as defined by the ACQ, as well correlated them to the ACQ score.

In the **group 1** analysis (controlled versus uncontrolled asthmatic patients), there were no significant differences in the levels of any of the TGFβ subclasses when the controlled group of patients was compared with the uncontrolled group of patients (Table 9.1; Figure 9.1). There was also no correlation between the ACQ score and any of the TGFβ subclasses as well (Table 9.1; Figure 9.2.) For the **group 2** analysis (totally controlled versus well-controlled versus uncontrolled asthmatics patients), there were no significant differences in the levels of any of the TGFβ subclasses when compared to the different categories of asthma control (Table 9.2; Figure 9.3).

However, equally important in the present study was that the levels of TGFβ1 and TGFβ2 were significantly higher in the patients using high-dose inhaled corticosteroids (ICS) when compared with those patients not using high-dose ICS ($p = 0.01$ and $p = 0.001$ respectively), and by comparison, significantly lower in the patients using moderate-dose ICS when compared with those patients not using moderate-dose ICS ($p = 0.02$ and $p = 0.001$ respectively) (Table 9.4; Figures 9.7, 9.8). A likely explanation for this finding is that the patients using high-dose ICS were more likely to be uncontrolled or difficult to control asthmatic patients, and therefore had elevated TGFβ levels. In support for this explanation, is the documentation that only 15% (8/52) of the controlled group of asthmatic patients were using moderate-dose ICS, whereas

29% (7/24) of the uncontrolled group of asthmatic patients were using high-dose ICS (Tables 5.7, 9.4). Therefore, the interpretation of this data analysis is that TGFβ levels were elevated in a subgroup of uncontrolled asthmatic patients not defined by the ACQ, but rather categorised by their use or not of high-doses of ICS.

A postulated pathogenesis for the significantly higher TGFβ levels in the above group of uncontrolled asthmatic patients, might be that in these patients, airway inflammation is modulated by the upregulation of Treg cells by TGFβ. Treg cells have a significant immunosuppressive potential within the airways in patients with asthma (48, 103). Their function is regulated by the intracellular transcription factor forkhead box P3 (Foxp3) (101), which is the main control gene in the development of these cells (102), and Treg cells limit airway inflammation via the production of cytokines IL-10 and TGFβ (100). Furthermore, via a positive feedback loop, TGFβ plays an important role in increasing the expression of Foxp3, which promotes the transition of naïve T cells toward a Treg endotype. This Treg cell endotype has the potential to modulate other T cell activation, including Th1 and Th2 cells, by inhibiting the expression of effector cytokines including IL-4, TNFα, IFNγ, IL-17 and IL-21 (104).

The second molecule measured as a potential biomarker of airway remodelling was vascular endothelial growth factor (VEGF). VEGF is a potent angiogenic factor and histology specimens of patient with severe asthma who have airway remodelling demonstrate significant neovascularisation within the airways (457). In the **group 1** analysis, there was no significant difference in the levels of VEGF when the controlled group of patients was compared with the uncontrolled group of patients. There was also no significant correlation between the ACQ score and VEGF levels. For the **group 2** analysis, there was a trend to lower levels of VEGF in the totally controlled group when compared with the well-controlled group ($p = 0.05$), as well as when the totally control group was compared with the uncontrolled group ($p = 0.07$). However, this was not significant following the Bonferroni correction (Table 9.2; Figure 9.3).

In summary, although the serum biomarkers TGFβ and VEGF did not demonstrate any significant association with the level of asthma control as defined by the ACQ, they have nonetheless, provided us with potential tools for a more specific and non-invasive assessment of airway inflammation in patients with asthma.

10.6 Potential strengths of the study

This study was designed as a prospective study with the patient numbers estimated from a previous publication from South Africa. The patient numbers that needed to be included into the study to adequately power the study were based on assumptions that were supported by data from this recent publication from asthma clinics in South Africa undertaken by Greenblatt *et al.* (339).

In this study, we have explored the various different pathogenic mechanism in asthma with a focus on the analysis of the small airways, asthma endotypes, asthma phenotypes and in particular a Th2 high phenotype and airway remodelling. These experiments were performed on the same study population and the outcomes throughout all the experiments conducted, were uniformly compared to a robust predefined parameter, being asthma control as defined by the ACQ. This reduced the heterogeneity that potentially dilutes the results when a study such as this present study is conducted on an illness as complicated as asthma. Furthermore, by keeping the population cohort constant and the outcome parameter standard, it allowed the investigator to compare all the different variables measured and correlated the association of these variables without diluting the analysis by the use of different population cohorts.

Another strength of this thesis was the application of strict inclusion and exclusion criteria in screening for the study population group to reduce the "signal to noise" trends that have confounded the interpretation of the published literature thus far in the assessment of inflammation in patients with asthma.

10.7 Potential Limitations of the study

This study has some potential limitations. It was a single centre study and therefore the results may not be extrapolated to the South African population or to the populations of other countries**.**

The number of patients included in the study was relatively small, but the study was adequately powered prior to the initiation of the investigation, to give a reasonable chance that the desired end-points would be achieved. Over 3000 patients were screened for inclusion into the study but the majority were excluded as they did not meet the strict predefined inclusion and exclusion criteria chosen to reduce the statistical "noise" and confounding bias from the inclusion of questionable factors that might dilute the final data analysis. However, inclusion of more patients may have resulted in some comparisons, which were not significantly different in the

analyses performed, becoming statistically significant (Type II error). Another limitation of the study was the cross-sectional design. This limited us in assessing whether the levels of biomarkers measured responded to titration of medication and variation in asthma control within individual patients over time. However, the aim of this thesis was to try to identify a point-of-care test that would aid in the assessment of airway inflammation in patients with asthma. With the significant heterogeneity in asthma symptoms, the cross-sectional design would provide an assessment of the state of airway inflammation at a particular point in time, as the nature of asthma is an illness with significant variability in symptoms and asthma control. Furthermore, the spread of the medication used by the patients included in the current study makes direct comparison of levels of biomarkers complicated, as some might be more and others less immune modulating. To mitigate against this, the levels of the cytokines measured were compared to the various medications used, and some significant difference were observed between the level of the cytokines measured and the medication used. These differences were documented and discussed above.

Another potential limitation of the study was the choice of the ACQ as the gold standard in this research. The major reason for using the ACQ was to make the statistical comparisons between the groups of controlled and uncontrolled patients viable, as the categorical cut-offs for asthma control defined by the ACQ are well defined and robust (59). However, potential limitations in using the ACQ is that it might not necessarily be the gold standard in assessment of asthma control but rather that it may just be identifying a phenotype of asthmatic patients whose control can be accurately categorised by the ACQ.

The majority of the biomarkers measured were serum biomarkers and not specific lung biomarkers obtained from the lungs directly, such as via bronchoscopy with bronchoalveolar lavage. The major drawback with bronchoscopy is that it is an invasive procedure and poses potential harm to a patient with “at risk inflamed airways”. Theoretically, this procedure might precipitate an acute bronchospastic response in patients with hyper-reactive airways leading to an acute asthma exacerbation and potentially status asthmaticus. Furthermore, invasive procedures cannot be performed as a routine investigation at repeated visits, nor are they easily accessible to practitioners outside specialised respiratory units. (73, 75).

The main objective of the present study was to identify simple, safe and easy to perform biomarkers (i.e. blood biomarker or FeNO) that could be performed by any health care

professional looking after patients with asthma. However, there was a poor correlation between these biomarkers and the ACQ score. Although this has not been mentioned previously in the text, a correlation matrix analysis was also performed to identify if there were any associations between these biomarkers of airway inflammation themselves, as well as between the biomarkers and any of the lung function parameters (Appendix 12). No additional associations between any the parameters measured (i.e. all the serum and airway biomarkers and the lung function parameters) in relation to each other as well as to the ACQ score were identified. The clinically relevant associations were extracted and reported in detail in the various chapters of this thesis.

10.8 Future recommendations

The analysis of this present study as well as the extensive literature review used as the basis of this thesis has led to the following recommendations for the identification, as well as application of an ideal inflammatory biomarker:

Firstly, it should characteristically only be raised in patients with asthma when endobronchial inflammation is present.

Secondly, this biomarker should demonstrate a linear relationship with the use of anti-inflammatory therapy, demonstrating a clear dose-response effect.

Finally, this biomarker should provide superior clinical information as to the state of inflammation in the airways when used along with conventional measurements like spirometry and control questionnaires (i.e. the ACQ).

These recommendations have been highlighted by Currie *et al.* (469) when reviewing the initial publications on FeNO. However, as reported in studies by Wu *et al.* (470), Brasier *et al.*(471) and Haldar *et al.* (472), as well as data from this present study, serum and blood biomarkers have not consistently correlated with disease activity and paradoxically, that higher levels of some cytokines may be associated with milder asthma phenotypes. Nonetheless, in this present study we identified several potential biomarkers that appear to be helpful in assessing airway inflammation in patients with asthma. These include CRP, IL-2, RANTES, LTE4 and TGFβ as well as the application of IOS in the assessment of small airway calibre.

Of importance, in this present study we have documented that a poor relationship exists between disease control and Th2 biomarker levels. This, suggests that factors other than Th2 inflammation might drive disease activity in some patients with uncontrolled asthma (473). One

of these factors might be that the Th1-Th2 immunopathogenesis might be too simplistic. Of a greater importance is the role of the Treg cells in the immunopathogenesis of airway inflammation in patients with asthma. Treg cells have been identified to play a significant role in limiting airway inflammation via the production of the cytokines IL-10 and TGFβ (100) (Figures 2.1, 10.1). Furthermore, Th17 and possibly Group 2 Innate Lymphoid Cells (ILC2), and their interactions with the previously unidentified novel cytokines IL-25, IL-33 and TSLP may play a far greater role in the control of airway inflammation than what we first anticipated (108). Thus, these biomarkers, including new novel biomarkers such as periostin, need to be studied in a similar cohort of patients to determine their utility in assessing airway inflammation when compared to asthma control as defined by the ACQ (474).

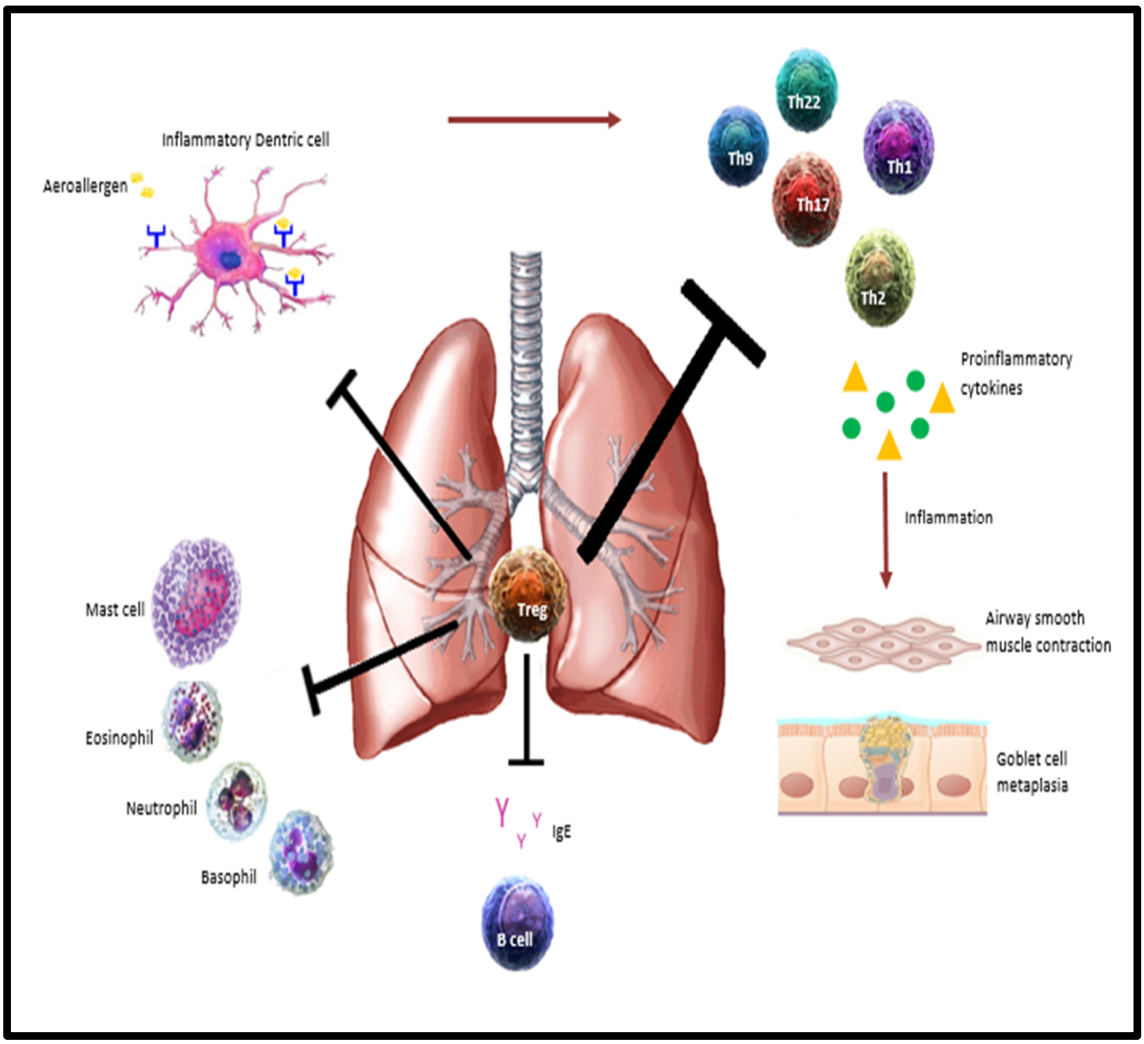

Figure 10.1: The anti-inflammatory interactions between Treg cells and effector T cells, mast cells, granulocytic cells and B cell in suppressing airway inflammation. Adapted from Seroogy *et al*. (100)
Treg: regulatory T cell, B cell: B lymphocyte, Th cell: T helper lymphocyte, IgE: Immunoglobulin E.

In summary, from the findings of this thesis we propose:

Firstly, that all patients with asthma be categorised according to their level of asthma control to appropriately risk stratify and titrate treatment. This assessment can be made using the ACQ6, ACQ7 or the ACT, as all are validated tools to assess asthma control.

Secondly, that the spirometric parameter FEF25-75 provided valuable information of the state of the small airways in patients with asthma, pre- and post-bronchodilation and that small airway inflammation as evidence by the presence of bronchial airway hyper-reactivity (BAH) was evident within the totally controlled and well-controlled groups, despite these groups of asthmatic patients being asymptomatic and having normal FEV1 values.

Thirdly, that IOS has a greater discriminating value in differentiating the controlled from the uncontrolled adult asthmatic patient with respect to the evaluation of the small airways.

Fourthly, that an elevated LTE4 coupled to a suppressed level of RANTES in the same patient might identify a yet undescribed asthma phenotype, and may provide us with a useful biomarker in assessing asthma control.

Finally, that a higher TGFβ level coupled with an elevated IL-2 level in the same patient might identify a yet undescribed asthma phenotype, and may further provide us with a useful biomarker in assessing asthma control. In addition, non-specific serum biomarkers like CRP when measured in the correct population cohort may prove to be a valuable biomarker of airway inflammation in the asthmatic patient.

These postulates are probably overly optimistic but the uniqueness of this study makes it possible for us to speculate that these finding document above may prove to be validated. This is the first study wherein all the biomarkers were measured in the same cohort of patients, categorised by their asthma control using an internationally validated asthma questionnaire i.e. the ACQ.

The next step will be to validate these findings in a larger and more heterogeneous patient cohort, mimicking a more real life scenario. Furthermore, these biomarkers documented above will need to be measured in a cohort of control subjects comparing them to asthma patients, to

try to determine appropriated cut-off values for these biomarkers to improve their clinical utility.

In conclusion, in our search for the panacea of a biomarker that will aid us identifying airway inflammation and titrating therapy to suppress this inflammation, we need to understand the heterogeneity of the inflammation in asthma. More importantly, it may be necessary to individualise and use multiple biomarkers profiles, based on different asthma endotypes as well as asthma phenotypes, to achieve our goal of being able to assess inflammation in asthma and more specifically persistent airway inflammation in the controlled asthmatic patient (421, 475).

CHAPTER 11:REFERENCES (EndNote Program X6)

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Appendix 1: ACQ by Juniper *et al.*

E.F. JUNIPER *et al.*

Appendix 1

ASTHMA CONTROL QUESTIONNAIRE©

Please answer questions 1-6
Circle the number of the response that best describes how you have been during the past week

I. On average, during the past week, how often were you woken by your asthma during the night?	0	Never
	1	Hardly ever
	2	A few minutes
	3	Several times
	4	Many times
	5	A great many times
	6	Unable to sleep because of asthma
2. On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?	0	No symptoms
	1	Very mild symptoms
	2	Mild symptoms
	3	Moderate symptoms
	4	Quite severe symptoms
	5	Severe symptoms
	6	Very severe symptoms
3. In general, during the past week, how limited were you in your activities because of your asthma?	0	Not limited at all
	1	Very slightly limited
	2	Slightly limited
	3	Moderately limited
	4	Very limited
	5	Extremely limited
	6	Totally limited
4. In general, during the past week, how much shortness of breath did you experience because of you asthma?	0	None
	1	A very little
	2	A little
	3	A moderate amount
	4	Quite a lot
	5	A great deal
	6	A very great deal
5. In general, during the past week, how much of the time did you wheeze?	0	Not at all
	1	Hardly any of the time
	2	A little of the time
	3	A moderate amount of the time
	4	A lot of the time
	5	Most of the time
	6	All the time
6. On average, during the past week, how many puffs of short-acting bronchodilator (e.g. Ventolin) have you used each day?	0	None
	1	1-2 puffs most days
	2	3-4 puffs most days
	3	5-8 puffs most days
	4	9-12 puffs most days
	5	13-16 puffs most days
	6	More than 16 puffs most days
To be completed by a member of the clinic staff		
7. FEV1 pre-bronchodilator:		
FEV1 predicted	0	>95% predicted
	1	95-90%
FEV1% predicted	2	89-80%
(Record actual values on the dotted lines and score the FEV1% predicted in the next column)	3	79-70%
	4	69--60%
	5	59--50%
	6	<50% predicted

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Appendix 2: ACT

Name: _____ Today's Date: _____

ASTHMA CONTROL TEST™

Know your score

The Asthma Control Test™ provides a numerical score to help you and your healthcare provider determine if your asthma symptoms are well controlled.

Take this test if you are 12 years or older. Share the score with your healthcare provider.

Step 1: Write the number of each answer in the score box provided.

Step 2: Add up each score box for the total.

Step 3: Take the completed test to your healthcare provider to talk about your score.

If your score is 19 or less, your asthma symptoms may not be as well controlled as they could be. No matter what the score, bring this test to your healthcare provider to talk about the results.

					SCORE
1. In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school or at home?	All of the time [1]	Most of the time [2]	Some of the time [3]	A little of the time [4]	None of the time [5]	.....
2. During the past 4 weeks, how often have you had shortness of breath?	More than Once a day [1]	Once a day [2]	3 to 6 times a week [3]	Once or twice a week [4]	Not at all [5]	.....
3. During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?	4 or more nights a week [1]	2 to 3 nights a week [2]	Once a week [3]	Once or twice [4]	Not at all [5]	.....
4. During the past 4 weeks, how often have you used your rescue inhaler or nebulizer medication (such as albuterol)?	3 or more times per day [1]	1 or 2 times per day [2]	2 or 3 times per week [3]	Once a week or less [4]	Not at all [5]	.....
5. How would you rate your asthma control during the past 4 weeks?	Not Controlled at All [1]	Poorly Controlled [2]	Somewhat Controlled [3]	Well Controlled [4]	Completely Controlled [5]	.....

If your score is 19 or less, your asthma symptoms may not be as well controlled as they could be. No matter what your score is, share the results with your healthcare provider.

TOTAL: .....

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This material was developed by GSK.

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Appendix 3: Ethics Clearance certificate

R14/49 Dr I. S. Kalla

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1706114

NAME: (Principal Investigator)	Dr I. S. Kalla
DEPARTMENT:	Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Association Between Inflammation and Control of Asthma
DATE CONSIDERED:	05/05/2006 (Initial Approval 03/07/2017)
DECISION:	Approved unconditionally
CONDITIONS:	Renewal for 5 Years Valid for the Period 01 June 2017 - 31 June 2022 Previously M060401
SUPERVISOR:	Prof Charles Feldman
APPROVED BY:	Professor P. Cleaton-Jones Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/06/2006

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review June and will therefore be due in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 01/08/2017

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 4: Participant information sheet and Consent form - Patient arm

PARTICIPANT INFORMATION SHEET
Participants with diagnosed asthma

TITLE OF PROJECT: **Aspects of inflammation in asthma**

NAME OF RESEARCHER: **Dr. I. S. Kalla**

INSTITUTION: **Department of Pulmonology, Johannesburg Hospital**

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL) NO.: **M060401**

PARTICIPANT REFERENCE NUMBER:

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S): **083 622 6888**

Invitation: Hello. My name is Dr Ismail S. Kalla. I am a Physician in the Department of Pulmonology at the Johannesburg Hospital and I am inviting you to take part in a research study. This study is voluntary. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study? Our research aims to assess the presence of inflammation in asthma. This will improve our understanding of asthma and aid us in better treating our patients.

Why have I been chosen? You have been invited to take part in this research project because you have Asthma.

Do I have to take part? It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part? If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study. This assessment is needed because we need to determine how well controlled your asthma currently is. You will be asked to give a blood test and a urine specimen. This involves inserting a needle into a vein on your arm and taking about 25 ml of blood (5 teaspoons). Venepunctures (i.e. drawing of blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A chest X-ray, if necessary, is a

commonly used diagnostic procedure that exposes you to a small amount of radiation. Although all radiation is cumulative over your lifetime, small doses from X-rays should not create a significant risk to your health. You will also be required to breathe into three machines. These machines will measure the airways and a by product of inflammation in your breath. These tests are non invasive and no different from the routine tests that have been previously carried out on you.

What do I have to do? Participation in this research project involves undergoing an examination, giving blood tests and a urine specimen for analysis of inflammation, and breathing into lung function machines to assess for airway inflammation. None of these procedures will have any effect on your normal daily life. The total amount of time required for your participation in this study will be a maximum of two hours. You will only be asked to visit me once during the study.

What is being tested? The presence of ongoing airway inflammation and its association with asthma symptoms.

What are the possible disadvantages and risks of taking part? There might be slight bruising after taking blood. There are no risks involved with any of the tests.

What are the possible benefits of taking part? It is likely that participation in this research project will not be of immediate benefit to you. The research should be able to tell us how well controlled your asthma is. This may allow us to plan new treatments guidelines for the future.

What happens when the research study stops? When the research is completed, information resulting from the study will be given to you by the doctors carrying out the research. Completion of this research project will not affect the clinical care you receive.

Rights as a participant in this study: Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. Your withdrawal will not affect your access to other medical care.

Will my taking part in this study be kept confidential? All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study. Any information uncovered regarding your test results or state of heath as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this study but this information will not be disclosed to any third party.

What will happen to the results of the research study? The results will be published in a scientific/medical journal, as well as being presented at national and international scientific/medical meetings. However, any written or illustrative material will not identify you in any report/presentation. All information obtained is strictly confidential – your individual information will be available only to the doctors doing the research. Any results shared with the medical profession will be grouped; it will not be possible to identify any individual.

Who is organising the research? This research project is being jointly organised by the Departments of Pulmonology and Immunology, Johannesburg Hospital, University of the Witwatersrand, Johannesburg.

Who has reviewed the study? This research project has been reviewed by the Human Research Ethics Committee (Medical) - HREC of the University of the Witwatersrand, Johannesburg. The study has been structured in accordance with the Declaration of Helsinki (last updated: October 2000), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from me should you wish to review it.

If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Professor Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2229.

Contact for further information:
Dr. Ismail S. Kalla
Specialist Physician
Department of Pulmonology
Johannesburg Hospital
University of the Witwatersrand
24-hour telephone number: 083 622 6888

You will be given a copy of the information sheet and a signed consent form to keep.

Thank you for taking part in this study.

CONSENT FORM

Project reference number:

Participant Identification Number for this study:

Human Research Ethics Committee (Medical) No.: **M060401**

Title of project: **Aspects of inflammation in asthma**

Name of Researcher: **Dr. I. S. Kalla**

Please initial box

1. I confirm that I have read and understand the information sheet dated _____ for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals or regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study. ☐

_____ _____ _____

Name of Patient Date Signature

_____ _____ _____

Name of Person taking consent Date Signature

_____ _____ _____

Researcher Date Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 5: Participant information sheet and Consent form - control arm

PARTICIPANT INFORMATION SHEET
Participant in control arm

TITLE OF PROJECT: **Aspects of inflammation in asthma**

NAME OF RESEARCHER: **Dr. I. S. Kalla**

INSTITUTION: **Department of Pulmonology, Johannesburg Hospital**

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL) NO.: **M060401**

PARTICIPANT REFERENCE NUMBER:

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S): **083 622 6888**

Invitation: Hello. My name is Dr Ismail S. Kalla. I am a Physician in the Department of Pulmonology at the Johannesburg Hospital and I am inviting you to take part in a research study. This study is voluntary. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study? Our research aims to assess the presence of inflammation in asthma. This will improve our understanding of asthma and aid us in better treating our patients.

Why have I been chosen? You have been invited to take part in this research project because you do not have any symptoms of asthma. Furthermore we need to compare your results with patients who are suffering from asthma to give us a better understanding of inflammation in asthma.

Do I have to take part? It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part? If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study. This assessment is needed because we need to determine how well controlled your asthma currently is. You will be asked to give a blood test and a urine specimen. This involves inserting a needle into a vein on your arm and taking about 25 ml of blood (5 teaspoons). Venepunctures (i.e. drawing of blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A chest X-ray, if necessary, is a commonly used diagnostic procedure that exposes you to a small amount of radiation.

Although all radiation is cumulative over your lifetime, small doses from X-rays should not create a significant risk to your health. You will also be required to breathe into three machines. These machines will measure the airways and a by product of inflammation in your breath. These tests are non invasive and no different from the routine tests that have been previously carried out on you.

What do I have to do? Participation in this research project involves undergoing an examination, giving blood tests and a urine specimen for analysis of inflammation, and breathing into lung function machines to assess for airway inflammation. None of these procedures will have any effect on your normal daily life. The total amount of time required for your participation in this study will be a maximum of two hours. You will only be asked to visit me once during the study.

What is being tested? The presence of ongoing airway inflammation and its association with asthma symptoms.

What are the possible disadvantages and risks of taking part? There might be slight bruising after taking blood. There are no risks involved with any of the tests.

What are the possible benefits of taking part? It is likely that participation in this research project will not be of immediate benefit to you. The research should be able to tell us how well controlled your asthma is. This may allow us to plan new treatments guidelines for the future.

What happens when the research study stops? When the research is completed, information resulting from the study will be given to you by the doctors carrying out the research. Completion of this research project will not affect the clinical care you receive.

Rights as a participant in this study: Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. Your withdrawal will not affect your access to other medical care.

Will my taking part in this study be kept confidential? All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study. Any information uncovered regarding your test results or state of heath as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this study but this information will not be disclosed to any third party.

What will happen to the results of the research study? The results will be published in a scientific/medical journal, as well as being presented at national and international scientific/medical meetings. However, any written or illustrative material will not identify you in any report/presentation. All information obtained is strictly confidential – your individual information will be available only to the doctors doing the research. Any results shared with the medical profession will be grouped; it will not be possible to identify any individual.

Who is organising the research? This research project is being jointly organised by the Departments of Pulmonology and Immunology, Johannesburg Hospital, University of the Witwatersrand, Johannesburg.

Who has reviewed the study? This research project has been reviewed by the Human Research Ethics Committee (Medical) - HREC of the University of the Witwatersrand, Johannesburg. The study has been structured in accordance with the Declaration of Helsinki (last updated: October 2000), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from me should you wish to review it.

If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Professor Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC),which is an independent committee established to help protect the rights of research participants at (011) 717 2229.

Contact for further information:
Dr. Ismail S. Kalla
Specialist Physician
Department of Pulmonology
Johannesburg Hospital
University of the Witwatersrand
24-hour telephone number: 083 622 6888

You will be given a copy of the information sheet and a signed consent form to keep.

Thank you for taking part in this study.

CONSENT FORM

Project reference number:

Participant Identification Number for this study:

Human Research Ethics Committee (Medical) No.: **M060401**

Title of project: **Aspects of inflammation in asthma**

Name of Researcher: **Dr. I. S. Kalla**

Please initial box

5. I confirm that I have read and understand the information sheet dated _____ for the above study and have had the opportunity to ask questions. ☐

6. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

7. I understand that sections of any of my medical notes may be looked at by responsible individuals or regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

8. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study. ☐

_____ _____ _____

Name of Patient Date Signature

_____ _____ _____

Name of Person taking consent Date Signature

_____ _____ _____

Researcher Date Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

APPENDIX 6: METHODS APPLIED FOR THE USE OF MULTIPLEX ASSAYS TO MEASURE THE NON-SPECIFIC INFLAMMATORY BIOMARKERS CRP AND SAAP

A6.1 Methodology

Cytokines, chemokines, non-specific biomarkers of inflammation as well as markers of fibrosis and angiogenesis were analysed using microplate-based multiplex immunoassay systems. The multiplex systems are designed using microscopic microspheres (beads), each with a different colour code (spectral address) to permit discrimination of different assays. The beads are dyed with different ratios of two fluorophores and are thus differentiated into one hundred unique bead regions. The multiplex assays use a selection of beads with different spectral addresses, each coupled to antibodies against a different target. In the case of the study population used in this study, these targets were various cytokines and chemokines. After a rigorous process of mixing and washing (outlined below) the beads bound to their targets within the multiplex plate were read with a multiplex reader.

The bead reader classifies the beads according to their spectral addresses assigned as per the manufacturer's information sheet, thus identifying the specific protein bound to the bead. The beads with their antibody-target complex are then fluorescently labelled with a streptavidin reporter, which binds to the biotin labelled detection antibodies. This allows for a quantification of the associated reporter signal intensity for each bead class. The microspheres (beads) are allowed to pass rapidly through a laser, which excites the internal dyes marking the microsphere (bead) set i.e. identifying the bead-target protein complex. A second laser excites streptavidin, a fluorescent dye on the reporter microsphere. Finally, several high-speed digital-signal processors identify each individual microsphere (bead) and quantify the result of the bioassay based on fluorescent reporter signal. The dyed beads can then be specifically identified and quantified by the intensity of their internal fluorescent signature, which is simultaneously read by the multiplex reader. This method enables scientists to differentiate and quantify several different proteins simultaneously.

The levels of all biomarkers measured via the bioplex assays were reported as fluorescent intensity units (FU). When the level of a biomarker was below the lower limit of detection, it was assigned half the value of lowest limit of detection. When the level of a biomarker was

above the upper limit of detection, it was assigned double the value of the uppermost limit of detection. This adjustment for outlying data has been verified in the literature as an acceptable method for data correction (337, 338), and was recommended by the manufacturers of the analyte kits as well.

The reason for reporting in fluorescent intensity units (FU) as opposed to standard international units (SI) was that fluorescent intensity is the actual measurement (raw data) obtained from the microplate-based multiplex immunoassay system. By using the raw data in FU as opposed to diluting the data with several correction steps, using pre-defined manufacturer provided control samples to determine the equivalent SI units, the statistical analysis and results obtained were improved (Appendix 6 and 7).

The reason for this is that the conversion of FU values to SI units required a computerised mathematical correction model as recommended by the manufacturer of the biomarker kits. However, the correction algorithm is not linear but it is a logistic mathematical correction algorithm utilizing a sigmoid shaped curve. Hence, the conversion of actual FU values to SI units would have resulted in some of the values of the analytes measured being assigned the same SI unit if the variations in the FU measured was minor. For example, analytes with FU values measured between 5 and 15 FU may have all been allocated the same SI unit of 10 pg based on the mathematical algorithm. Thus, if a significant number of the patients had a biomarker level that fell within this band of 5 to 15 FU, they would have been all allocated the same SI unit of 10 pg. This conversion, by estimating the value of the SI unit based on a logistic mathematical algorithm would have resulted in the unintentional creation of categorical values in SI units. These categorical values would have decreased the spread of the SI units, and made the statistical analysis more difficult by diluting and combining different FU values into a single SI unit.

A6.2 Methods

The Millipore human cardiovascular disease panel 2 (HCVD2 – 62 BK) bioplex assay was used according to manufacturer's recommendations.

A6.2.1 Preparation of samples (Serum)

1. Diluted serum samples to a 1:2 000 dilution as follows:

   5 µl serum: 495 µl of assay buffer [1 in 100]

10 µl diluted serum [1 in 100]: 190 µl assay buffer (1 in 2 000)

A6.2.2 Preparation of immobilised beads (CRP and SAAP)

1. Each bead vial was sonicated for 30 sec.
2. Then each vial was vortex for 1 min.
3. 150 µl CRP bead mix obtained from manufacturer was added to brown mixing bottle.
4. 150 µl SAAP bead mix obtained from manufacture was added to CRP bead mix.
5. 2700 µl bead diluents was added to make up 3000 µl.
6. The vials were re-vortex for 1 min.
7. The unused portion was stored at 2-8 ℃.

A6.2.3 Preparation of Quality Control (QC) (provided by manufacturer of multiplex kit used)

1. QC1 and QC2 were reconstituted with 250 µl deionised water (dH_2O).
2. Each vial was inverted several times to mix the solutions.
3. Then each vial was vortex for 1 min.
4. Left to settle for 10 min.
5. The final QC solution was transferred via an Eppendorf to the plate.
6. Unused portion was stored at -20 ℃.

The following quality control assays provided were used:

1. CRP:QC 1:0.12 – 0.25 ng/ml
   QC 2:1.2 – 2.4 ng/ml

2. SAAP: QC 1:0.7 – 1.4 ng/ml
   QC 2:6.2 – 12.9 ng/ml

A6.2.4 Preparation of wash buffer

30 ml of the 10x concentration wash buffer concentrate provided by the manufacturer was reconstituted with 270 ml of dH_2O to make 300 ml of 1 x concentration wash buffer.

A6.2.5 Preparation of standards (provided by manufacturer of multiplex kit used)

1. Reconstituted the standards cocktail with 250 µl dH_2O making final concentrations of: 50 ng/µl CRP and

250 ng/µl SAAP

2. Each vial was inverted several times and left to settle for 10 minutes.

3. All standards solution weretransferred via an Eppendorf into separate containers used for the preparation of the standards, with the first solution prepared being Standard (Std) 6(Figure A 6.1).

A6.2.6 Preparation of further dilutions of standards solution

A schematic diagram of the preparation of the dilutions of the standards solution used is summarised in Figure A 6.1.

	Assay buffer	Vol. of std.	Dilution	CRP ng/ml	SAAP ng/ml
Std 6	0 µl	250 µl (step 6.7)	1	50. 00	250. 00
Std 5	200 µl	50 µlStd 6	5x	10. 00	50. 00
Std 4	200 µl	50 µlStd 5	25x	2. 00	10.00
Std 3	200 µl	50 µlStd 4	125x	0.4	2.00
Std 2	200 µl	50 µlStd 3	625x	0.08	0.4
Std 1	200 µl	50 µlStd 2	3125x	0.016	0.08
Blank	200 µl				

Figure A 6.1: Schematic used for the preparation of dilutions of standards
Vol: Volume, Std: Standard, CRP: C-Reactive Protein, SAAP: Serum Amyloid Associated Protein, ng/ml: Nanogram per millilitre, µl: Microliter

A6.2.7 Preparation of the CRP-SAAP multiplex plate according to manufacturer's instructions (Millipore)

1. Reagents and samples were allowed to warm to room temperature (RT).
2. The filter plate was placed on the plate holder at all times during reagent dispensing and incubation so that the bottom of the plate did not touch any surface.
3. All excess buffer solution was removed from the bottom of the plate with blotting paper.
4. Plate was washed with 200 µl wash buffer which was pipetted into each well and agitated for 10 min. at room temperature.
5. Plate was vacuumed to remove wash buffer.
6. Standards and patients serum prepared as above were pipetted into wells as follows:
7. 25 µl of the standard or control solutions were added to each of wells A1 to H2 - Standards were assayed in duplicate
8. 25 µl of the sample was added to each of appropriate cells to wells A3 to H40 - Samples were assayed induplicate.

9. 25 µl of assay buffer was added to all wells
10. 25 µl of mixed beads was added to each well
11. Excess fluid was removed by vacuum.
12. Plate was washed twice with 200 µl wash buffer per well.
13. Excess buffer was removed by vacuum between washes and blot excess liquid from bottom of plate.
14. 25 µl of detection antibodies was added to each well(provided in kit) and sealed with foil.
15. The plates containing a combination of standard and patents serum in the individual wells were incubated at RT for 30 mins with shaking but no vacuuming.
16. 25 µl of streptavidin/ phycoerythrin was added to each well and wells were re-sealed.
17. The plate was re-incubated for 30 mins at RT with shaking.
18. Excess fluid was removed by vacuum.
19. The plate was washed twice with 200 µl wash buffer per well.
20. Buffer was removed by vacuum between washes and excess liquid was blotted from bottom of plate.
21. 100 µl of sheath fluid was added to all wells.
22. Plate containing the wells were sealed and re-shook for 5 mins.
23. Plate analysed on the Bio-plex 200 analyser. (Figure A 6.2)
24. The above process was repeated for specimens 41-80.

A6.2.7.1 CRP-SAAP multiplex plate schematic

The multiplex plate was prepared as described above and the specimens to be analysed were pipetted into each wells on the plate as the per the diagram schematic in Figure A 6.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B 1	Std 4	1	5	9	13	17	21	25	29	33	37
B	B 1	Std 4	1	5	9	13	17	21	25	29	33	37
C	Std 1	Std 5	2	6	10	14	18	22	26	30	34	38
D	Std 1	Std 5	2	6	10	14	18	22	26	30	34	38
E	Std 2	Std 6	3	7	11	15	19	23	27	31	35	39
F	Std 2	Std 6	3	7	11	15	19	23	27	31	35	39
G	Std 3	QC 1	4	8	12	16	20	24	28	32	36	40
H	Std 3	QC 2	4	8	12	16	20	24	28	32	36	40

Figure A 6.2: Plate schematic used for analysis of standards as well as for the biomarkers CRP and SAAP
B = Blank, Std = Standard, QC= Quality control

A6.2.8 Equipment settings

The specifications of the Luminex reader used to analyse the multiplex plates was 100 IS version (v). 2.1/2.2 using the Luminex High Throughput Screening (HTS) system. The settings, as per manufacturer's instructions, used for the Luminex reader are summarised in Table A 6.1.

Table A 6.1: Equipment specifications used for Luminex reader for the biomarkers CRP and SAAP

Events:	50 per bead	
Sample size:	50 µl	
Gate settings:	8,000 to 15, 000	
Reporter gain:	default	
Time out:	60 seconds	
Bead set:	2- Plex beads	
	CRP	48
	SAAP	67

CRP: C-reactive protein, SAAP: Serum amyloid associated protein, µl: Microliter

A6.2.9 Results

After the plate was processed, concentrations of the standards and the analytes were determined using 5PL algorithm (Bioplex 5.0 software) and stored for statistical analysis. Results as discussed above were reported as FU.

APPENDIX 7: METHODS APPLIED FOR THE USE OF MULTIPLEX ASSAYS TO MEASURE SERUM CYTOKINES (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IFNγ and TNFα), CHEMOKINES (eotaxin and RANTES) AND THE GROWTH FACTOR (VEGF)

A7.1 Methodology

The theory explaining the concept of multiplex assay has been described in Appendix 6. The preparation of bioplex plates is discussed in detail in Appendix 6. Only steps unique to the plating of the cytokine and chemokine multiplex assay will be elaborated on in Appendix 7.

A7.2 Methods

The Bioplex pro™ cytokine, chemokine and growth factor assay (Bio-Rad M500AD1ZL6) was used according to manufacturer's recommendations.

A7.2.1 Preparation of standards(provided by manufacturer of multiplex kit used)

Lyophilised standards as provided by the manufacturer were reconstituted as follows:

1. The vial containing the lyophilised standards was tapped on a solid surface to dislodge the pellets that may have settled to bottom of vial.
2. The pellets was reconstituted with 500 µl of standard diluents - provided by manufacturers.
3. The reconstituted solution was vortexed for 3 seconds and then incubated on ice for 30 min.
4. A new Eppendorf pipette tip was used to transfer the solution to the wells on the plate (Figure A 7.1).
5. Standards were vortexed for 3 seconds before transfer.
6. Bioplex pro™ human cytokine standard (Lot #5023534) was used and was stored at 4℃ prior to reconstitution.
7. Dilution series using diluents and standards provided were prepared. Discussed in detail in Appendix 6.

A7.2.2 Preparation of further dilutions of standards solution

A schematic diagram of the preparation of the dilutions of the standards solution used is summarised in Figure A 7.1.

Standard vial	Standard diluting	Volume standard
S1	72 µl diluent	128 µl of reconstituted std
S2	150 µl diluents	50 µl of S1
S3	150 µl diluents	50 µl of S2
S4	150 µl diluents	50 µl of S3
S5	150 µl diluents	50 µl of S4
S6	150 µl diluents	50 µl of S5
S7	150 µl diluents	50 µl of S6
S8	150 µl diluents	50 µl of S7
Blank	150 µl diluent	-

Figure A 7.1: Schematic used for the preparation of dilutions of standards
Std: Standard, µl: Microliter

A7.2.3 Preparation of patient's specimen samples

Patient's serum was diluted 1 in 4 as per the manufacturers recommendations as follows:

1. 50 µl serum was added to 150 µl of the sample diluent (provide by manufacturer's).
2. Samples were kept on ice until plated.

A7.2.4 Preparation of immobilised beads

Preparation of coupled beads:

1. The beads were placed in a 15 ml polypropylene tube.
2. The beads were than vortexed for 30 seconds.
3. 5175 µl assay buffer was added to 575 µl of the coupled bead solution.
4. The solution was covered with aluminium foil to protect from light and allowed to equilibrate at room temperature.

A7.2.5 Preparation of detection antibodies (provide by manufacture)

Detection antibodies were placed in a 15 ml polypropylene tube and 2700µl of the detection antibody diluent was added to 300 µl of the detection antibodies and vortexed for 30 seconds.

A7.2.6 Preparation of streptavidin/phycoerythrin (provide by manufacture)

A solution of 15 ml streptavidin/phycoerythrin was placed in a 15 ml polypropylene tube:

1. Solution vortex for 30 sec
2. 5940 µl assay buffer was added to 60 µl streptavidin/phycoerythrin

A7.2.7 Preparation of multiplex plate according to manufacturer's instructions (Bioplex pro™)

1. Filter plate was pre-wet with 100 ul assay buffer per well.
2. The plate was vacuumed and the bottom of the plate blotted.
3. Coupled beads were vortexed for 30 seconds.
4. 50 µl of coupled beads was added to each well.
5. The plate was vacuumed and the bottom of plate blotted.
6. The plate was washed twice with 100 µl wash buffer.
7. The plate was vacuumed and the bottom of the plate blotted after each wash.
8. 50 µl of samples, standards and control solution were added as per plate schematic.
9. The plate was incubated on a shaker at room temperature for 30 minutes in the dark.
10. The plate was washed three times with 100 µl wash buffer.
11. The plate was vacuumed and the bottom of the plate blotted after each wash.
12. 25 µl of detection antibodies were added
13. The plate was covered and incubated in a dark at room temperature, after being agitated at 300 rpm for 30 minutes.
14. Steps 10 and 11 were repeated. Plate vacuumed and bottom of plate blotted after each wash.
15. 50 µl streptavidin/ phycoerythrin was added to each well.
16. The plate was covered and incubated in the dark at room temperature, after being agitated at 300 rpm for 10 minutes.
17. Steps 10 and 11 were repeated. Plate vacuumed and bottom of plate blotted after each wash.
18. Beads were re-suspended by adding 125 µl assay buffer to each well before reading.
19. The plate was agitated at 300 rpm for 30 sec and then read with a setting of 100 beads per region. (Figure A 7.2)
20. The above process was repeated for specimens 41-80.

A7.2.7.1 Cytokine, chemokine and growth factor Multiplex plate schematic

The multiplex plate was prepared as described above and the specimens to be analysed were pipetted into each wells on the plate as the per the diagram schematic in Figure A 7.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	S4	1	5	9	13	17	21	25	29	33	37
B	Blank	S4	1	5	9	13	17	21	25	29	33	37
C	Std 8*	S3	2	6	10	14	18	22	26	30	34	38
D	S7	S3	2	6	10	14	18	22	26	30	34	38
E	S6	S2	3	7	11	15	19	23	27	31	35	39
F	S6	S2	3	7	11	15	19	23	27	31	35	39
G	S5	S1***	4	8	12	16	20	24	28	32	36	40
H	S5	S7**	4	8	12	16	20	24	28	32	36	40

Figure A 7.2: Plate schematic used for analysis of standards as well as for serum cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, INFγ and TNFα), chemokines (eotaxin and RANTES) and the growth factor (VEGF)
Std: Standard, ***S1 = Concentrated, **S7 = Control, *S8 = Diluent

A7.2.8 Equipment Settings

The specifications used for the Luminex reader to analyse the multiplex plates: 100 IS v.1.7 or Luminex 100 IS v2.1/2.2, Luminex 200 v2.3 x potent, and Luminex HTS as per manufacturer's instructions are indicated in Table A 7.1.

Table A 7.1: Equipment specifications used for Luminex reader for Bio-Rad M500AD1ZL6 multiplex plate

Analyte	Concentration of S1 (pg/ml)	Bead Position
IL-1β	35 640	39 #
IL-2	16 139	38
IL-4	3 928	52
IL-5	35 782	33
IL-6	20 077	19
IL-8	30 993	54
IL-12	36 970	75
IL-13	34 712	57
EOTAXIN	22 002	43
IFNγ	29 436	21
RANTES	30 454	37
TNFα	77 264	36
VEGF	38 375	45

#The BIO RAD program had IL-1β at bead 32 that was incorrect and changed to position 39 after discussions with the local Company Representative.
IL: Interleukin, IFNγ: Interferon gamma, RANTES: Regulated on Activation, Normal T Expressed and Secreted, TNFα: Tumour necrosis factor alpha, VEGF: Vascular endothelial growth factor.

A7.2.9 Results

After the plate was processed and analysed on the luminex analyser the results were obtained by an automated program and stored for statistically analysis. The results are presented in the chapter detailing the results.

APPENDIX 8: MEASUREMENT OF TOTAL SERUM IGE LEVELS IN SERUM

A8.1 Methodology

Serum IgE levels were determined by a chemiluminescent detection system. The IMMULITE 2000 Total IgE solid phase, chemiluminescent immunometric assay was used. Chemiluminescence is the emission of light as the result of a chemical reaction. In chemiluminescence, a chemical reaction, instead of absorption of radiation, generates an organic molecule in an electronic excited state. Chemiluminescence differs from fluorescence in that the electronic excited state is derived from the product of a chemical reaction rather than the more typical way of creating electronic excited states, namely absorption.

A8.2 Methods

The Immulite detection system is a fully automated, random access immunoassay analyser that has a unique solid-phase washing process and a chemiluminescent detection system. The Immulite total IgE assay has a monoclonal antibody specific for IgE coated on a 6 mm polystyrene bead as a solid phase. The beads were sealed in a patented test unit into which 5µl of the patient's serum and an alkaline phosphatase (bovine calf intestine) conjugated to a polyclonal goat anti-IgE antibody were pipetted.

After incubation at 37C° with intermittent agitation, the IgE in the sample was bound by the antibody to form a sandwich complex. The unbound conjugate was removed by a centrifugal wash accomplished by spinning the tube on its vertical axis. After four such washes, the chemiluminescent substrate was added to the test unit and incubated for a further 10 mins. The chemiluminescent substrate PPD [4-methoxy-4-(3-phosphatephenyl)-spiro-(l, 2-dioxetane-3,2-adamantane)] a phosphate ester of adamantyl dioxetane, is hydrolysed by alkaline phosphatase to an unstable anion. This hydrolysis process is accompanied by the emission of light. The light is measured by a luminometer. Relative light units (RLUs) reflect the number of photons detected by the photomultiplier tube, which is proportional to the concentration of IgE in the sample. The Immulite 2000 instrument automatically handled the addition of the serum samples and reagents, the incubation under intermittent agitation, the four spin-wash steps, and the measurement of IgE by the temperature-controlled luminometer (476, 477).

APPENDIX 9: MEASUREMENT SERUM LEUKOTRIENE LEVELS

A9.1 Optimisation of Measuring Serum Leukotriene Levels – Pilot study with one LT analyte

Currently there are minimal published data on the appropriate methodology of measuring serum leukotriene levels using Enzymatic Immuno-Assays (EIA) kits. Most of the data thus far published are on the measurement of urinary leukotriene levels (406, 412-415). Furthermore, LTB4 is not excreted in the urine (476). Hence, a pilot study to determine the optimal method of measuring serum leukotriene level was performed (416-418).

The assay is based on the competition between serum LT and a LT–acetylcholinesterase (AChE) conjugate called an LT Tracer (416). Since the concentration of the LT Tracer is held constant while the concentration of the LT varies, the amount of LT Tracer that is able to bind to the LT Antiserum will be inversely proportional to the concentration of LT in the well (419). This antibody-LT complex binds to a mouse monoclonal anti-rabbit IgG that has been attached to the well by the manufacturers. The plate is washed to remove any unbound reagents and Ellman's Reagent, which contains the substrate to AChE is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs light strongly at 412 nm. The intensity of this colour, determined spectrophotometrically is proportional to the amount of free LT Tracer bound to the well. This value is inversely proportional to the amount of free LT present in the well (Figure 8.1)

The problem with the measurement of serum LT concentration is twofold:

- The concentration of serum LT levels in serum is minuscule. Thus to improve detection of LT in serum it is proposed by the manufacturer's to spike the specimens with a known concentration of LT supplied by the manufacturer's – a method called a cold spike. This process allows for the subtraction of the additional spike to get the true concentration of the analyte.
- The assay uses the intensity of a colour reaction determined spectrophotometrically to infer the concentration of the analyte. Thus the serum/urine from which the analyte is being measured has to be purified of any substances that might interfere with the reading process. The complication of the purification process is that the purification might leach the analyte as well and thus decrease the final concentration of the analyte.

A9.1.1 Development of Optimisation Experiments: Interpretation of results of purification, recovery and spiking process rational:

A9.1.1.1 Purification vs non-purification of samples

The purpose of this experiment was to pass standards provided by the manufactures of the kit through the purification columns to assess if the columns were leaching out analyte. Thus, the study was designed to determine the recovery factor and thus calculate if purification was leaching analyte. Standards with a known concentration were passed through the cartridges and compared with standards that had not gone through the purification column. This experiment allowed us to calculate of how much of the leukotrienes were being bound to the purification column, and what the recovery factor was post-purification. Furthermore, if there was no significant difference in measurements between purified and non-purified samples then the purification step could be omitted.

A9.1.1.2 Spiking samples to assess range of detection of kits

The purpose of this experiment was to determine the optimal range of analyte detection of the kits. Samples were spiked at the high end (Std 1 or Std 2) and low end (Std 7 or Std 8) of the range and repeated 5 times. The rational for this was to determine if the analyte in the samples could be detected at the high and low ranges of their concentrations by the kits used. Furthermore, the detection yield of the analyte was assessed depending on increased or lowered analyte concentration values.

A9.1.1.3 Spiking samples to increase readability

The rational for this was to determine if samples with very low analyte concentrations could be adequately assessed by spiking the samples with a known concentration of standard analyte provided by the manufactures. This was performed so that any minute concentrations of native analyte present in the serum could be read accurately by the analyser. The rational was that the difference between the measured value minus the known concentration of the spiked value would yield the actual serum LT concentration.

This rationale and the performing of these experiments allowed us to develop the final process of optimisation and the optimal dilutions for the samples that was used in the final protocol.

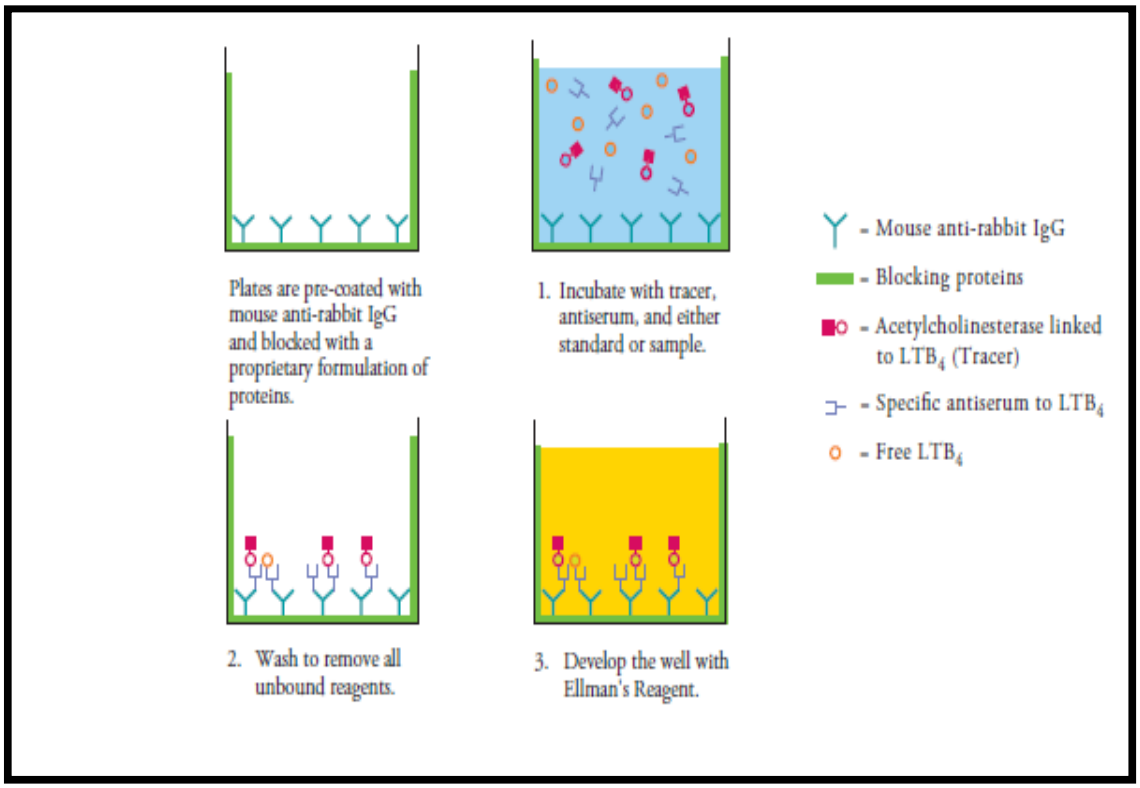


Figure A9.1: Schematic diagram of the technique of the ACE™ EIA kit. Figure courtesy of Caymen Chemicals.
Step 1: Incubation of patient serum with manufacturer provided tracer and antiserum in wells pre-coated with IgG antibodies that are fixed to the wells, and are targeted against antiserum that binds leukotriene molecules and the free leukotriene molecules
Step 2: washing of well to remove all unbound reagents
Step 3: developing the well with Ellman's reagent and measuring the chemiluminescence emitted as a measure of the serum leukotriene concentration.
LTB_4: leukotriene B4

Thus, optimisation studies following the hypothesis described above with serum from five of the final group of patients was performed. The patient samples were not randomly chosen but were chosen depending on their ACQ stratification i.e. patients samples with an ACQ < 0.75; between 0.75 and 1.5 & an ACQ > 1.5 to answer the following questions:

1. Was it necessary to spike the sample or could we derive the same results without spiking?
2. Was it necessary to column purify the serum sample or would the analyte cleave to the column, reducing the final concentration of the analyte being measured?
3. What is the optimal dilution to obtain results of measured LT at both spectrums of the kit sensitivity?

A9.2 Preparation of Leukotriene kit according to manufacturer's instructions (Cayman chemicals ™)

Kit used: Leukotriene C4 E/A kit # 520211 Cayman chemicals ACE

A9.2.1 Preparation Process - Day 1

Step 1: Preparation Process

- Preparation of Enzyme Immuno-Assay (EIA) buffer
  - 10 ml of EIA buffer concentrate [10X] provided by manufacturer's was diluted with 90 ml of ultra-pure H2O
- Preparation of wash buffer
  - 5 ml wash buffer concentrate [400X] provided by manufacturer's was diluted with 2000 ml with ultra-pure H2O
  - 1 ml of Tween 20 (detergent) was added to the total volume of wash buffer as per manufacturer's instructions
- Preparation of samples– plasma
  - Plasma samples were collected in vacutainers containing EDTA and the processing of the serum involved the following steps:

Step 2: Purification process:

- Consumables used:
  - Hyper sep C8 SPE column separation cartridge with a 3 ml capacity purchased from Thermo Scientific was used (ref no: # 66108-309)
  - Molar 1.0 M HCL
  - Methanol
  - Ethanol
  - Hexane
  - 3 ml glass tubes (collect sample)

- Process of purification:
  - Samples were aliquoted into different Hyper sep C8 SPE separation cartridge columns as follows:
    - 500 µl of plasma, and
    - 500 µl of plasma and spiked with 125 ng/ml LTC4
  - Plasma pH was reduced to 4.0 using 1.0 molar concentration of HCL.

- The separation cartridge was prepared by adding 2 ml methanol and followed by 2 ml ultrapure H_2O.
- The cartridge was not allowed to dry.
- The sample was passed through the cartridge.
- The separation cartridge columns was then rinsed with ultra-pure H_2O.
- The separation cartridge columns was then rinsed again with 2 ml hexane.
- The cartridge was then allowed to dry and rinsed again with ultra-pure H_2O.
- Remaining LTC4 was eluted from the separation cartridge columns with 2 ml methanol.
- The methanol was evaporated under a stream of dry nitrogen dryer at 50℃ for 40 minutes.
- 250 µl of EIA buffer was added to each sample, which were then vortexed.

Step 3: Preparation of Standards (Figure A 9.2)

- The pipette tip was equilibrated in ethanol.
- Pipette tip used to transfer 100 µl of 100 ng/ml standard supplied by manufacture to 900 µl ultra-pure H_2O making a final concentration of 10 ng/ul
- Further dilutions of standard were prepared as follows:

	Conc.	EIA buffer	Vol standard
Bulk std	10 ng/ml	As above	
S1	1000 pg/ml	900 ul	100 ul of bulk std
S2	500 pg	500 ul	500 ul of S1
S3	250 pg/ml	500 ul	500 ul of S2
S4	125 pg/ml	500 ul	500 ul of S3
S5	62.5 pg/ml	500 ul	500 ul of S4
S6	31.3 pg/ml	500 ul	500 ul of S5
S7	15.6 pg/ml	500 ul	500 ul of S6
S8	7.8 pg/ml	500 ul	500 ul of S7
Bo	0 pg/ml	500 ul	-

Figure A9.2: Plate schematic used for the preparation of dilutions for standards as well as for the analyte LTC4

Conc: Concentration, EIA: Enzyme Immuno-Assay, Vol: Volume, Std: Standard, ng/ml: Nanogram per millilitre, µl: microliter, pg/ml: Picogram per millilitre

Step 4: Preparation of AChE Tracer

100 ml lyophilised LTC4 tracer was reconstituted with 6 ml EIA buffer

Step 5: Preparation of Tracer Dye

60 µl tracer dye (pink) was added to 6 ml of tracer

Step 6: Preparation of LTC4 EIA antiserum

100 ml lyophilised LTC4 antiserum was reconstituted with 6 ml EIA buffer

Step 7: Preparation of Anti Serum Dye

100 ml LTC4 antiserum dye was reconstituted with 6 ml EIA buffer

Step 8: Preparation of Serum Samples (Figure A 9.3):

Several different samples were prepared and analysed to determine the concentration of LTC4 in the optimisation experiments. The five different samples were prepared as follows:

1) Purified non-spiked, undiluted samples were neither spiked with LTC4, nor diluted

2) Purified spiked samples were prepared by adding 100 µl of serum to 100 µl of LTC4 with a known concentration of 250 ng/ml (provided by the manufacturers) to give a final concentration 125 ng/ml

3) Purified non-spiked diluted samples were prepared by adding 100 µl of serum to 100 µl of EIA buffer (diluted 1 in 2)

4) Non-purified, non-spiked serum but diluted samples were prepared by adding 100 µl of serum to 100 µl of EIA buffer (diluted 1 in 2) and were not spiked.

5) Non-purified, spiked serum samples were prepared by adding 100 µl of serum to 100 µl of LTC4 with a known concentration of 250 ng/ml (provided by the manufacturers) to give a final concentration 125 ng/ml

	1	2	3	4	5	6	
A	BLK	S1	1a	2a	3a	4a	purified, non- spiked, undiluted
B	BLK	S2	1a	2a	3a	4a	purified, non- spiked, undiluted
C	NSB	S3	1b	2b	3b	4b	purified, spiked
D	NSB	S4	1b	2b	3b	4b	purified, spiked
E	B_O	S5	1c	2c	3c	4c	purified, non- spiked, diluted 1:2
F	B_O	S6	1c	2c	3c	4c	purified, non- spiked, diluted 1:2
G	B_O	S7	1d	2d	3d	4d	non- purified, non- spiked, diluted 1:2
H	TA	S8	1e	2e	3e	4e	non- purified, spiked

Figure A9.3: Plate schematic used for analysis of initial pilot study of LTC4
NSB: Non-specific binding, B0: Maximum binding between tracer and antibody without any analyte, BLK: Blank, TA: Total activity.

Step 9: Plate reconstitution:

- 100 µl of EIA buffer was pipetted into wells C1-D1
- 50 µl of standard was pipetted into wells A2 – H2
- 50 µl serum sample (prepared as step 8 detailed above)was pipetted into wells A3 – H6
- 50 µl LTC4 tracer was additionally pipetted into wells A2 – H6
- 50 µl LTC4 antiserum additionally pipetted into wells A2 – H6
- A1 - B1: BLK left as an empty well
- C1 – D1: NSB : 100 ul EIA buffer + 50 µl LTC4 tracer were added
- E1 – G1: B0 : 50 ul EIA buffer + 50 µl LTC4 tracer + 50 µl LTC4 antiserum added
- H1 : TA: only 5 µl LTC4 tracer added

Step 10: Plate covered and incubated at room temperature for 18 hours

A9.2.2 Day 2 - Analysis of data

- Reconstituted Ellman's reagent
- Emptied the wells and wash 6 times with a plate washer
- Added 200 µl of Ellman's reagent to each well
- Covered plate with a foil film and allowed reaction to develop while incubated for 60-90 mins the dark on a shaker
- Bottom of plate wiped
- Measured at A405 wave-length (B_O should be between 0.3 and 1.0 after subtracting the blank)
- Results documented, analysed and discussed in section A9.3 (Figure A 9.4):

ASSAY: Leukotriene C4

Dilution Factor
Needed for proper calculations, use 1 for undiluted samples, 2 for 2 fold, etc.

LAYOUT	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std01	Std01	6a	9a	34a	36a	6e	Smpl21	Smpl25	Smpl29	Smpl33
B	Blank	Std02	Std02	2	2	2	2	2	2	2	2	2
C	NSB	Std03	Std03	6b	9b	34b	36b	9e	Smpl22	Smpl26	Smpl30	Smpl34
D	NSB	Std04	Std04	2	2	2	2	2	2	2	2	2
E	Bo	Std05	Std05	6c	9c	34c	36c	34e	Smpl23	Smpl27	Smpl31	Ctrl1
F	Bo	Std06	Std06	2	2	2	2	2	2	2	2	1
G	Bo	Std07	Std07	6d	9d	34d	36d	36e	Smpl24	Smpl28	Smpl32	Ctrl2
H	TA	Std08	Std08	2	2	2	2	2	2	2	2	1

RAW DATA	1	2	3	4	5	6	7	8	9	10	11	12
A	0.120	0.356	0.356	0.335	0.299	0.290	0.282	0.455				
B	0.121	0.422	0.422	0.340	0.310	0.282	0.295	0.455				
C	0.122	0.504	0.504	0.304	0.213	0.231	0.241	0.404				
D	0.125	0.517	0.517	0.302	0.219	0.242	0.249	0.404				
E	0.604	0.514	0.514	0.467	0.401	0.360	0.383	0.442				
F	0.585	0.559	0.559	0.468	0.417	0.380	0.388	0.442				
G	0.636	0.575	0.575	0.445	0.417	0.422	0.388	0.420				
H	1.285	0.542	0.542	0.445	0.417	0.422	0.388	0.420				

AvgRawBlnk= 0.1205

SUBTRACTED	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.001	0.236	0.236	0.215	0.179	0.170	0.162	0.335	-0.121	-0.121	-0.121	-0.121
B	0.001	0.302	0.302	0.220	0.190	0.162	0.175	0.335	-0.121	-0.121	-0.121	-0.121
C	0.002	0.384	0.384	0.184	0.093	0.111	0.121	0.284	-0.121	-0.121	-0.121	-0.121
D	0.005	0.397	0.397	0.182	0.099	0.122	0.129	0.284	-0.121	-0.121	-0.121	-0.121
E	0.484	0.394	0.394	0.347	0.281	0.240	0.263	0.322	-0.121	-0.121	-0.121	-0.121
F	0.465	0.439	0.439	0.348	0.297	0.260	0.268	0.322	-0.121	-0.121	-0.121	-0.121
G	0.516	0.455	0.455	0.325	0.297	0.302	0.268	0.300	-0.121	-0.121	-0.121	-0.121
H	1.165	0.422	0.422	0.325	0.297	0.302	0.268	0.300	-0.121	-0.121	-0.121	-0.121

AvgBlank= 0.0000
AvgTA= 1.1645
AvgNSB= 0.0030
AvgBo= 0.4848

B/Bo	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.007	0.480	0.480	0.436	0.362	0.343	0.327	0.684	-0.255	-0.255	-0.255	-0.255
B	-0.005	0.616	0.616	0.447	0.385	0.327	0.354	0.684	-0.255	-0.255	-0.255	-0.255
C	-0.003	0.785	0.785	0.372	0.185	0.222	0.242	0.579	-0.255	-0.255	-0.255	-0.255
D	0.003	0.812	0.812	0.368	0.197	0.244	0.259	0.579	-0.255	-0.255	-0.255	-0.255
E	0.991	0.805	0.805	0.708	0.572	0.488	0.535	0.657	-0.255	-0.255	-0.255	-0.255
F	0.952	0.898	0.898	0.711	0.605	0.529	0.546	0.657	-0.255	-0.255	-0.255	-0.255
G	1.057	0.931	0.931	0.663	0.605	0.616	0.546	0.612	-0.255	-0.255	-0.255	-0.255
H	2.396	0.863	0.863	0.663	0.605	0.616	0.546	0.612	-0.255	-0.255	-0.255	-0.255

>80% B/Bo <20% B/Bo

LOGIT	1	2	3	4	5	6	7	8	9	10	11	12
A		-0.082	-0.082	-0.256	-0.567	-0.648	-0.722	0.771	#NUM!	#NUM!	#NUM!	#NUM!
B		0.471	0.471	-0.215	-0.470	-0.722	-0.603	0.771	#NUM!	#NUM!	#NUM!	#NUM!
C		1.294	1.294	-0.522	-1.485	-1.256	-1.140	0.317	#NUM!	#NUM!	#NUM!	#NUM!
D		1.461	1.461	-0.540	-1.405	-1.129	-1.052	0.317	#NUM!	#NUM!	#NUM!	#NUM!
E		1.421	1.421	0.888	0.291	-0.049	0.141	0.650	#NUM!	#NUM!	#NUM!	#NUM!
F		2.178	2.178	0.898	0.428	0.116	0.183	0.650	#NUM!	#NUM!	#NUM!	#NUM!
G		2.606	2.606	0.677	0.428	0.471	0.183	0.454	#NUM!	#NUM!	#NUM!	#NUM!
H		1.842	1.842	0.677	0.428	0.471	0.183	0.454	#NUM!	#NUM!	#NUM!	#NUM!

CONC	1	2	3	4	5	6	7	8	9	10	11	12
A		2217.6	2217.6	6485.7	12742.7	15209.9	17870.2	693.1	#NUM!	#NUM!	#NUM!	#NUM!
B		665.4	665.4	5921.4	10317.6	17870.2	13778.3	693.1	#NUM!	#NUM!	#NUM!	#NUM!
C		111.1	111.1	11569.3	94105.8	57064.0	44350.9	1862.5	#NUM!	#NUM!	#NUM!	#NUM!
D		77.3	77.3	12023.5	79036.9	43282.9	36629.7	1862.5	#NUM!	#NUM!	#NUM!	#NUM!
E		84.3	84.3	537.2	1968.0	4127.6	2729.7	902.7	#NUM!	#NUM!	#NUM!	#NUM!
F		16.2	16.2	525.7	1462.6	2881.4	2493.8	902.7	#NUM!	#NUM!	#NUM!	#NUM!
G		6.4	6.4	850.1	1462.6	1330.9	2493.8	1382.3	#NUM!	#NUM!	#NUM!	#NUM!
H		33.7	33.7	850.1	1462.6	1330.9	2493.8	1382.3	#NUM!	#NUM!	#NUM!	#NUM!

STANDARDS	Conc	Avg	B/Bo	Logit	Recalc
Std01	1000.00	0.233	0.480	-0.082	2217.64
Std02	500.00	0.299	0.616	0.471	665.45
Std03	250.00	0.381	0.785	1.294	111.06
Std04	125.00	0.394	0.812	1.461	77.27
Std05	62.50	0.391	0.805	1.421	84.29
Std06	31.25	0.436	0.898	2.178	16.22
Std07	15.63	0.452	0.931	2.606	6.39
Std08	7.81	0.419	0.863	1.842	33.69

STATS	
y-int=	3.4581
slope=	-0.4595
R^2=	0.7995

CURVE	Value	%B/Bo	Logit
IC20=	37916.39	20.00	-1.3863
IC50=	1855.74	50.00	0.0000
IC80=	90.83	80.00	1.3863

FILENAME: 16 aug 11 c4

VERSION: 2008D235

	LTC4	pg/mL	%B/Bo	Dilution	StdDev	%CV	SEM
6a	6203.5	44.1	1	399.04	6.43	282.17	
6b	11796.4	37.0	2	321.15	2.72	227.08	
6c	531.4	71.0	2	8.18	1.54	5.79	
6d	850.1	66.3	1	0.00	0.00	0.00	
9a	11530.1	37.3	1	1714.82	14.87	1212.56	
9b	86571.3	19.1	2	10655.29	12.31	7534.42	
9c	1715.3	58.9	2	357.36	20.83	252.69	
9d	1462.6	60.5	1	0.00	0.00	0.00	
34a	16540.1	33.5	1	1881.08	11.37	1330.12	
34b	50173.4	23.3	2	9744.72	19.42	6890.56	
34c	3504.5	50.8	2	881.19	25.14	623.10	
34d	1330.9	61.6	1	0.00	0.00	0.00	
36a	15824.3	34.0	1	2893.36	18.28	2045.91	
36b	40490.3	25.1	2	5459.74	13.48	3860.62	
36c	2611.7	54.0	2	166.78	6.39	117.93	
36d	2493.8	54.6	1	0.00	0.00	0.00	
6e	693.1	68.4	2	0.00	0.00	0.00	
9e	1862.5	57.9	2	0.00	0.00	0.00	
34e	902.7	65.7	2	0.00	0.00	0.00	
36e	1382.3	61.2	2	0.00	0.00	0.00	
Smpl21	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl22	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl23	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl24	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl25	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl26	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl27	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl28	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl29	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl30	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl31	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl32	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl33	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
Smpl34	#NUM!	-25.5	2	#NUM!	#NUM!	#NUM!	
							% Error
Ctrl1	#NUM!	-25.5	1	#NUM!	#NUM!	#NUM!	#NUM!
Ctrl2	#NUM!	-25.5	1	#NUM!	#NUM!	#NUM!	#NUM!

=Data outside 20-80% B/Bo range

Figure A9.4: Screen-capture of computer readout of EIA well analysis for initial LT optimisation experiment using LTC4

A9.3 Optimisation of Measuring Serum Leukotriene Levels – Pilot study with all 3 LT analytes

After completing the initial purification and spiking tests and analysing the data, the above experiment was repeated using each of the LTB4, LTC4, and LTE4 kits to determine if process was repeatable for all 3 kits i.e. optimisation of LTB4, LTC4, and LTE4 on patient samples. The patient samples were not randomly chosen but were chosen depending on their ACQ stratification i.e. 2 patients samples with an ACQ < 0.75; 1 with an ACQ between 0.75 and 1.5 & 2 patients with an ACQ > 1.5 each.

Kits used: - Leukotriene B4 EIA kit, # 520111, Caymans chemicals
- Leukotriene C4 EIA kit, #520211, Caymans chemicals
- Leukotriene E4 EIA kit, #520411, Caymans chemicals

A9.3.1 Brief synopsis of sample preparation with detailed principles discussed in section 9.2 (Figure A 9.5)

- **LTB4:**
  - Sample consisted of 500 µl plasma
  - Spiked with known amount of LTB4
  - Adjusted to pH 4.0 using 2 µl HCL added to 25 µl of sample
  - Prepared separation cartridge by adding 2 ml methanol and 2 ml ultra-pure H_2O
  - Did not allow cartridge to dry.
  - Applied pH adjusted sample to purification cartridge
  - Passed sample through the cartridge
  - Washed with ultra-pure H_2O
  - Eluted and rinsed LTB4 with 2 ml ethanol
  - Evaporated ethanol under a stream of dry nitrogen dryer at 50℃ for 40 mins
  - Reconstituted recovered sample with 500 µl of EIA buffer and vortexed

- **LTC4:**
  - Sample consisted of 500 µl plasma
  - Spiked with known amount of LTC4
  - Adjusted to pH 4.0 using 2 µl HCL added to 25 µl sample

- Prepared separation cartridge by adding 2 ml methanol and 2 ml ultra-pure H_2O
- Did not allow cartridge to dry
- Applied pH adjusted sample to purification cartridge
- Passed sample through the cartridge
- Washed with ultra-pure H_2O
- Washed a second time with hexane
- Eluted and rinsed LTC4 with 2 ml methanol
- Evaporated methanol under a stream of dry nitrogen dryer at 50℃ for 40 mins
- Reconstituted recovered sample with 500 µl of EIA buffer and vortexed

- **LTE4:**
  - Sample consisted of 500 µl plasma
  - Spiked with known amount of LTE4
  - Adjusted to pH 4.0 using 2 µl HCL added to 25 µl sample
  - Prepared separation cartridge by adding 2 ml methanol and 2 ml ultrapure H_2O
  - Did not allow cartridge to dry
  - Applied pH adjusted sample to purification cartridge
  - Passed sample through the cartridge
  - Washed with ultra-pure H_2O
  - Washed a second time with hexane
  - Eluted and rinsed LTE4 with 2 ml methanol
  - Evaporated methanol under a stream of dry nitrogen dryer at 50℃ for 40 mins
  - Reconstituted recovered sample with 500 µl of EIA buffer and vortexed

A9.3.2 Spiked Samples Prepared as Follows:

- **LTB4:** 75 µl plasma added to 75 µl of Standard 4 prepared as above and then eluted with 150 µl EIA
- **LTC4:** 75 µl plasma added to 75 µl of Standard 4 prepared as above and then eluted with 150 µl EIA

- **LTE4:** 75 µl plasma added to 75 µl of Standard 4 prepared as above and then eluted with 150 µl EIA

A9.3.3 Non - Spiked Samples Prepared as Follows:

- **LTB4:** 300 µl plasma eluted with 300 µl EIA
- **LTC4:** 400 µl plasma eluted with 400 µl EIA
- **LTE4:** 400 µl plasma eluted with 400 µl EIA

All above samples further diluted to 1:2 and 1:4 by mixing with equal quantities of EIA

A9.3.4 Preparation of Standards as per manufacturer's instructions (Figure A 9.5):

- **LTB4:** standard vial = 50 ng/ml
- **LTC4:** standard vial = 100 ng/ml
- **LTE4:** standard vial = 100 ng/ml

Standard were prepared in bulk as discussed in 9.2

A pipette tip was equilibrate in ethanol and use to transfer 100 µl of standard solution provided by the manufacturers to 900 µl ultrapure H_2O

	EIA buffer	Standard	Conc. LTB4	Conc. LTC4	Conc. LTE4
Bulk std	As in above 9.2	As in above 9.2	5 ng/ml	10 ng/ml	10 ng/ml
S1	900 µl	100 µl of bulk std	500 pg/ml	1000 pg/ml	1000 pg/ml
S2	500 µl	500 µl of S1	250 pg/ml	500 pg/ml	500 pg/ml
S3	500 µl	500 µl of S2	125 pg/ml	250 pg/ml	250 pg/ml
S4	500 µl	500 µl of S3	62.5 pg/ml	125 pg/ml	125 pg/ml
S5	500 µl	500 µl of S4	31.3 pg/ml	62.5 pg/ml	62.5 pg/ml
S6	500 µl	500 µl of S5	15.6 pg/ml	31.3 pg/ml	3.3 pg/ml
S7	500 µl	500 µl of S6	7.8 pg/ml	15.6 pg/ml	15.6 pg/ml
S8	500 µl	500 µl of S7	3.9 pg/ml	7.8 pg/ml	7.8 pg/ml
BO	500 ul	-	0	0	0

Figure A9.5: Plate schematic used for the preparation of further dilutions for standards for LTB4, LTC4 and LTE4

Conc: Concentration, EIA: Enzyme Immuno-Assay, Vol: Volume, Std: Standard, ng/ml: Nanogram per millilitre, µl: Microliter, pg/ml: Picogram per millilitre, LT: Leukotriene.

A9.3.5 Plate Reconstitution- discussed in detail in 9.2 step 9 (Figure A 9.6):

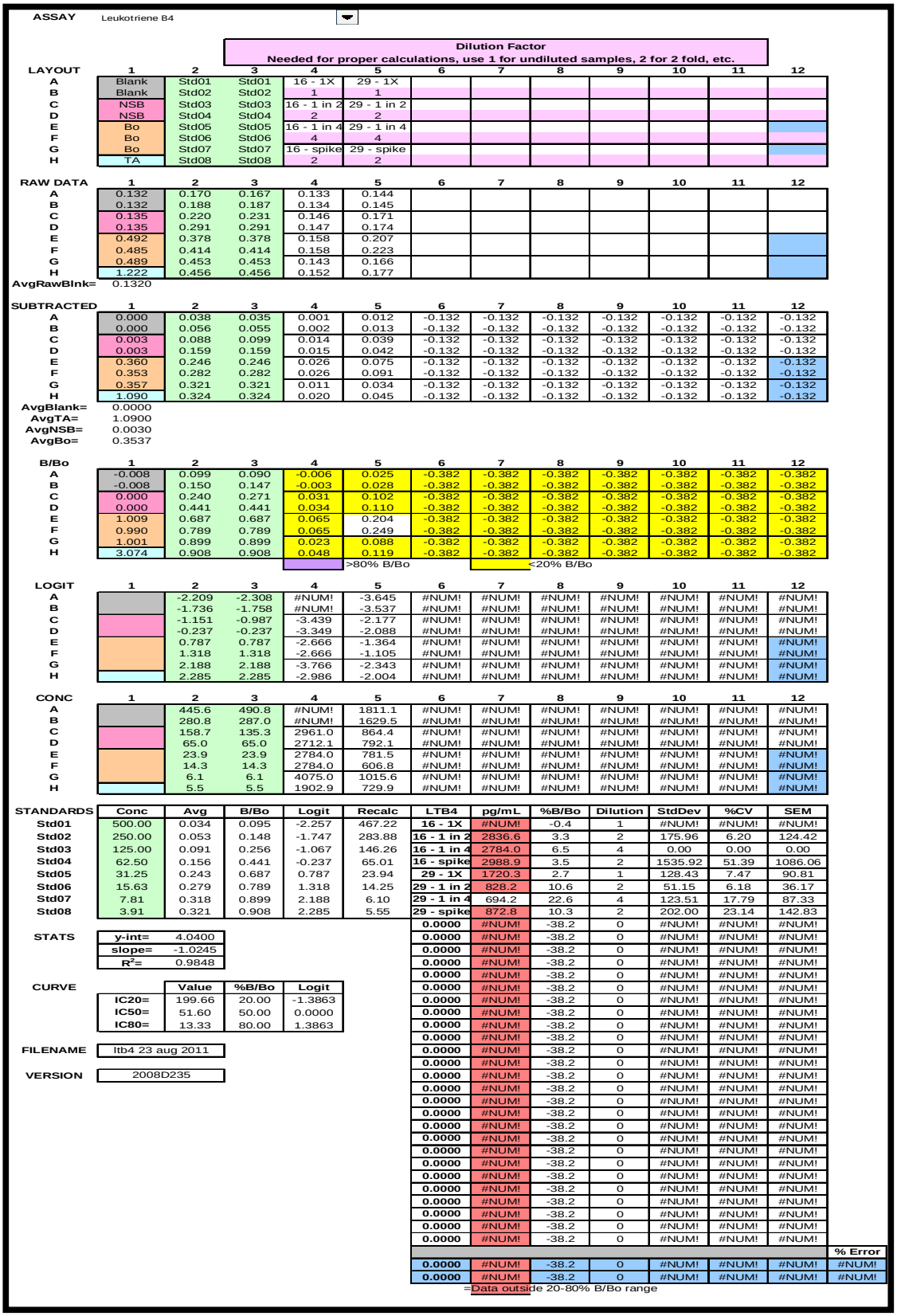
- Standard wells consisted of 50 µl standard with 50 µl tracer and 50 µl antiserum added
- Sample wells consisted of 50 µl standard with 50 µl tracer and 50 µl antiserum added
- BLK empty well
- NSB consisted of 100 ul EIA buffer with 50 µl LTC4 tracer added
- TA consisted of only 5 µl LTC4 tracer added
- Plate covered and incubated at room temperature for 18 hours

	1 LTB4	2 LTB4	3 LTB4	4 LTB4	5 LTC4	6 LTC4	7 LTC4	8 LTC4	9 LTE4	10 LTE4	11 LTE4	12 LTE4
A	BLK	S6	Sample1 [1:1]	Sample2 [1:1]	BLK	S5	Sample1 [1:1]	Sample2 [1:1]	BLK	S5	Sample1 [1:1]	Sample2 [1:1]
B	NSB	S7	Sample1 [1:1]	Sample2 [1:1]	NSB	S6	Sample1 [1:1]	Sample2 [1:1]	NSB	S6	Sample1 [1:1]	Sample2 [1:1]
C	BO	S8	Sample1 [1:2]	Sample2 [1:2]	BO	S7	Sample1 [1:2]	Sample2 [1:2]	BO	S7	Sample1 [1:2]	Sample2 [1:2]
D	S1	BO	Sample1 [1:2]	Sample2 [1:2]	BO	S8	Sample1 [1:2]	Sample2 [1:2]	BO	S8	Sample1 [1:2]	Sample2 [1:2]
E	S2	S1	Sample1 [1:4]	Sample2 [1:4]	S1	S1	Sample1 [1:4]	Sample2 [1:4]	S1	S1	Sample1 [1:4]	Sample2 [1:4]
F	S3	S2	Sample1 [1:4]	Sample2 [1:4]	S2	S2	Sample1 [1:4]	Sample2 [1:4]	S2	S2	Sample1 [1:4]	Sample2 [1:4]
G	S4	S3	Sample1 Spiked	Sample2 Spiked	S3	S3	Sample1 Spiked	Sample2 Spiked	S3	S3	Sample1 Spiked	Sample2 Spiked
H	S5	TA	Sample1 Spiked	Sample2 Spiked	S4	TA	Sample1 Spiked	Sample2 Spiked	TA	TA	Sample1 Spiked	Sample2 Spiked

Figure A9.6: Plate schematic used for analysis of the optimisation study using all 3 leukotrienes i.e. LTB4, LTC4 and LTE4
LT: Leukotriene, Std: Standard, B0: Maximum binding between tracer and antibody without any analyte.

A9.3.6 Day 2 - Analysis of data revealed the following:

- **LTB4 (Figure A 9.7):**
  - All of the sample are out of range except for sample 2 [1:4]
  - Need to dilute samples further
- **LTC4 (Figure A 9.8):**
  - Do not need to dilute samples
- **LTE4 (Figure A 9.9):**
  - Dilute samples 1:2 or 1:4



ASSAY: Leukotriene B4

Dilution Factor
Needed for proper calculations, use 1 for undiluted samples, 2 for 2 fold, etc.

LAYOUT	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std01	Std01	16 - 1X	29 - 1X							
B	Blank	Std02	Std02	1	1							
C	NSB	Std03	Std03	16 - 1 in 2	29 - 1 in 2							
D	NSB	Std04	Std04	2	2							
E	Bo	Std05	Std05	16 - 1 in 4	29 - 1 in 4							
F	Bo	Std06	Std06	4	4							
G	Bo	Std07	Std07	16 - spike	29 - spike							
H	TA	Std08	Std08	2	2							

RAW DATA	1	2	3	4	5	6	7	8	9	10	11	12
A	0.132	0.170	0.167	0.133	0.144							
B	0.132	0.188	0.187	0.134	0.145							
C	0.135	0.220	0.231	0.146	0.171							
D	0.135	0.291	0.291	0.147	0.174							
E	0.492	0.378	0.378	0.158	0.207							
F	0.485	0.414	0.414	0.158	0.223							
G	0.489	0.453	0.453	0.143	0.166							
H	1.222	0.456	0.456	0.152	0.177							

AvgRawBlnk= 0.1320

SUBTRACTED	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.038	0.035	0.001	0.012	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
B	0.000	0.056	0.055	0.002	0.013	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
C	0.003	0.088	0.099	0.014	0.039	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
D	0.003	0.159	0.159	0.015	0.042	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
E	0.360	0.246	0.246	0.026	0.075	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
F	0.353	0.282	0.282	0.026	0.091	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
G	0.357	0.321	0.321	0.011	0.034	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132
H	1.090	0.324	0.324	0.020	0.045	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132	-0.132

AvgBlank= 0.0000
AvgTA= 1.0900
AvgNSB= 0.0030
AvgBo= 0.3537

B/Bo	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.008	0.099	0.090	-0.006	0.025	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
B	-0.008	0.150	0.147	-0.003	0.028	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
C	0.000	0.240	0.271	0.031	0.102	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
D	0.000	0.441	0.441	0.034	0.110	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
E	1.009	0.687	0.687	0.065	0.204	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
F	0.990	0.789	0.789	0.065	0.249	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
G	1.001	0.899	0.899	0.023	0.088	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382
H	3.074	0.908	0.908	0.048	0.119	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382	-0.382

>80% B/Bo <20% B/Bo

LOGIT	1	2	3	4	5	6	7	8	9	10	11	12
A		-2.209	-2.308	#NUM!	-3.645	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B		-1.736	-1.758	#NUM!	-3.537	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C		-1.151	-0.987	-3.439	-2.177	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D		-0.237	-0.237	-3.349	-2.088	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E		0.787	0.787	-2.666	-1.364	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F		1.318	1.318	-2.666	-1.105	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G		2.188	2.188	-3.766	-2.343	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H		2.285	2.285	-2.986	-2.004	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

CONC	1	2	3	4	5	6	7	8	9	10	11	12
A		445.6	490.8	#NUM!	1811.1	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B		280.8	287.0	#NUM!	1629.5	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C		158.7	135.3	2961.0	864.4	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D		65.0	65.0	2712.1	792.1	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E		23.9	23.9	2784.0	781.5	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F		14.3	14.3	2784.0	606.8	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G		6.1	6.1	4075.0	1015.6	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H		5.5	5.5	1902.9	729.9	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

STANDARDS	Conc	Avg	B/Bo	Logit	Recalc
Std01	500.00	0.034	0.095	-2.257	467.22
Std02	250.00	0.053	0.148	-1.747	283.88
Std03	125.00	0.091	0.256	-1.067	146.26
Std04	62.50	0.156	0.441	-0.237	65.01
Std05	31.25	0.243	0.687	0.787	23.94
Std06	15.63	0.279	0.789	1.318	14.25
Std07	7.81	0.318	0.899	2.188	6.10
Std08	3.91	0.321	0.908	2.285	5.55

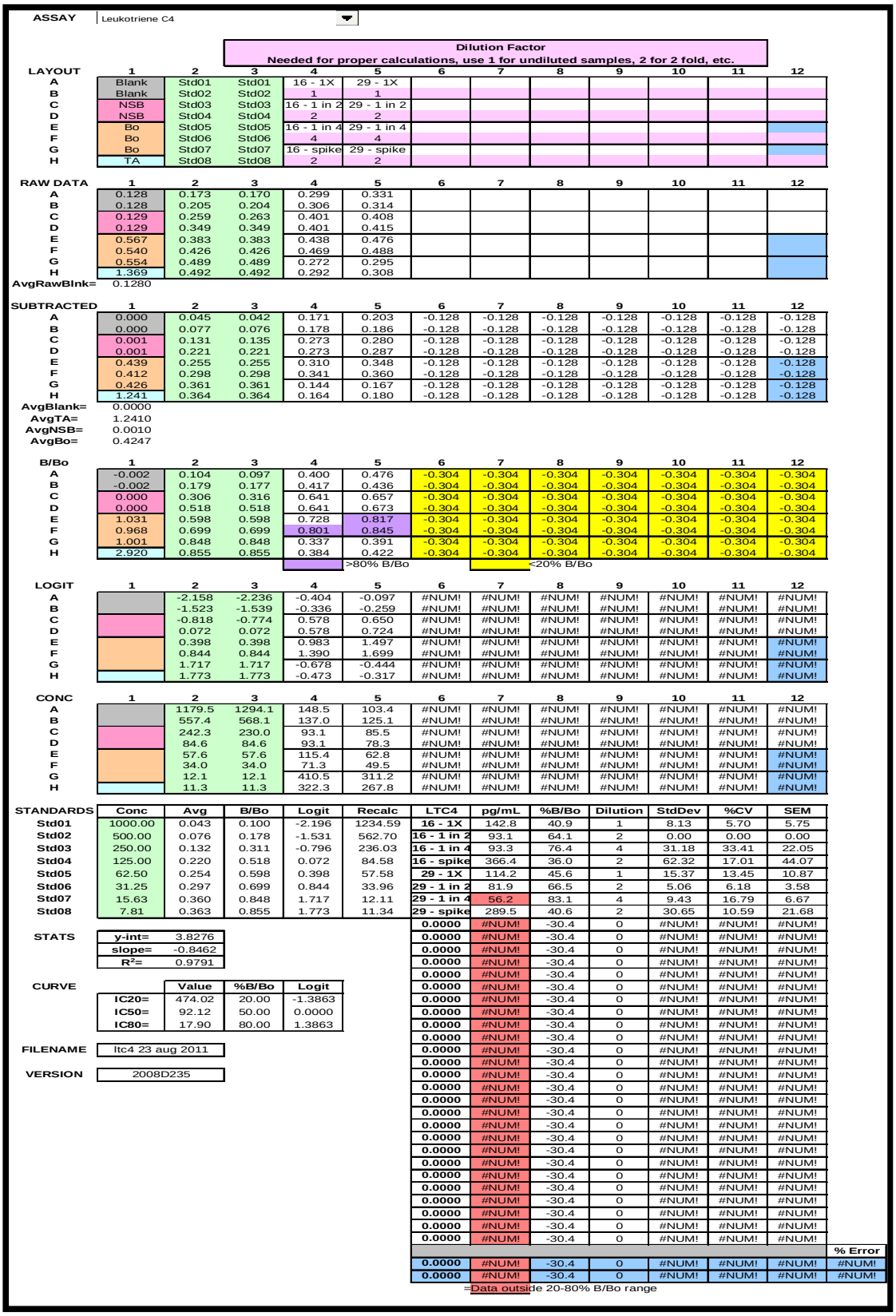
STATS	
y-int=	4.0400
slope=	-1.0245
R^2=	0.9848

CURVE	Value	%B/Bo	Logit
IC20=	199.66	20.00	-1.3863
IC50=	51.60	50.00	0.0000
IC80=	13.33	80.00	1.3863

FILENAME: ltb4 23 aug 2011

VERSION: 2008D235

LTB4	pg/mL	%B/Bo	Dilution	StdDev	%CV	SEM
16 - 1X	#NUM!	-0.4	1	#NUM!	#NUM!	#NUM!
16 - 1 in 2	2836.6	3.3	2	175.96	6.20	124.42
16 - 1 in 4	2784.0	6.5	4	0.00	0.00	0.00
16 - spike	2988.9	3.5	2	1535.92	51.39	1086.06
29 - 1X	1720.3	2.7	1	128.43	7.47	90.81
29 - 1 in 2	828.2	10.6	2	51.15	6.18	36.17
29 - 1 in 4	694.2	22.6	4	123.51	17.79	87.33
29 - spike	872.8	10.3	2	202.00	23.14	142.83
0.0000	#NUM!	-38.2	0	#NUM!	#NUM!	#NUM!



ASSAY: Leukotriene C4

Dilution Factor
Needed for proper calculations, use 1 for undiluted samples, 2 for 2 fold, etc.

LAYOUT	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std01	Std01	16 - 1X	29 - 1X							
B	Blank	Std02	Std02	1	1							
C	NSB	Std03	Std03	16 - 1 in 2	29 - 1 in 2							
D	NSB	Std04	Std04	2	2							
E	Bo	Std05	Std05	16 - 1 in 4	29 - 1 in 4							
F	Bo	Std06	Std06	4	4							
G	Bo	Std07	Std07	16 - spike	29 - spike							
H	TA	Std08	Std08	2	2							

RAW DATA	1	2	3	4	5	6	7	8	9	10	11	12
A	0.128	0.173	0.170	0.299	0.331							
B	0.128	0.205	0.204	0.306	0.314							
C	0.129	0.259	0.263	0.401	0.408							
D	0.129	0.349	0.349	0.401	0.415							
E	0.567	0.383	0.383	0.438	0.476							
F	0.540	0.426	0.426	0.469	0.488							
G	0.554	0.489	0.489	0.272	0.295							
H	1.369	0.492	0.492	0.292	0.308							

AvgRawBlnk= 0.1280

SUBTRACTED	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.045	0.042	0.171	0.203	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
B	0.000	0.077	0.076	0.178	0.186	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
C	0.001	0.131	0.135	0.273	0.280	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
D	0.001	0.221	0.221	0.273	0.287	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
E	0.439	0.255	0.255	0.310	0.348	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
F	0.412	0.298	0.298	0.341	0.360	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
G	0.426	0.361	0.361	0.144	0.167	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128
H	1.241	0.364	0.364	0.164	0.180	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128	-0.128

AvgBlank= 0.0000
AvgTA= 1.2410
AvgNSB= 0.0010
AvgBo= 0.4247

B/Bo	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.002	0.104	0.097	0.400	0.476	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
B	-0.002	0.179	0.177	0.417	0.436	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
C	0.000	0.306	0.316	0.641	0.657	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
D	0.000	0.518	0.518	0.641	0.673	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
E	1.031	0.598	0.598	0.728	0.817	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
F	0.968	0.699	0.699	0.801	0.845	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
G	1.001	0.848	0.848	0.337	0.391	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304
H	2.920	0.855	0.855	0.384	0.422	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304	-0.304

>80% B/Bo
<20% B/Bo

LOGIT	1	2	3	4	5	6	7	8	9	10	11	12
A		-2.158	-2.236	-0.404	-0.097	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B		-1.523	-1.539	-0.336	-0.259	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C		-0.818	-0.774	0.578	0.650	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D		0.072	0.072	0.578	0.724	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E		0.398	0.398	0.983	1.497	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F		0.844	0.844	1.390	1.699	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G		1.717	1.717	-0.678	-0.444	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H		1.773	1.773	-0.473	-0.317	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

CONC	1	2	3	4	5	6	7	8	9	10	11	12
A		1179.5	1294.1	148.5	103.4	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B		557.4	568.1	137.0	125.1	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C		242.3	230.0	93.1	85.5	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D		84.6	84.6	93.1	78.3	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E		57.6	57.6	115.4	62.8	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F		34.0	34.0	71.3	49.5	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G		12.1	12.1	410.5	311.2	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H		11.3	11.3	322.3	267.8	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

STANDARDS	Conc	Avg	B/Bo	Logit	Recalc	LTC4	pg/mL	%B/Bo	Dilution	StdDev	%CV	SEM
Std01	1000.00	0.043	0.100	-2.196	1234.59	16 - 1X	142.8	40.9	1	8.13	5.70	5.75
Std02	500.00	0.076	0.178	-1.531	562.70	16 - 1 in 2	93.1	64.1	2	0.00	0.00	0.00
Std03	250.00	0.132	0.311	-0.796	236.03	16 - 1 in 4	93.3	76.4	4	31.18	33.41	22.05
Std04	125.00	0.220	0.518	0.072	84.58	16 - spike	366.4	36.0	2	62.32	17.01	44.07
Std05	62.50	0.254	0.598	0.398	57.58	29 - 1X	114.2	45.6	1	15.37	13.45	10.87
Std06	31.25	0.297	0.699	0.844	33.96	29 - 1 in 2	81.9	66.5	2	5.06	6.18	3.58
Std07	15.63	0.360	0.848	1.717	12.11	29 - 1 in 4	56.2	83.1	4	9.43	16.79	6.67
Std08	7.81	0.363	0.855	1.773	11.34	29 - spike	289.5	40.6	2	30.65	10.59	21.68
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
												% Error
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!
						0.0000	#NUM!	-30.4	0	#NUM!	#NUM!	#NUM!

=Data outside 20-80% B/Bo range

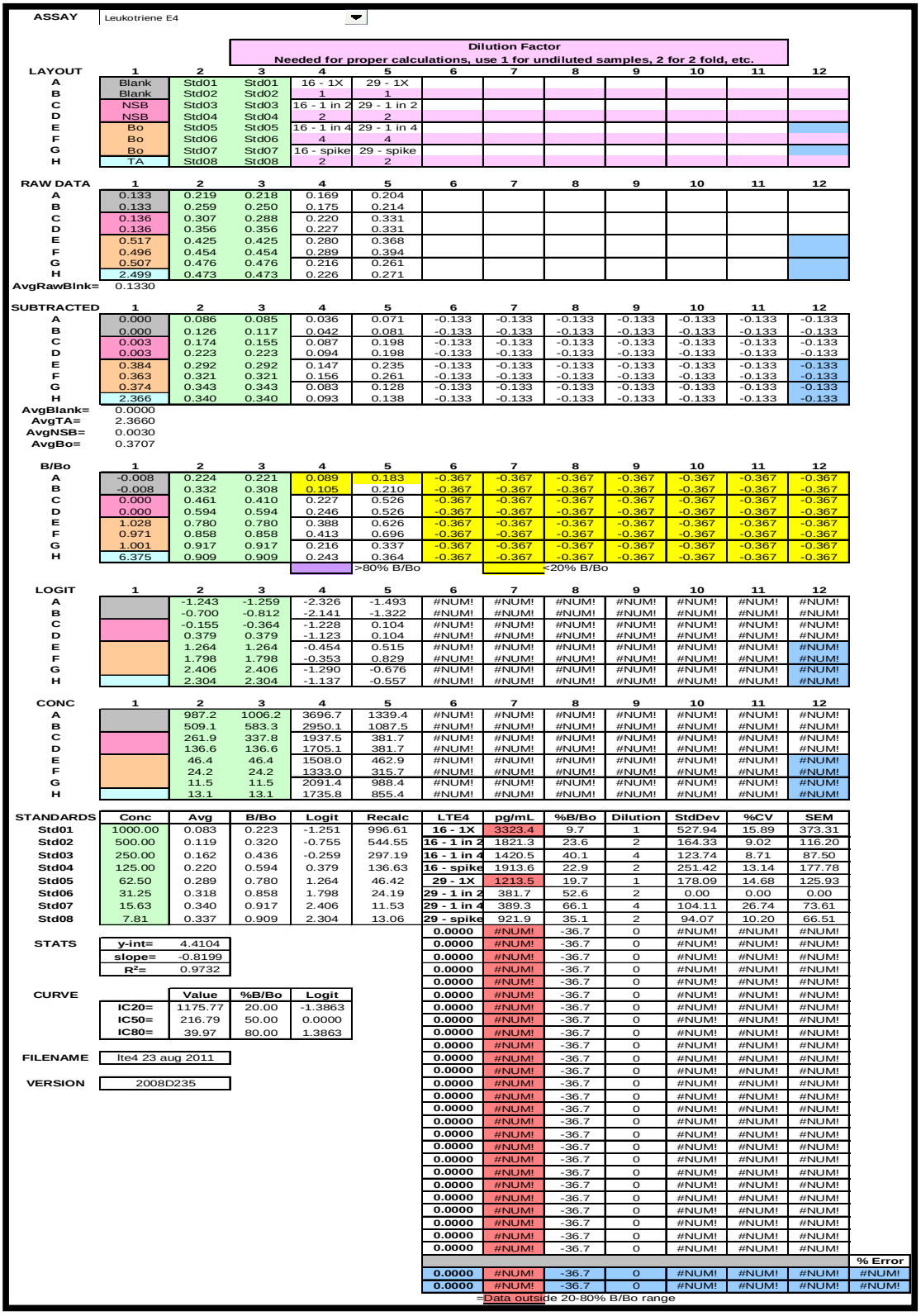
STATS	
y-int=	3.8276
slope=	-0.8462
R^2=	0.9791

CURVE	Value	%B/Bo	Logit
IC20=	474.02	20.00	-1.3863
IC50=	92.12	50.00	0.0000
IC80=	17.90	80.00	1.3863

FILENAME: ltc4 23 aug 2011

VERSION: 2008D235

Figure A9.8: Screen-capture of computer readout of EIA well analysis for secondary LT optimisation experiment using LTC4.



ASSAY: Leukotriene E4

Dilution Factor
Needed for proper calculations, use 1 for undiluted samples, 2 for 2 fold, etc.

LAYOUT	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std01	Std01	16 - 1X	29 - 1X							
B	Blank	Std02	Std02	1	1							
C	NSB	Std03	Std03	16 - 1 in 2	29 - 1 in 2							
D	NSB	Std04	Std04	2	2							
E	Bo	Std05	Std05	16 - 1 in 4	29 - 1 in 4							
F	Bo	Std06	Std06	4	4							
G	Bo	Std07	Std07	16 - spike	29 - spike							
H	TA	Std08	Std08	2	2							

RAW DATA	1	2	3	4	5	6	7	8	9	10	11	12
A	0.133	0.219	0.218	0.169	0.204							
B	0.133	0.259	0.250	0.175	0.214							
C	0.136	0.307	0.288	0.220	0.331							
D	0.136	0.356	0.356	0.227	0.331							
E	0.517	0.425	0.425	0.280	0.368							
F	0.496	0.454	0.454	0.289	0.394							
G	0.507	0.476	0.476	0.216	0.261							
H	2.499	0.473	0.473	0.226	0.271							

AvgRawBlnk= 0.1330

SUBTRACTED	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.086	0.085	0.036	0.071	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
B	0.000	0.126	0.117	0.042	0.081	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
C	0.003	0.174	0.155	0.087	0.198	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
D	0.003	0.223	0.223	0.094	0.198	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
E	0.384	0.292	0.292	0.147	0.235	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
F	0.363	0.321	0.321	0.156	0.261	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
G	0.374	0.343	0.343	0.083	0.128	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133
H	2.366	0.340	0.340	0.093	0.138	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133	-0.133

AvgBlank= 0.0000
AvgTA= 2.3660
AvgNSB= 0.0030
AvgBo= 0.3707

B/Bo	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.008	0.224	0.221	0.089	0.183	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
B	-0.008	0.332	0.308	0.105	0.210	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
C	0.000	0.461	0.410	0.227	0.526	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
D	0.000	0.594	0.594	0.246	0.526	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
E	1.028	0.780	0.780	0.388	0.626	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
F	0.971	0.858	0.858	0.413	0.696	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
G	1.001	0.917	0.917	0.216	0.337	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367
H	6.375	0.909	0.909	0.243	0.364	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367	-0.367

>80% B/Bo <20% B/Bo

LOGIT	1	2	3	4	5	6	7	8	9	10	11	12
A		-1.243	-1.259	-2.326	-1.493	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B		-0.700	-0.812	-2.141	-1.322	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C		-0.155	-0.364	-1.228	0.104	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D		0.379	0.379	-1.123	0.104	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E		1.264	1.264	-0.454	0.515	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F		1.798	1.798	-0.353	0.829	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G		2.406	2.406	-1.290	-0.676	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H		2.304	2.304	-1.137	-0.557	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

CONC	1	2	3	4	5	6	7	8	9	10	11	12
A		987.2	1006.2	3696.7	1339.4	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B		509.1	583.3	2950.1	1087.5	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C		261.9	337.8	1937.5	381.7	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D		136.6	136.6	1705.1	381.7	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E		46.4	46.4	1508.0	462.9	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F		24.2	24.2	1333.0	315.7	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G		11.5	11.5	2091.4	988.4	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H		13.1	13.1	1735.8	855.4	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

STANDARDS	Conc	Avg	B/Bo	Logit	Recalc
Std01	1000.00	0.083	0.223	-1.251	996.61
Std02	500.00	0.119	0.320	-0.755	544.55
Std03	250.00	0.162	0.436	-0.259	297.19
Std04	125.00	0.220	0.594	0.379	136.63
Std05	62.50	0.289	0.780	1.264	46.42
Std06	31.25	0.318	0.858	1.798	24.19
Std07	15.63	0.340	0.917	2.406	11.53
Std08	7.81	0.337	0.909	2.304	13.06

STATS	
y-int=	4.4104
slope=	-0.8199
R^2=	0.9732

CURVE	Value	%B/Bo	Logit
IC20=	1175.77	20.00	-1.3863
IC50=	216.79	50.00	0.0000
IC80=	39.97	80.00	1.3863

FILENAME: lte4 23 aug 2011

VERSION: 2008D235

LTE4	pg/mL	%B/Bo	Dilution	StdDev	%CV	SEM
16 - 1X	3323.4	9.7	1	527.94	15.89	373.31
16 - 1 in 2	1821.3	23.6	2	164.33	9.02	116.20
16 - 1 in 4	1420.5	40.1	4	123.74	8.71	87.50
16 - spike	1913.6	22.9	2	251.42	13.14	177.78
29 - 1X	1213.5	19.7	1	178.09	14.68	125.93
29 - 1 in 2	381.7	52.6	2	0.00	0.00	0.00
29 - 1 in 4	389.3	66.1	4	104.11	26.74	73.61
29 - spike	921.9	35.1	2	94.07	10.20	66.51
0.0000	#NUM!	-36.7	0	#NUM!	#NUM!	#NUM!

A9.4 Final Optimisation Experiments For: LTB4, LTC4, LTE4

A9.4.1 Aim:

- Compare standards processed through the C8 purification cartridges to standards that have not undergone purification through the cartridges. This was to calculate the recovery factor of the analyte measured.
- Compare serum samples that have been through the C8 purification cartridges to the same serum specimens that have not been purified through the cartridges. This was to calculate the recovery factor of the analyte being measured in the serum after being passed through the purification column.
- Determine the best dilution of serum for the test used.
- Kits used: - LTB4 EIA kit, # 520 111, Cayman chemicals

  - LTC4 EIA kit, # 520 211, Cayman chemicals

  - LTE4 EIA kit, # 520 411, Cayman chemicals

A.9.4.2 Methodology of sample preparation - Day 1

A detailed description of these steps has been discussed above.

A.9.4.2.1 Preparation of Standards:

- Standards were prepared as per manufacturer's instructions (paragraph 9.2)
- The standards (LTB4, LTC4& LTE4)were divided into two 250 µl aliquots of each analyte (i.e. LTB4, LTC4& LTE4).
- Both the aliquots were passed through the purification columns, but the second 250 µl aliquot was treated with 30 µl 1.0 molar HCL first as per manufactures recommendations
- All of the standards were eluted out of the purification column using methanol
- After dissipation the standards were reconstituted in a further 250 µl EIA buffer
- One 500 µl aliquot was left untreated
- The second 500 µl aliquot was treated as follows:

- LTC4 & LTE4: aliquot treated with 60 µl 1.0m HCL, purified via the C8 column and eluted in methanol, desiccated with N2 and reconstituted in 500 ml EIA buffer
- LTB4: aliquot treated with 60 µl 1.0m HCL, purified via the C8 column and eluted in ethanol, desiccated with N2 and reconstituted in 500 ml EIA buffer

A.9.4.2.2 Plate reconstitution (Figure A 9.10):

- Standard wells consisted of 50 µl standard with 50 µl tracer and 50 µl antiserum added
- Sample wells consisted of 50 µl standard with 50 µl tracer and 50 µl antiserum added
- BLK empty well
- NSB consisted of 100 ul EIA buffer with 50 µl LTC4 tracer added
- TA consisted of only 5 µl LTC4 tracer added
- Plate covered and incubated at room temperature for 18 hours

	1	2 LTE4 Not Purified	3 LTE4 Purified	4 LTE4 Methanol Sample	5 LTC4 Not Purified	6 LTC4 Purified	7 LTC4 Methanol Sample	8 LTC4 Not Purified	9 LTB Purified	10 LTB4 Methanol Sample	11 LTB4
A	BLK	S1	S1	1X	S1	S1	1X	S1	S1	2X	16XEthanol Sample
B LTE_4	E4 BO	S2	S2	2X	S2	S2	2X	S2	S2	4X	32XEthanol Sample
C LTE_4	E4 BO	S3	S3	4X	S3	S3	4X	S3	S3	8X	1X Serum sample
$DLTC_4$	C4 BO	S4	S4	8X	S4	S4	8X	S4	S4	16X	2X Serum sample
E LTC_4	C4 BO	S5	S5	1X	S5	S5	1X	S5	S5	32X	4X Serum sample
F LTB_4	B4 BO	S6	S6	2X	S6	S6	2X	S6	S6	2X	8X Serum Sample
G LTB_4	B4 BO	S7	S7	4X	S7	S7	4X	S7	S7	4X	16XSerum Sample
H	NSB	S8	S8	8X	S8	S8	8X	S8	S8	8X	32X Serum sample

Figure A 9.10: Plate schematic used for analysis of the final optimisation study using all 3 leukotrienes i.e. LTB4, LTC4 and LTE4
LT: Leukotriene, NSB: Non-specific binding, B0: Maximum binding between tracer and antibody without any analyte, BLK: Blank, TA: Total activity.

A9.4.3 Day 2

- Reconstituted Ellman's reagent
- Emptied the wells and wash 6 times with a plate washed

- Added 200 µl of Ellman's reagent to each well
- Covered plate with a foil film and allowed reaction to develop while incubated for 60-90 mins the dark on a shaker
- Bottom of plate wiped
- Measured at A405 wave-length (BO was between 0.3 and 1.0 after subtracting the blank)

A9.4.3.1 Discussion of Optimisation Results:

The above experiments allowed for the development of a protocol for the final testing of serum leukotriene levels. These optimisations experiments allowed us to determine the necessity for spiking the specimens and or purification of the specimens, as well as determining the optimal dilution factors for the samples to be analysed.

The results of these studies informed us that spiking of the serum was unnecessary. Based on the results obtained which are tabulated and documented above the following recommendations for each of the leukotrienes measured were proposed:

A9.4.3.2 LTB4

It was difficult to measure the concentration of the leukotriene lost due to the purification steps as poor results were obtained from the purified specimens, as well as the spiked specimens that were passed through the purification cartridges. This implied that the purification process was leaching concentration of the analyte measured.

As the recovery concentration of the analyte measured was less than expected, the standard curve, which is a computerised program that mathematically converts the spectrophotometrically obtained value for LTB4 into the true serum concentration, had to be modified. This was because results obtained were out of range, and were a poor fit for the appropriate extrapolation of LTB4 values.

A rough estimate of the analyte lost due to the purification process was approximately 80 – 85% at higher concentrations (100-1000 pg/ml) and about 60-70% at the lower concentrations (3.9 - 62.5 pg/ml). When comparing the results from the purified and non-purified, as well as the spiked and un-spiked samples, an eight times dilution factor without spiking was determined as the most appropriate for this assay.

Thus, the final recommendation for the preparation for measurement of LTB4 was that non-purified serum samples with a factor of eight dilution should be used, and that Std 7 (15.6 pg/ml) and Std 8 (7.8 pg/ml) should be omitted from the standard curve for interpretation of results.

Final recommendation for the preparation of LTB4:

1) use non purified serum samples at a 8 X dilution
2) spiking not necessary
3) omit S 8 (7.8 pg/ml) & S7 (15.6 pg/ml) from standard curve for interpretation of results

A9.4.3.3 LTC4

The standard curve for LTC4 had very few results out of range and the standard curve was a good fit for the extrapolation of serum LTC4 concentration. There was no need for a mathematical modification of the computer algorithm. Loss due to purification was about 80% at higher concentrations (100-1000 pg/ml) and about 60-70% at lower concentrations (30-100 pg/ml). When comparing the purified and non-purified, as well as the spiked and un-spiked samples, a two times dilution factor without spiking was determined as the most appropriate for this assay.

Thus, the final recommendation for the preparation for measurement of LTC4 was that non-purified serum samples with a factor of two dilution should be used, while spiking was not necessary and that Std 7 (15.6 pg/ml) and Std 8 (7.8 pg/ml) should be omitted from the standard curve for interpretation of the results.

The standard curve for LTC4 had some results out of range, but standard curve was a good fit for extrapolation of further values. S7 (15.6 pg/ml) and S8 (7.8 pg/ml) may be omitted from the STD curves

Final recommendation for the preparation of LTC4:

1) use non purified serum samples at a 2X dilution
2) spiking not necessary
3) omit S8 (7.8 pg/ml) & S7 (15.6 pg/ml) from standard curve for interpretation of results

A9.4.3.4 LTE4

The standard curve for LTE4 had very few results out of range and the standard curve was a good fit for the extrapolation of serum LTE4 concentration. There was no need for a mathematical modification of the computer algorithm. Loss due to purification was about 80% at higher concentrations (100 - 1000 pg/ml) and 50 - 60% at lower concentrations (7.8 – 100 pg/ml). When comparing the purified and non-purified, as well as the spiked and un-spiked samples, a four times dilution factor without spiking was determined as the most appropriate for this assay.

Thus, the final recommendation for the preparation for measurement of LTE4 was that non-purified serum samples with a factor of four dilution should be used, while spiking was not necessary, and that Std 7 (15.6 pg/ml) and Std 8 (7.8 pg/ml) should be omitted from the standard curve for interpretation of results.

The standard curve for LTE4 had some results out of range, but standard curve was a good fit for extrapolation of further values. S7 (15.6 pg/ml) and S8 (7.8 pg/ml) may be omitted from the STD curves.

Final recommendation for the preparation of LTE4:

1) use non purified serum samples at a 4X dilution
2) spiking not necessary
3) omit S 8 (7.8 pg/ml) & S7 (15.6 pg/ml) from standard curve for interpretation of results

In summary, these optimisation experiments allowed us to develop the protocol and methodology necessary to carry out the tests on measuring serum leukotriene levels in asthmatic patients. Enzyme-linked immunosorbent assays (ELISA) were used to determine the serum concentrations of leukotriene B4, C4 and E4 (Cayman Chemicals, #520111; 520211; 520411 respectively). Sera were diluted 1 in 8, 1 in 2 and 1 in 4 for LTB4, LTC4 and LTE4 respectively and assayed according to the manufacturer's recommendations (Appendix 9 - optimisation of LTB4, LTC4, LTE4). Optical density of the analytes was measured at 405 nm on a microplate reader (Elx800, BioTek, USA).

APPENDIX 10: METHODS APPLIED FOR THE USE OF MULTIPLEX ASSAYS TO MEASURE BIOMARKERS OF REMODELLING TGFβ SUBSETS 1, 2, & 3

A10.1 Methodology

The theory explaining the concept of multiplex assay has been described in Appendix 6. The preparation of bioplex plates is discussed in detail in Appendix 6. Only steps unique to the plating of the TGFβ 1, 2, 3 multiplex assay will be elaborated on in Appendix 10.

A10.2 Methods

The Millipore Kit: TGFβ 1, 2, 3 multiplex kits, TGFβ- 64k-03 was used according to manufacturer's recommendations.

A10.2.1 Day 1

A10.2.1.1 Preparation of immobilised beads

1. The samples were stored in polypropylene tubes, not in glass as per the manufacturer's recommendations.
2. The beads were placed in a 15 ml polypropylene tube.
3. Each bead vial was sonicated for 30 sec.
4. Thereafter, each vial vortexed for 1 min.

A10.2.1.2 Preparation of Quality Controls (CQ) (provided by manufacturers of multiplex kit used)

1. The samples were stored in polypropylene tubes, not in glass.
2. Reconstituted the QC1 and QC2 with 250 µl dH_2O.
3. Inverted the vial several times to mix the solutions.
4. Then vortexed the vial for 1 min.
5. Left to settle for 10 min.
6. Final QC solutions were transferred via an Eppendorf to the plate.
7. Unused portion was stored at -20 ℃.

The following quality control assays provided were used:

TGFβ1:- control 1: 148 – 308 pg/ml

- control 2: 417 – 867 pg/ml

TGFβ2:- control 1: 162 – 336 pg/ml

- control 2: 495 – 1028 pg/ml

A10.2.1.3 Preparation of wash buffer

1. 30 ml of the 10x concentration wash buffer concentrate provided by the manufacturer was reconstituted with 270 ml of dH_2O to make 300 ml of 1x concentration wash buffer.

A10.2.1.4 Preparation of serum matrix for standards and controls

1. This process was necessary to remove debris and excess lipids from serum samples only.
2. 1 ml dH_2O and 4.0 ml assay buffer were added to the vial containing the lyophilised serum matrix.
3. Solution mixed and left to settle at RT for 10 min and then mixed again.
4. In a separate tube, 0.5 ml of the reconstituted serum matrix was added to 0.5 ml assay buffer to make a [1:10] concentration of the standards solution.
5. The tube was agitated for 10 minutes.

A10.2.1.5 Treatment of serum samples

1. This process was necessary to remove debris and excess lipids from serum samples only.
2. The serum samples were diluted by mixing 20 µl serum with 80 µl sample diluents (provided in kit) to make a [1:4] concentration of the serum solution.
3. Thereafter, 2 µl of HCL was then added to 25 µl of each of the diluted samples and agitated for 1 hour at room temperature (RT).
4. Then the acid treated samples were further diluted to a [1:6] concentration by adding 100 µl of the assay buffer to 20 µl serum-acid sample prepared above.
5. This process yielded a final dilution of [1:36].

A10.2.1.6 Preparation of TGFβ1, β2, β3 Standard (provided by manufacturer)

1. Standards were reconstituted with 250 µl assay buffer to make a [1:10 000 pg/ml] solution.

2. The vial containing the solution was inverted several times to mix it.
3. Thereafter the vial was vortexed for 10 sec.
4. The vial was allowed to settle for 10 mins.
5. The solution was then transferred via Eppendorf to standard well 6 on the analysing plate (Figure A 10.1).
6. Standards solution was further diluted 4 times.
7. A clean Eppendorf tip was used before transferring each of standards.
8. The standards solutions were vortexed before transferring to the plates.
9. Unused portions were stored at ≤ -20 degrees for 1 month.

A10.2.1.7 Preparation of further dilutions of standards solution

A schematic diagram of the preparation of the dilutions of the standards solution used is summarised in Figure A 10.1.

Standard	Conc. pg/ml	Vol. assay buffer	Vol. standard
Std 6	10 000	250 µl	0
Std 5	2 500	150 µl	50µl of Std 10 000
Std 4	625	150µl	50µl of Std 2 500
Std 3	156	150µl	50µl of Std 625
Std 2	39	150µl	50µl of Std 156
Std 1	9.8	150µl	50µl of Std 39
Blank	0	150µl	

Figure A 10.1: Schematic used for the preparation of dilutions of standards
Std: Standard, µl: Microliter, pg/ml: Picogram per millilitre.

A10.2.1.8 Preparation of the TGFβ1, β2, β3 multiplex plate according to manufacturer's instructions (Millipore)

1. Reagents were allowed to warm to RT.
2. A filter plate was placed on the plate holder at all times during reagent dispensing and incubation so that the bottom of the plate did not touch any surface.
3. All excess buffer solution was removed from the bottom of the plate with blotting paper.
4. The plate was washed with 200 µl wash buffer which was pipetted into each well and agitated for 10 min. at RT.
5. Standards and specimens pipetted to wells as follows:
   Added 25 µl standard or control to wells A1 to H2
   Added 25 µl serum matrix to wells A1 to H2

Added 25 µl of sample to wells A3 to H40

Added 25 µl of assay buffer to wells A3 to H40

Added 25 µl of vortex beads to each well

6. The plate was then sealed and incubated overnight on an agitator at 4℃. (Figure A 10.2)

A10.2.1.8.1 TGFβ1, β2, β3 multiplex plate schematic

The multiplex plate was prepared as described above and the specimens to be analysed were pipetted into each wells on the plate as the per the diagram schematic in Figure A 10.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	Std 625	1	5	9	13	17	21	25	29	33	37
B	B1	Std 625	1	5	9	13	17	21	25	29	33	37
C	Std 9.8	Std 2500	2	6	10	14	18	22	26	30	34	38
D	Std 9.8	Std 2500	2	6	10	14	18	22	26	30	34	38
E	Std 39	Std 10 000	3	7	11	15	19	23	27	31	35	39
F	Std 39	Std 10 000	3	7	11	15	19	23	27	31	35	39
G	Std 156	QC1	4	8	12	16	20	24	28	32	36	40
H	Std 156	QC2	4	8	12	16	20	24	28	32	36	40

Figure A 10.2: Plate schematic used for analysis of standards as well as for the biomarkers **TGFβ1, β2, β3**
B = Blank, Std = Standard, QC= Quality control

A10.2.2 Day 2

A10.2.2.1 Multiplex plate preparation prior to analysis

1. All excess fluid was removed by vacuuming the plate.
2. Thereafter, the plate was washed twice with 200 µl wash buffer per well.
3. The excess buffer was removed by vacuuming the plate between washes and blotting the excess liquid from bottom of plate.
4. Then 25 µl of detection antibody solution was added to each well and the plate was sealed with foil.
5. The plate was then incubated at RT for 30 mins with shaking but no vacuuming.
6. Then 25 µl of streptavidin/phycoerythrin was added to each well and the plate re-sealed.
7. The plate was re-incubated for 30 mins at RT with shaking.
8. All the excess fluid was removed by vacuuming the plate.
9. The plate was washed twice with 200 ul wash buffer per well.

10. Removed buffer by vacuuming between washes and blotting of the excess liquid from bottom of plate.
11. Then added 100 µl sheath fluid to all wells.
12. The plate was sealed and shaken for 5 mins.
13. The plate was placed into the luminex analyser for analysis.
14. Above process was repeated for specimens 41-80.

A10.2.2.2 Equipment Settings

The specifications of the Luminex reader used to analyse the multiplex plates was 100 IS version (v). 2.1/2.2 using the Luminex High Throughput Screening (HTS) system. The settings, as per manufacturer's instructions, used for the Luminex reader are summarised in in Table A 10.1.

Table A 10.1: Equipment specifications used for Luminex reader for the biomarkers TGFβ1, β2, β3

Events:	50 per bead	
Sample size:	50 ul	
Gate settings:	8,000 to 15, 000	
Reporter gain:	default	
Time out:	60 seconds	
Bead set:	Premixed TGFβ 1,2,3	Bead Region
	TGFβ1	19
	TGFβ2	41
	TGFβ3	79

TGFβ: Transforming Growth Factor Beta,

A10.2.2.3 Results

After the plate was processed and analysed on the luminex analyser the results were obtained by an automated program and stored for statistically analysis. The results are presented in the chapter detailing the results.

APPENDIX 11: CUMULATIVE OF ALL VARIABLES MEASURED

Accumulative data tables of all variables measured and analysed.

The legend for all the tables been included in a text box here as there are no space for it on pages below.

Table A11.1: ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, SBP: systolic blood pressure, DBP: diabolic blood pressure, Abd Cirm: abdominal circumference, BMI: body mass index, ACQ: asthma control questionnaire, AST: Physicians Asthma Rating, sd: standard dose, ACT tot: Asthma control test as actual score, ACT cat: Asthma control test as a categorical variable less than or greater than 19, hd: high dose, LABA: long acting Beta 2 agonist, SABA: short acting β2 agonist, SAMA: short acting anti-muscarinic antagonist, PPI: proton pump inhibitor, H2 R blocker: Histamine 2 receptor blocker

Table A11.2: ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, Pre: pre bronchodilator, FVC: forced vital capacity, FEV_1: forced expiratory volume in one second, FEV_1/FVC: percentage of the FVC expired in one second, PEFR: peak expiratory flow, FEF: forced expiratory flow related to a specific portion of the FVC, FEF25-75: forced expiratory flow during the middle half of the FVC, FVCin: Forced inspiratory vital capacity, FIF 50: Forced inspiratory flow at 50% or mid inspiration, Post: post bronchodilator, %Δ: % change post bronchodilator

Table A11.3: ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, Pre: pre bronchodilator, vt actual: R5: total airway resistance, R20: central airway resistance, R5-R20: resistance of the small airways, R35: airway resistance at 35Hz, zbei5: respiratory impedance at 5 Hz, Hz: Hertz, X5: peripheral capacitive reactance, X20: reactance at 20Hz, X35: reactance at 35Hz, AX: reactance area, res freq: resonant frequency, Post: post bronchodilator, %: percentage of predicted, %Δ: % change post bronchodilator

Table A11.4: ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, IL: interleukin, IFNγ: interferon gamma, TNFα: tumour necrosis factor alpha, FU: fluorescent intensity units, pg/ml: picograms per millilitre

Table A11.5: ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, FENO 1: fractional exhaled nitric oxide initial test, FENO 2: fractional exhaled nitric oxide 2nd test, FENO avg: fractional exhaled nitric oxide average, FENO cat: fractional exhaled nitric oxide as categorical value less then or greater then 25, RANTES: Regulated on activation normal T expressed and secreted, LT: Leukotriene, IgE: Immunoglobulin E, FU: fluorescent intensity units, kIU/L: kilo international units per litre, pg/ml: picograms per millilitre

Table A11.6: ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, VEGF: vascular endothelial growth factor, CRP: C-reactive protein, SAAP: serum amyloid associated protein, TGFβ: transforming growth factor beta, FU: fluorescent intensity units, pg/ml: picograms per millilitre

Table A 11.1: Cumulative data of asthma control values and categories, patient demographics and medication used

ID	Group 1	Group 2	SBP	DBP	Abd Cirm	Gender	Age	Race	Height	Weight	BMI	ACQ score	AST Rating	ACT tot	ACT cat	Budeflam sd	Budeflam hd	LABA Foratec	Prednisone hd	Prednisone ld	Singulair	SABA	Nasal steroid	SAMA	PPI	Serevent	Theophyllin	Antihistamine	H2 R blocker	Seretide
1	0	0	123	75	90	1	51	0	175	86	28	0.57	0	18	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0
3	0	0	130	80	93	0	69	1	191	82	22	0.14	0	18	0	1	0	0	1	1	0	1	1	0	0	1	0	1	0	0
5	0	0	180	66	90	0	85	0	169	80	28	0.57	0	20	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
8	0	0	107	75	77	1	27	0	152	52	22	0.43	0	22	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
10	0	0	113	73	74	0	42	0	178	63	19	0.57	0	23	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
11	0	0	121	76	90	0	56	0	174	79	26	0.57	0	23	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
12	0	0	110	70	82	0	21	0	174	73	24	0.71	2	9	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0
15	0	0	130	85	90	1	47	0	160	72	28	0.71	1	15	1	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0
18	0	0	136	87	67	1	55	0	153	50	21	0	0	19	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
19	0	0	114	86	83	1	43	0	157	69	28	0.43	0	22	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
20	0	0	130	89	81	0	24	0	165	66	24	0	0	23	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
21	0	0	119	72	78	1	21	0	164	67	25	0.57	1	18	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
22	0	0	121	65	74	1	21	0	162	57	21	0.14	0	21	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0
23	0	0	114	65	88	1	39	0	166	79	28	0.43	1	18	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1	0
24	0	0	112	69	70	1	16	0	157	51	20	0.57	1	18	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
25	0	0	133	82	88	1	48	0	160	72	28	0.71	1	19	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0
41	0	0	150	89	100	0	45	0	177	104	33	0.57	0	20	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0
42	0	0	116	73	69	0	34	0	172	65	22	0.14	0	22	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
43	0	0	106	62	70	1	37	0	152	48	20	0.57	0	22	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0
44	0	0	148	85	80	1	67	0	152	59	25	0.29	0	20	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0
45	0	0	138	76	89	0	37	0	168	76	27	0	0	23	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
46	0	0	130	85	92	0	55	0	174	66	21	0.57	1	17	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0
48	0	0	117	72	87	1	59	0	162	67	25	0.43	1	16	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
49	0	0	104	62	64	0	20	1	150	53	23	0.29	0	19	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
50	0	0	139	85	92	0	38	0	167	84	30	0.57	1	21	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0
54	0	0	96	64	73	0	42	0	178	63	19	0.57	0	23	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
59	0	0	135	80	94	0	69	0	169	66	23	0.29	0	21	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0
61	0	0	122	73	75	0	19	0	164	56	20	0.43	1	22	0	1	0	1	0	0	1	1	1	0	0	0	0	1	0	0
63	0	0	120	67	88	0	55	0	174	66	21	0.43	0	23	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
64	0	0	138	80	75	1	16	0	161	61	23	0.71	1	14	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0
2	0	1	138	72	77	1	28	0	164	74	27	1	1	15	1	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0
4	0	1	110	75	92	0	38	0	163	71	26	1	1	17	1	0	1	1	0	0	0	1	1	0	1	0	0	1	0	0
6	0	1	104	69	78	1	65	0	158	72	28	1	1	11	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
7	0	1	160	95	85	0	64	0	158	63	25	0.86	1	18	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0
9	0	1	121	56	75	0	19	0	164	56	20	1.43	1	21	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0
13	0	1	140	80	89	1	35	0	167	62	22	1.43	2	11	1	1	0	1	0	0	1	1	1	0	1	0	0	1	0	0
14	0	1	110	70	70	0	64	1	164	51	19	0.86	1	13	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	0
16	0	1	136	88	87	0	77	1	157	58	23	1.14	1	13	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
17	0	1	109	79	70	1	24	0	163	57	21	1.43	1	16	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
26	0	1	115	54	73	0	42	0	178	63	19	1.14	1	21	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
47	0	1	135	68	90	1	52	0	168	87	30	1.43	1	13	1	1	0	0	0	0	0	1	1	0	1	1	1	0	0	0
51	0	1	106	69	100	0	70	0	174	88	29	1.29	1	19	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
52	0	1	110	75	90	1	42	0	163	75	28	1	1	11	1	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0
53	0	1	140	98	96	0	39	0	163	73	27	1.29	1	18	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0	0
55	0	1	110	70	71	0	42	0	178	63	19	1.29	2	13	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
56	0	1	135	99	83	1	59	0	158	68	27	1.14	0	16	1	1	0	1	0	0	0	1	1	0	1	0	1	0	0	0
57	0	1	146	95	83	1	59	0	158	66	26	1	1	18	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1	0
58	0	1	138	86	83	1	59	0	158	62	25	0.86	1	15	1	1	0	1	0	0	1	1	1	0	0	0	0	1	1	0
62	0	1	138	81	83	1	31	0	168	73	25	0.86	1	13	1	0	1	1	0	0	0	1	1	0	0	0	1	0	0	0
65	0	1	105	79	72	1	30	0	148	49	22	1.43	1	13	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	0
66	0	1	95	60	76	1	33	0	164	53	19	0.86	2	16	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
75	0	1	145	95	83	1	29	0	161	74	28	1.43	2	13	1	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0
27	1	2	139	72	72	1	81	0	149	43	19	2.29	1	15	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0
28	1	2	146	84	87	0	69	0	169	66	23	2.43	2	13	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
29	1	2	98	69	75	1	25	0	163	71	26	3.29	2	13	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0
30	1	2	121	76	67	0	26	0	163	48	18	2.71	1	21	0	0	1	0	0	1	1	1	1	0	1	1	1	1	0	0
31	1	2	105	79	74	1	30	0	148	45	20	4.14	2	14	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
32	1	2	118	84	82	1	31	0	168	73	25	3.29	2	10	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0
33	1	2	110	63	83	1	21	0	155	64	26	3.29	2	11	1	0	1	1	0	0	1	1	1	0	1	0	0	1	0	0
34	1	2	140	90	84	1	59	0	158	62	24	2.14	2	12	1	1	0	1	0	0	0	1	1	0	0	0	0	0	1	0
35	1	2	133	70	90	1	44	0	152	60	25	2.43	2	14	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0
36	1	2	162	83	88	1	64	0	150	62	27	5.14	2	5	1	0	1	0	0	1	1	1	0	0	1	1	0	0	0	0
37	1	2	142	99	83	1	29	0	161	74	28	2.14	2	17	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
38	1	2	100	60	72	1	21	0	165	56	20	1.86	2	9	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	0
60	1	2	115	70	89	0	69	0	169	61	21	3.29	1	15	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
67	1	2	155	90	90	1	42	0	160	71.5	27	3.29	2	14	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0
68	1	2	105	70	73	1	33	0	164	60	22	2	2	18	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
69	1	2	94	60	70	0	19	0	180	56	17	2.29	2	12	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0
70	1	2	111	82	77	1	43	0	166	66	24	2.14	2	9	1	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0
71	1	2	120	74	89	1	51	0	150	64	28	1.86	1	21	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0
72	1	2	130	80	88	1	47	0	160	74	28	1.57	2	13	1	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0
73	1	2	128	87	94	0	38	0	163	73	27	1.71	1	22	0	1	0	1	0	0	0	1	1	0	1	0	0	1	0	0
74	1	2	153	96	83	1	59	0	158	69	27	1.57	1	18	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0
76	1	2	110	63	83	1	21	0	155	64	26	3.43	2	13	1	0	1	1	0	1	1	1	1	0	1	0	0	1	0	0
77	1	2	132	71	90	0	48	0	166	75	27	4.29	2	7	1	0	1	1	0	1	1	1	1	0	0	0	0	0	0	0
78	1	2	138	75	81	1	16	0	161	61	23	2.43	2	12	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0

Table A 11.2: Cumulative data of asthma control categories and spirometry parameters used

ID	Group 1	Group 2	Pre FVC	Pre FEV_1	Pre FEV_1/FVC	Pre PEFR	Pre FEF25	Pre FEF50	Pre FEF75	Pre FEF25-75	Pre FVCin	Pre FIF50	Post FVC	FVC %Δ	Post FEV_1	FEV_1 %Δ	Post FEV_1/FVC	Post PEFR	PEFR %Δ	Post FEF25	FEF25 %Δ	Post FEF50	FEF50 %Δ	Post FEF75	FEF75 %Δ	Post FEF25-75	FEF25-75 %Δ	Post FVCin	FVCin %Δ	Post FIF50	FIF 50 %Δ
1	0	0	96	76	68.2	113	61	41	22	31	98	5.27	91	-5	77	2	73.3	126	11	80	32	51	24	33	50	43	42	93	-5	5.03	-5
3	0	0	104	97	71.9	100	94	72	43	72	103	4.14	105	1	104	7	76.2	100	0	110	18	90	24	60	41	90	25	103	0	4.29	4
5	0	0	86	79	66.4	91	60	38	28	36	69	1.49	80	-7	80	2	72.91	94	4	69	14	51	34	45	60	54	49	69	1	2.22	49
8	0	0	111	86	67.5	96	67	43	27	34	112	3.25	106	-4	87	1	71.41	109	14	69	4	46	6	27	1	36	5	110	-2	4.72	45
10	0	0	98	62	51.9	76	32	20	13	18	96	7.83	100	3	72	16	58.67	91	19	43	36	29	47	19	42	26	47	97	1	7.97	2
11	0	0	122	88	57.6	93	58	35	20	30	112	3.2	125	2	91	3	58.19	97	4	61	6	38	9	20	1	32	5	116	4	2.85	-11
12	0	0	107	98	78.8	112	106	82	58	72	107	7.48	106	0	107	9	86.4	116	4	115	8	107	30	85	46	96	33	110	3	8.37	12
15	0	0	94	60	54.4	93	29	16	8	13	96	6.1	122	29	70	17	49.4	103	12	30	3	19	20	10	21	15	11	110	15	6.66	9
18	0	0	178	107	50.7	119	42	25	24	27	180	6.55	188	6	123	15	55.18	121	2	56	33	41	66	33	40	36	33	187	3	6.69	2
19	0	0	121	87	61.5	93	50	37	25	30	107	3.52	123	1	97	12	68.24	95	3	66	33	51	41	32	26	42	43	114	6	3.66	4
20	0	0	103	99	82.9	114	91	90	82	78	99	8.17	105	2	99	0	81.48	122	8	93	3	78	-12	71	-14	75	-4	100	0	7.77	-5
21	0	0	111	83	65.6	100	57	46	30	39	112	6.49	112	1	102	23	79.62	122	21	93	63	75	63	62	108	70	80	112	0	7.21	11
22	0	0	95	90	83.3	116	110	77	59	71	94	5.12	97	3	95	6	86	119	2	118	8	90	17	65	12	80	13	95	1	5.09	-1
23	0	0	105	83	69	112	66	48	34	43	102	5.49	115	9	99	18	74.43	119	6	88	33	70	45	42	23	58	37	114	12	6.03	10
24	0	0	98	90	87.2	106	100	80	78	77	92	4.34	101	2	100	12	95.13	124	16	148	48	119	48	117	51	113	46	94	3	5.32	23
25	0	0	115	65	48.7	89	26	16	8	11	111	6.19	117	2	66	1	48.13	89	-1	32	23	15	-6	8	0	12	6	122	10	5.79	-6
41	0	0	83	65	63.9	86	43	35	19	28	85	7.55	96	15	71	9	60.42	86	1	46	7	33	-5	17	-7	26	-7	92	9	7.02	-7
42	0	0	101	90	74.5	130	89	62	47	57	98	7	107	5	94	4	73.67	135	4	97	10	62	-1	48	2	56	-1	99	1	8.45	21
43	0	0	94	88	81.1	110	111	76	40	61	89	3.57	88	-6	90	2	87.67	118	7	122	10	110	44	66	65	88	45	82	-8	2.55	-29
44	0	0	114	92	67	116	74	36	21	28	107	3.77	120	6	104	13	71.34	133	15	96	31	49	34	26	24	36	31	122	14	3.73	-1
45	0	0	116	100	72.9	113	81	66	38	53	116	8.37	119	3	109	8	77.02	124	10	99	22	82	24	61	60	75	42	112	-3	7.58	-9
46	0	0	113	77	54	88	44	28	12	19	101	4.2	107	-6	84	10	63.03	99	12	62	41	42	49	22	79	35	80	103	2	3.49	-17
48	0	0	137	117	72.3	157	133	70	37	55	138	4.34	138	1	116	-1	71.18	165	5	126	-5	66	-5	31	-16	51	-7	133	-4	4.52	4
49	0	0	85	83	88	68	67	67	73	58	77	3.44	92	8	91	9	89.08	81	19	80	19	77	15	100	38	71	22	85	12	4.8	39
50	0	0	109	78	59.8	93	60	34	16	24	103	6.14	106	-3	92	18	72.67	107	15	95	58	62	82	37	131	52	109	103	-1	6.72	9
54	0	0	112	71	51.9	98	35	26	16	23	106	8.61	113	1	74	5	54.08	95	-2	40	14	30	14	19	17	26	11	104	-2	8.47	-2
59	0	0	143	81	43.6	101	26	13	16	10	147	7.71	157	10	91	13	45	106	5	38	47	20	51	17	0	20	91	150	3	7.34	-5
61	0	0	103	88	74.3	107	73	56	45	49	104	8.85	106	3	97	10	79.76	106	-1	109	50	77	37	62	39	69	41	102	-2	9.14	3
63	0	0	106	76	57.5	91	46	29	13	21	106	4.05	109	3	81	6	59.39	94	4	53	15	35	20	16	27	27	28	108	3	4.01	-1
64	0	0	91	83	86.3	115	138	98	74	89	79	5.88	95	4	90	9	90.56	123	7	151	9	123	26	99	34	117	31	83	5	6.9	17
2	0	1	108	88	70.9	103	73	48	29	41	109	5.53	112	3	80	-9	62.54	94	-8	0.63	-14	43	-11	25	-13	34	-18	112	3	5.3	-4
4	0	1	151	99	55.4	99	54	40	29	35	147	6.6	145	-4	64	-35	37.36	67	-32	25	-54	17	-57	13	-55	14	-61	136	-8	5.58	-15
6	0	1	124	92	61.6	108	51	34	17	27	125	2.14	129	5	110	20	70.94	129	20	80	55	54	59	28	65	42	56	133	7	3.65	70
7	0	1	137	106	61.7	125	88	40	28	32	134	6.3	136	0	112	5	65.06	127	2	108	23	48	20	38	36	41	27	136	1	5.43	-14
9	0	1	104	94	78.1	122	85	65	60	60	100	7.01	105	0	92	-2	76.11	121	0	102	19	60	-7	46	-23	52	-13	1.01	1	8.77	25
13	0	1	128	101	68.4	106	78	54	33	48	131	6.05	132	3	108	7	71.07	113	7	93	19	66	23	44	32	59	24	132	1	5.98	-1
14	0	1	143	127	70.1	104	77	74	80	76	142	5.49	142	-1	120	-6	66.49	108	4	93	20	52	-30	69	-13	61	-21	139	-3	4.57	-17
16	0	1	111	83	56.7	73	36	29	37	26	105	3.98	117	5	95	14	61.53	88	21	48	32	37	26	21	-42	27	4	119	14	5.52	39
17	0	1	109	99	79.6	117	120	78	51	68	108	3.59	109	1	108	9	86.6	138	17	150	26	113	45	61	19	86	26	109	1	3.78	5
26	0	1	105	60	46.8	80	32	19	12	17	103	5.41	103	-2	71	17	56.1	88	9	44	36	30	61	22	80	27	56	97	-5	7.24	34
47	0	1	120	83	59	114	53	32	13	24	110	5.64	126	5	89	8	60.13	124	9	66	25	37	15	14	7	25	7	119	8	6.35	13
51	0	1	70	54	59.4	67	28	24	12	20	72	2.46	74	6	63	16	65.16	75	13	42	50	28	17	10	-20	17	-15	76	5	3.73	51
52	0	1	100	38	32.5	49	13	8	13	7	104	3.94	115	15	54	43	40.33	65	33	24	88	14	89	13	0	14	114	113	9	5.41	37
53	0	1	122	56	38.9	65	23	13	8	10	125	5.26	134	10	69	22	43.3	80	23	31	37	21	69	14	82	19	82	127	1	5.5	5
55	0	1	100	71	58	89	49	29	19	26	103	8.03	102	1	78	10	62.93	94	6	57	15	37	29	23	23	33	27	103	-1	8.06	0
56	0	1	116	88	63.8	97	58	37	13	25	111	3.59	118	2	88	0	62.78	95	-1	57	-2	35	-5	12	-3	22	-11	118	6	4.56	27
57	0	1	100	65	54.9	80	37	19	19	20	97	4.15	108	8	79	21	61.75	89	10	48	31	31	63	19	4	26	34	99	2	4.26	3
58	0	1	105	74	59.8	89	43	28	15	20	102	3.76	101	-3	82	10	68.13	98	10	48	12	40	43	19	32	28	41	106	4	3.35	-11
62	0	1	98	73	64.9	103	54	37	17	27	100	3.93	105	7	88	20	73.36	107	5	79	47	52	42	42	156	52	91	103	3	4.43	13
65	0	1	86	39	39.6	36	15	12	10	10	82	2.46	82	-4	41	5	43.2	37	4	17	13	12	5	12	16	12	18	84	1	2.51	2
66	0	1	131	101	67.3	124	93	55	31	45	124	4.26	131	0	124	22	81.96	148	19	156	68	116	112	73	135	100	124	126	2	5.82	37
75	0	1	111	90	70.8	104	63	53	40	47	98	6.23	109	-2	100	12	80.37	126	21	89	41	71	35	55	36	65	39	102	3	4.36	-30
27	1	2	99	79	62.8	64	35	18	18	20	89	2.44	100	1	75	-4	59.49	68	7	30	-14	17	-6	18	18	15	-23	89	0	2.65	8
28	1	2	117	53	35	69	13	10	10	10	123	6.65	140	20	68	29	37.56	79	14	21	55	12	21	10	0	12	23	146	18	7.76	17
29	1	2	110	41	32.8	45	17	9	8	9	105	3.33	120	9	54	30	39.17	58	30	24	45	12	32	9	13	12	32	124	18	3.71	11
30	1	2	94	66	60.6	90	46	29	20	24	95	7.03	101	8	69	4	58.88	90	-1	43	-5	30	3	20	0	24	-1	96	1	6.83	-3
31	1	2	73	30	35.3	34	11	8	7	7	65	1.66	84	15	39	30	39.71	38	13	15	41	11	47	13	91	11	60	78	20	2.51	51
32	1	2	88	51	50.7	67	31	17	13	17	80	3.27	88	0	61	20	60.57	82	22	44	43	27	56	21	57	25	53	83	4	3.38	4
33	1	2	71	47	57.8	67	27	16	12	14	76	2.42	82	16	66	41	70.54	101	52	63	131	29	80	21	67	26	83	81	7	2.55	5
34	1	2	98	62	52.8	67	28	17	11	15	96	4.32	111	13	82	33	61.91	80	19	49	73	33	92	30	164	28	85	105	9	4.31	0
35	1	2	115	38	27.9	52	9	6	8	6	113	3.5	130	13	47	26	31.17	56	8	16	66	8	32	8	0	8	35	121	7	3.9	11
36	1	2	82	50	49.7	37	18	16	19	16	66	1.54	92	12	63	27	56.51	55	47	27	53	17	2	17	-8	16	-4	90	37	1.79	17
37	1	2	108	91	73.5	106	64	54	46	52	109	5.6	108	0	102	11	81.84	121	15	90	40	74	37	64	40	70	36	88	-19	6.08	9
38	1	2	80	58	63.5	68	43	28	23	26	78	4.61	82	2	75	29	79.89	92	37	65	51	53	88	49	120	50	94	80	3	4.91	6
60	1	2	131	68	40.3	76	24	9	10	9	139	7.55	145	11	81	19	43.03	87	14	27	11	18	98	10	0	14	61	155	12	7.01	-7
67	1	2	77	49	54.6	59	29	16	9	9	72	4.07	68	-11	53	8	66.51	66	11	35	22	20	23	9	0	14	49	74	2	3.67	-10
68	1	2	118	92	68.2	116	81	53	31	43	115	5.2	123	5	110	19	77.46	135	16	137	68	89	69	46	45	70	63	120	4	6.28	21
69	1	2	88	73	69.9	88	49	47	46	43	87	6.86	87	-2	81	12	79.97	97	11	67	36	56	20	69	50	60	39	86	-2	7.37	7
70	1	2	148	112	65.2	123	70	56	26	46	141	8.07	145	-2	115	3	68.21	119	-3	90	28	64	15	37	41	56	23	142	0	7.63	-6
71	1	2	72	61	71.5	66	34	28	16	21	72	2.03	72	-1	63	3	74.2	72	9	45	33	32	12	17	2	25	18	76	4	2.16	7
72	1	2	103	69	57.2	102	37	22	13	19	106	4.95	127	24	75	9	50.44	114	12	33	-12	18	-19	12	-9	15	-19	122	15	4.55	-8
73	1	2	119	49	34.9	49	17	13	13	14	131	5.39	127	8	61	24	40.43	59	20	24	40	19	40	17	28	17	24	127	-4	5	-7
74	1	2	105	75	60.2	75	42	29	19	23	101	4.51	108	3	82	10	64.42	84	11	52	25	32	9	11	-41	21	-8	106	5	4.09	-9
76	1	2	63	39	54.4	71	23	12	8	10	56	2.43	65	4	43	11	58.28	84	19	33	41	14	22	9	15	13	24	63	12	3.12	28
77	1	2	68	23	28.5	38	7	5	7	5	71	3.31	89	32	33	42	30.62	52	36	10	39	7	35	7	7	6	28	91	27	3.86	17
78	1	2	90	82	87.1	121	126	88	55	81	91	6.22	93	4	88	7	89.94	123	2	143	14	112	28	86	56	104	30	88	-3	6.27	

Table A 11.3: Cumulative data of asthma control categories and IOS parameters used

ID	Group 1	Group 2	Pre vt actual	Pre R5	Pre R20	Pre R5-R20	Pre R35	Pre zbei5	Pre X5	Pre X20	Pre X35	Pre AX	Pre res_freq	Post vt actual	vt %Δ	Post R5	R5 %Δ	Post R20	R20 %Δ	Post R5-R20	R5-R20 %Δ	Post R35	R35 %Δ	Post zbei5	zbei5 %Δ	Post X5	X5 %Δ	Post X20	X20 %Δ	Post X35	X35 %Δ	Post res_freq	Res freq %Δ	Post AX	AX %Δ
1	0	0	0.68	111	103	21.75	153	117	262	71	69	0.74	16.3	0.62	-8	72	-35	69	-33	18.59	-15	107	-30	82	-30	264	1	144	104	99	44	13.81	-15	0.54	-28
3	0	0	1.06	93	94	12.09	122	95	228	99	117	0.18	13	1.38	31	98	5	101	8	9.94	-18	141	15	100	6	280	23	149	50	136	16	11.24	-14	0.18	0
5	0	0	0.51	78	66	26.45	85	85	264	9	85	0.65	19.6	0.47	-8	77	-2	68	3	22.74	-14	79	-7	78	-8	135	-49	-59	-719	50	-41	23.35	19	0.5	-23
8	0	0	1.08	196	142	40.7	221	224	-3700	-48	42	2.96	23.7	1.12	4	136	-31	116	-18	29.79	-27	168	-24	142	-37	-1400	62	7	-115	70	67	19.2	-19	0.9	-69
10	0	0	1.04	161	143	23.95	166	170	-2614	-68	25	1.46	29.5	0.96	-7	110	-31	112	-21	12.58	-47	152	-9	122	-28	-2566	-2	28	-142	79	211	16.11	-45	0.51	-65
11	0	0	1.95	173	128	36.19	149	191	2225	-117	74	2.12	27	2.54	30	206	19	164	28	31.29	-14	197	33	221	16	2254	1	-137	18	44	-41	29.69	10	2.39	13
12	0	0	1.4	99	106	9.68	157	103	-271	-9	2	0.53	24.2	0.94	-33	91	-8	98	-7	9.08	-6	147	-6	97	-6	-289	7	0	105	14	620	19.78	-18	0.44	-16
15	0	0	0.79	119	111	22.18	152	123	252	-12	52	0.85	20.9	0.67	-15	129	8	113	2	26.49	19	147	-3	131	7	230	-9	-69	475	38	-27	25.36	21	1.23	44
18	0	0	0.98	111	81	38.8	139	115	186	-65	57	1.26	21.4	1.44	46	109	-2	96	19	25.57	-34	135	-3	114	0	212	14	-75	15	64	11	23.05	8	1.09	-13
19	0	0	1.48	151	107	41.03	155	166	627	-35	17	2.09	23.4	1.02	-31	106	-30	89	-16	29.48	-28	131	-16	117	-29	456	-27	50	-241	64	265	16.74	-28	1	-52
20	0	0	0.54	176	175	16.09	242	185	-574	3	49	0.83	19.6	0.76	41	119	-33	121	-31	13.67	-15	190	-21	125	-32	-394	-31	55	1541	73	47	13.08	-33	0.33	-60
21	0	0	0.69	160	137	29.97	186	169	-1634	-48	27	1.71	25.1	0.55	-21	128	-20	132	-4	15.85	-47	183	-2	135	-20	-1299	-21	9	-120	49	82	18.23	-27	0.65	-62
22	0	0	0.66	142	143	17.84	183	151	-1478	-4	34	0.94	20.9	0.62	-6	114	-20	121	-16	13.96	-22	203	11	121	-20	-1201	-19	63	-1663	62	82	13.24	-37	0.44	-53
23	0	0	1.02	153	141	23.18	162	164	698	-75	56	1.8	27.7	0.9	-12	108	-29	123	-13	5.84	-75	179	10	118	-28	539	-23	67	-189	68	20	13.83	-50	0.51	-72
24	0	0	0.68	165	128	36.93	188	175	-1435	-54	50	2.11	24.9	0.67	-1	127	-23	118	-8	24.54	-34	171	-9	133	-24	-972	-32	8	-114	62	25	18.77	-25	0.85	-60
25	0	0	0.9	153	121	33.59	160	165	461	-146	26	2.39	27.9	0.84	-7	155	1	107	-12	42.24	26	154	-4	172	4	562	22	-174	19	27	4	26.38	-5	2.87	20
41	0	0	0.5	158	139	24.61	178	168	-1600	-21	70	1.11	21.8	0.51	1	131	-17	117	-15	23.16	-6	161	-10	136	-19	-1000	38	36	-273	100	43	16.87	-22	0.53	-52
42	0	0	0.8	151	154	13.15	211	160	-948	36	80	0.57	15.5	0.92	15	124	-18	127	-17	12.29	-7	193	-8	128	-20	-622	-34	82	130	109	36	11.75	-24	0.27	-53
43	0	0	0.24	105	99	21.26	147	108	372	28	45	0.51	16.5	0.34	42	84	-20	97	-3	4.25	-80	164	12	118	9	1100	196	72	161	109	141	15.31	-7	0.95	88
44	0	0	0.66	150	134	24.06	177	153	195	-847	10	1.77	30	0.55	-17	113	-25	106	-21	20.12	-16	144	-18	117	-23	177	-9	-98	-88	53	439	20.69	-31	1.03	-42
45	0	0	0.63	137	135	16.03	164	139	-493	1	60	0.38	19.9	0.84	33	108	-21	114	-15	9.99	-38	157	-4	111	-20	-561	14	60	6842	103	70	12.27	-38	0.16	-56
46	0	0	1.65	161	141	24.44	186	185	2828	-66	99	1.73	23.8	2.19	33	139	-14	120	-15	25.65	5	155	-17	155	-16	2129	-25	0	-101	113	15	19.98	-16	1.07	-38
48	0	0	0.63	78	75	18.71	115	81	138	181	104	0.4	13.9	0.73	17	70	-10	62	-17	24.87	33	102	-11	69	-15	28	-79	171	-6	83	-20	3.71	-73	0.4	0
49	0	0	1.38	180	179	15.92	238	187	-451	17	63	0.64	17.2	1.4	1	146	-19	145	-19	16.23	2	215	-10	152	-18	-372	-18	63	281	107	71	12.14	-29	12.14	-29
50	0	0	0.59	179	163	22.27	196	184	-1105	-54	39	1.23	26.9	0.99	68	139	-22	134	-18	17.47	-22	183	-6	143	-22	-916	-17	26	-148	83	114	17.19	-36	0.5	-59
54	0	0	1.28	134	123	21.51	149	141	-2060	-13	47	0.74	22.1	0.98	-23	105	-22	104	-15	15.08	-30	142	-5	112	-21	-1830	-11	35	-376	75	61	15.06	-32	0.39	-48
59	0	0	2.11	176	108	46.54	146	193	981	-184	56	2.56	25.8	2.24	6	153	-13	117	9	33.49	-28	140	-4	174	-10	991	1	-172	-6	53	-4	28.1	9	2.26	-12
61	0	0	0.69	162	147	23.4	195	174	-530	1	72	0.92	19.9	0.83	19	115	-29	107	-27	21.55	-8	146	-25	128	-27	-450	-15	35	3369	75	5	14.94	-25	0.54	-42
63	0	0	1.83	148	119	30.25	156	162	2093	-20	115	1.22	21.4	1.58	-14	145	-2	117	-2	30.18	0	151	-3	163	0	2319	11	-37	81	106	-8	22.32	4	1.35	11
64	0	0	0.7	145	138	22.31	222	152	-982	40	72	0.77	15.62	0.75	7	138	-5	148	7	12.82	-43	258	16	146	-4	-984	0	68	72	84	17	13.58	-13	0.52	-33
2	0	1	0.57	92	100	10.8	150	100	2342	58	76	0.39	13.4	0.54	-4	80	-13	83	-18	15.54	44	125	-17	82	-18	1010	-57	25	-57	48	-36	14.39	7	0.21	-46
4	0	1	0.75	185	162	25.21	218	200	-2052	-35	103	1.5	22.8	0.9	21	168	-9	158	-2	19.82	-21	218	0	185	-8	-2056	0	-14	-61	90	-12	20.94	-8	1.21	-19
6	0	1	0.58	124	114	22.05	138	126	149	-394	30	1.33	28	0.64	11	117	-6	114	0	17.33	-21	153	11	117	-7	112	-25	157	-140	83	172	17.47	-38	0.58	-56
7	0	1	0.73	132	120	21.6	160	141	735	40	117	0.65	17.2	0.96	31	133	1	128	7	16.73	-23	169	6	146	4	902	23	45	12	137	17	17.01	-1	0.7	7
9	0	1	0.7	169	145	27.46	176	178	-465	-26	57	1.14	23.6	0.75	7	126	-26	112	-23	25.11	-9	164	-7	135	-24	-414	-11	30	-216	80	40	16.05	-32	0.57	-50
13	0	1	0.86	114	101	26.87	129	120	583	-29	34	1.09	24.5	0.84	-2	97	-15	100	-1	15.2	-43	147	14	101	-15	453	-22	45	-253	48	41	15.21	-38	0.42	-61
14	0	1	1.6	137	133	15.51	180	142	620	34	97	0.56	17.2	1.84	15	139	2	128	-4	19.86	28	165	-8	142	0	451	-27	48	41	100	3	16.39	-4	0.52	-6
16	0	1	0.75	100	86	24.88	113	109	414	63	128	0.68	17.7	0.75	0	73	-27	67	-22	19.83	-20	86	-24	81	-26	334	-19	97	55	139	8	15.37	-13	0.4	-42
17	0	1	0.92	124	124	18.5	156	132	-1500	-7	45	0.8	21.3	1.21	33	113	-9	116	-6			190	22	123	-7	-1600	7	32	-554	63	39	15.86	-26	0.57	-28
26	0	1	0.76	159	143	23.06	161	163	-1723	-70	11	1.37	32.1	0.77	1	135	-15	138	-3	12.73	-45	185	15	140	-14	-1749	2	34	-150	70	519	14.83	-54	0.39	-72
47	0	1	0.82	135	89	44.61	145	163	575	25	43	2.3	19	0.72	-13	126	-7	86	-4	42.83	-4	150	3	141	-13	407	-29	54	113	68	58	18.61	-2	1.71	-26
51	0	1	0.85	165	121	35.89	179	212	1541	-91	74	2.71	22.8	1	19	161	-2	112	-8	39.35	10	159	-11	183	-14	1006	-35	-23	-74	120	62	20.68	-9	1.83	-32
52	0	1	0.91	296	178	49.86	223	320	1194	-407	-3	7.27	35.7	0.73	-19	149	-50	143	-19	19.95	-60	223	0	152	-53	298	-75	52	-113	95	-3880	16.79	-53	0.59	-92
53	0	1	1.21	283	204	38.34	277	321	-4478	-170	7	4.48	33	0.89	-26	183	-35	194	-5	9.54	-75	257	-7	193	-40	-1840	-59	8	-105	108	1406	14.92	-55	0.66	-85
55	0	1	0.89	105	100	19.04	124	111	-1740	-53	0	1.18	35	0.93	4	93	-12	91	-9	16.28	-14	123	-1	99	-11	-1640	-6	-21	-61	16	18972	27.32	-22	0.66	-44
56	0	1	0.77	147	130	25.04	168	156	303	-187	72	1.82	25.2	0.97	26	135	-8	128	-1	19.7	-21	170	1	142	-9	258	-15	3	-101	85	17	19.9	-21	1.13	-38
57	0	1	0.84	153	126	30.48	150	163	325	-257	57	2.32	28.1	0.72	-15	125	-18	99	-22	33.27	9	127	-15	133	-19	257	-21	-149	-42	86	51	23.62	-16	1.63	-30
58	0	1	0.71	106	96	22.88	133	110	181	36	87	0.8	18.5	0.68	-4	121	15	107	11	25.06	10	138	4	128	16	237	31	-40	-211	108	24	21.6	17	1.18	47
62	0	1	1.03	218	166	37.47	197	233	2196	-131	-10	3.93	37.1	0.9	-12	147	-32	148	-11	17.34	-54	230	17	151	-35	852	-61	32	-124	50	-578	16.48	-56	0.56	-86
65	0	1	0.41	304	288	21.91	351	330	4362	-188	-29	5.8	41.4	0.44	7	234	-23	240	-16	15.23	-30	317	-10	250	-24	2982	-32	-85	-55	6	-121	32.64	-21	2.61	-55
66	0	1	0.92	252	154	49.48	197	302	3345	-263	-21	7.39	41.6	1.35	47	89	-65	86	-44	20.6	-58	126	-36	96	-68	697	-79	49	-119	78	-466	14.58	-65	0.45	-94
75	0	1	0.36	199	184	23.73	252	201	1311	-22	42	1.22	23.7	0.38	5	129	-35	132	-29	15.66	-34	195	-23	136	-32	1903	45	62	-381	77	84	14.2	-40	0.58	-53
27	1	2	0.68	174	118	41.76	141	189	311	530	-39	5.05	39.03	0.74	9	169	-3	119	1	39.43	-6	140	0	175	-8	227	-27	430	-19	-1	-98	35.09	-11	3.85	-24
28	1	2	1.73	300	140	59.36	155	366	2530	-673	-65	8.74	41	1.86	7	303	1	159	13	54.39	-8	717	10	351	-4	2143	-15	-620	-8	-57	-12	42	4	7.93	-9
29	1	2	0.86	284	202	41.81	232	311	4200	-192	-32	6.87	41.1	0.75	-12	172	-40	175	-13	16.65	-60	249	7	183	-41	-2100	-50	-13	-93	17	-152	21.83	-47	1.32	-81
30	1	2	1.15	168	158	20.31	228	176	-589	9	62	0.8	19	0.92	-20	120	-29	113	-29	20.03	-1	172	-25	128	-27	-513	-13	45	420	86	40	14.36	-25	0.46	-42
31	1	2	0.34	271	263	19.98	315	301	4383	-200	-31	5.77	42	0.41	22	223	-18	225	-15	16.96	-15	288	-9	235	-22	2506	-43	-76	-62	10	-133	32.22	-23	2.31	-60
32	1	2	0.63	229	178	36.01	204	256	3137	-168	-9	4.93	36.6	0.54	-15	138	-40	144	-19	13.85	-62	203	-1	148	-42	1450	-54	10	-106	38	-501	18.98	-48	0.84	-83
33	1	2	0.69	149	133	27.43	196	158	-1519	-10	48	1.13	21.3	0.54	-22	116	-23	119	-11	16.33	-40	196	-5	127	-20	-1528	1	43	-517	70	45	14.36	-33	0.59	-48
34	1	2	0.78	183	138	36.54	168	193	353	-410	26	3.31	30.9	0.65	-17	165	-10	137	0	29.87	-18	178	6	172	-11	290	-18	-167	-59	66	155	25.2	-19	1.96	-41
35	1	2	0.47	286	209	38.94	237	317	1197	-455	-98	11.09	53.56	0.76	62	331	16	203	-3	48.77	25	217	-8	369	16	1422	19	-527	16	-82	-16	53.56	0	11.09	0
36	1	2	0.45	245	123	57.28	163	351	1159	-2330	-84	3.78	32.13	0.49	10	178	-28	111	-10	46.73	-18	141	-13	189	-46	337	-71	-908	-61	20	-123	32.13	0	3.78	0
37	1	2	0.41	133	132	18.21	179	134	837	33	58	0.44	14.5	0.5	22	102	-23	100	-24	18.79	3	149	-17	107	-21	1360	62	44	32	55	-5	14.23	-2	0.45	3
38	1	2	0.6	215	157	40.1	233	236	-2909	-78	18	3.15	26.7	0.63	5	141	-34	138	-12	20.2	-50	200	-14	151	-36	-1629	-44	19	-124	64	266	17.33	-35	0.86	-73
60	1	2	1.54	159	108	40.88	136	170	745	-308	-72			1.6	4	113	-29	104	-4	20.35	-50	134	-1	119	-30	438	-41	-162	-47	-11	-85				
67	1	2	0.62	237	201	29.2	254	276	1356	-263	-47	6.36	47.1	0.74	19	221	-7	189	-6	28.68	-2	251	-1	241	-13	932	-31	-141	-46	-7	-85	37.04	-21	3.5	-45
68	1	2	0.91	172	123	41.06	182	193	1742	-70	36	2.63	25.5	1.13	23	107	-38	108	-12	16.59	-60	152	-17	118	-39	968	-44	19	-127	84	135	17.71	-31	0.68	-74
69	1	2	1.71	138	132	19.52	174	144	-359	13	66	0.54	18.1	1.75	3	108	-22	122	-8	5.11	-74	180	4	113	-22	-275	-23	72	472	77	17	10.1	-44	0.2	-63
70	1	2	1.18	154	133	27.8	178	162	474	-63	46	1.71	25.8	0.84	-29	117	-24	109	-18	22.07	-21	164	-8	118	-27	197	-58	34	-154	80	74	17.22	-33	0.51	-70
71	1	2	0.55	311	247	33.24	254	357	1153	-849	-112	11.99	56.6	0.54	-1	226	-27	200	-19	25.64	-23	252	-1	235	-34	466	-60	-188	-78	28	-125	31.25	-45	2.89	-76
72	1	2	0.63	99	83	29.66	118	101	196	1	52	0.78	20	0.71	12	101	2	98	18	18.47	-38	121	3	102	0	150	-23	-32	-5039	28	-47	24.01	20	0.66	-15
73	1	2	1.01	403	203	56.95	288	463	-5988	-266	0	7.48	35	0.7	-31	234	-42	205	1	25.48	-55	243	-16	254	-45	-2603	-57	-106	-60	47	47	29.5	-16	4.68	-64
74	1	2	1.03	163	138	28.49	164	175	363	-331	22	2.83	31.6	0.93	-10	138	-15	116	-16	29.25	3	152	-7	150	-14	326	-10	-157	-52	77	245	23.64	-25	1.89	-33
76	1	2	0.45	207	148	41.81	219	216	-1805	-69	48	2.53	25.3	0.38	-16	166	-20	146	-1	28.16	-33	198	-10	170	-21	-1099	-39	-32	-54	32	-33	24.47	-3	1.33	-48
77	1	2	0.85	312	196	45.84	238	350	-4600	-242	-65		39.35	0.84	-1	215	-31	147	-25	41.18	-10	176	-26	244	-30	-3300	28	-186	-230	-19	-71	39.35	0	4.13	
78	1	2	0.73	146	143	20.16	231	155	-1063	36	62	0.78	16	0.76	5	119	-18	121	-16	17.54	-13	198	-15	126	-19	-824	-22	58	60	68	9	13.51	-15	0.5	-37

Table A 11.4: Cumulative data of asthma control categories and cytokines measured in fluorescent intensity units (FU) and standard international units (SI)

ID	Group 1	Group 2	IL-1β (FU)	IL-2 (FU)	IL-4 (FU)	IL-5 (FU)	IL-6 (FU)	IL-8 (FU)	IL-12 (FU)	IL-13 (FU)	IFN γ (FU)	TNF α (FU)	IL-1β (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-12 (pg/ml)	IL-13 (pg/ml)	IFN γ (pg/ml)	TNF α (pg/ml)
1	0	0	13.75	18.5	8.5	18.25	20.25	30	102.5	59.25	18	10.75	2.12	0.665	0.02	6.2	0.95	8.04	26.55	3.08	21.12	2.21
3	0	0	11.5	18.5	10.5	11.75	21	22	45.25	20.75	17.25	6	1.82	0.665	0.04	4.37	0.95	3.35	1.165	0.405	18.46	2.21
5	0	0	12.5	21.75	17	10.75	16	33	25	12.25	23.25	17.75	1.96	0.665	0.78	4.07	0.95	9.71	1.165	0.405	39.24	2.21
8	0	0	10	16.25	15.25	12	21.25	16.25	47.5	17	22.5	8.25	1.62	0.665	0.59	4.45	0.95	0.425	1.165	0.405	36.69	2.21
10	0	0	10.5	17.75	11.5	8.5	16.5	21	84.25	25.75	12.5	13.75	1.69	0.665	0.16	3.34	0.95	2.72	18.8	0.405	0.56	2.21
11	0	0	8.25	12.5	11.25	8	12.75	26	33.5	12.25	16.75	7.25	1.38	0.665	0.14	3.17	0.95	5.76	1.165	0.405	16.67	2.21
12	0	0	9.5	14	15	20.75	16	18.25	73.75	25.75	21.75	13	1.55	0.665	0.56	6.86	0.95	0.85	13.82	0.405	34.13	2.21
15	0	0	13.25	53	11	9.25	26.25	22.5	62.25	35	20.25	10.25	2.06	0.665	0.11	3.59	0.95	3.66	7.24	0.405	28.98	2.21
18	0	0	9	36	16.5	9.5	24.25	27.5	85.25	26	19.5	9.5	1.48	0.665	0.73	3.67	0.95	6.63	19.24	0.405	26.37	2.21
19	0	0	11	17.75	11.75	16.25	13	14	67.5	18.75	17	12	1.76	0.665	0.19	5.66	0.95	0.425	10.49	0.405	17.57	2.21
20	0	0	11	14	10.5	9.25	20	23.5	67.75	30.5	17.5	6.75	1.76	0.665	0.04	3.59	0.95	4.27	10.63	0.405	19.35	2.21
21	0	0	14	19.25	9	6	14.75	13.5	28.5	15.5	9.5	31	2.15	0.665	0.02	2.43	0.95	0.425	1.165	0.405	0.28	17.7
22	0	0	11.25	18	10.5	6.5	20.5	19	113.75	31	16	7.25	1.79	0.665	0.04	2.63	0.95	1.39	31.03	0.405	13.97	2.21
23	0	0	16.75	16.25	13	12	14.5	21.25	65.25	42	19.5	14	2.51	0.665	0.34	4.45	0.95	2.88	9.17	0.05	26.37	2.21
24	0	0	7.25	12	7.5	12.75	18.5	16.5	32.25	18.75	16.5	8	1.24	0.665	0.02	4.67	0.95	0.425	1.165	0.405	15.78	2.21
25	0	0	12.75	52.5	14	7	45.5	28	41.5	34	12	5.5	1.99	0.665	0.45	2.81	9.27	6.91	1.165	0.405	0.28	2.21
41	0	0	10.5	20.75	14.25	8.75	20.75	24	52.25	21	20.75	15.25	1.38	0.515	1.56	1.075	2.03	7.78	22.01	1	0.895	27.58
42	0	0	9	14.5	14.75	8	11.25	23.5	33.75	13	14.25	17	1.2	0.515	1.64	1.075	0.025	7.56	8.94	1	0.895	32.58
43	0	0	11	19	9.5	13	19	26	56.5	39.75	16.25	9.25	1.44	0.515	0.66	1.075	1.53	8.7	24.61	1	0.895	10.19
44	0	0	10.25	7	9.25	13.75	18	23.5	50.5	21.25	15.5	17.25	1.35	0.515	0.61	1.075	1.24	7.56	20.92	1	0.895	33.3
45	0	0	12.25	10.25	21.25	10.5	13.5	27.25	20	12.75	11.75	13.75	1.58	0.515	2.73	1.075	0.05	9.27	0.26	1	0.895	23.28
46	0	0	10	23.25	14.25	11.75	13.5	25.25	47	40.5	14	9.5	1.32	0.515	1.56	1.075	0.05	8.36	18.68	1	0.895	3699.39
48	0	0	17.25	16	15.75	15.75	96.5	51.5	152.5	23	27.5	12.5	2.16	0.515	1.82	1.075	27.48	20.68	75.98	1	86.64	19.67
49	0	0	6.5	16.5	11	12.75	11.75	30.75	25.5	8.75	12	14	0.9	0.515	0.96	1.075	0.025	10.89	0.26	1	0.895	23.99
50	0	0	10.5	16.5	8.5	0.5	13	25.25	29	12.5	13	7	1.38	0.515	0.45	1.075	0.025	8.36	3.89	1	0.895	3.48
54	0	0	10	13.75	12.25	12.75	15	25	46.5	15	19.75	11	1.32	0.515	1.19	1.075	0.42	8.24	18.35	1	0.895	15.32
59	0	0	9.5	13	8.75	14.5	14.25	30.25	56.75	14.5	15.75	6.5	1.26	0.515	0.5	1.075	0.23	10.66	24.76	1	0.895	1.95
61	0	0	10.25	12.75	14.5	6.5	20.25	17.5	73.5	32	13.75	9	1.35	0.515	1.6	1.075	1.89	4.85	34.41	1	0.895	9.46
63	0	0	9.25	14.5	12.5	10.5	11	20.5	43.25	24.75	16.5	10	1.23	0.515	1.24	1.075	0.025	6.2	16.17	1	0.895	12.4
64	0	0	11.75	12	7.75	6.25	9.75	18.5	24	4.25	13.75	13.75	1.53	0.515	0.27	1.075	0.025	5.3	0.26	1	0.895	23.28
2	0	1	11.5	18.5	19.5	17.5	26.5	25	46	41	17.75	13.75	1.82	0.665	1.05	6	0.95	5.17	1.165	0.405	20.23	2.21
4	0	1	18	51	8.5	4		68	36.25	56	17.25	21.5	2.67	0.665	0.02	1.56		27.71	1.165	2.57	18.46	2.21
6	0	1	16.25	38.25	19	19.5	40.5	27.75	137.25	25	27	9.25	2.45	0.665	1	6.53	5.65	6.77	39.98	0.405	51.84	2.21
7	0	1	14	13.25	14	20.5	15.5	27	128.25	22.5	14.75	10.75	2.15	0.665	0.45	6.79	0.95	6.34	36.6	0.405	9.38	2.21
9	0	1	17.75	18.5	13.5	13	26	24.5	205.5	24.5	22	13.5	2.64	0.665	0.39	4.74	0.95	4.87	64.57	0.405	34.99	2.21
13	0	1	5	15.25	10.75	13	18.25	15.5	72	38.5	14.25	15.5	0.91	0.665	0.07	4.74	0.95	0.425	12.93	0.405	7.51	2.21
14	0	1	12	48.75	15.75	9.5	20.5	34	51	18	14.5	14	1.89	0.665	0.65	3.67	0.95	10.26	1.165	0.405	8.45	2.21
16	0	1	9.75	18.5	11.5	18	9.5	33.5	101.5	33.5	16.5	12.25	1.59	0.665	0.16	6.14	0.95	9.98	26.14	0.405	15.78	2.21
17	0	1	9	38	13.5	10.5	15.5	15.5	68.25	25	18.5	18.75	1.48	0.665	0.39	3.99	0.95	0.425	10.91	0.405	22.88	2.21
26	0	1	17.75	9.5	15.5	11	11.75	24	81.25	14.25	15.75	19	2.64	0.665	0.62	4.14	0.95	4.57	17.43	0.405	13.06	2.21
47	0	1	11.5	27.5	14.5	12.25	21.5	33.5	46	16.5	18.5	11.25	1.5	0.515	1.6	1.075	2.25	12.17	18.02	1	0.895	16.04
51	0	1	9.5	14	11.5	36	16.5	24.5	48.5	23.5	8	5	1.26	0.515	1.05	3.35	0.82	8.01	19.65	1	0.895	0.59
52	0	1	18.25	72.75	13	6	61.75	40	22	14.25	36	23	2.27	19.1	1.33	1.075	15.3	15.21	0.26	1	167.15	49.58
53	0	1	9.5	57.25	14	22	19.5	24.75	30	14.5	15	15	1.26	12.66	1.51	1.075	1.67	8.13	5.19	1	0.895	26.87
55	0	1	13.5	17.75	18.5	9.5	12	43.5	49	5.5	11.5	22.25	1.73	0.515	2.28	1.075	0.025	16.87	19.97	1	0.895	47.47
56	0	1	10.5	22.5	9	7.5	21.75	22	54.25	35.25	43.25	13	1.38	0.515	0.56	1.075	2.33	6.87	23.24	1	228.09	21.11
57	0	1	11	23	13	5	11	32.75	97.25	33.75	12	8.5	1.44	0.515	1.33	1.075	0.025	11.82	47.28	1	0.895	7.98
58	0	1	10.5	22.5	13.75	9	12	24.75	89.5	21	27.75	19	1.38	0.515	1.47	1.075	0.025	8.13	43.14	1	89.24	38.27
62	0	1											17.8	105.82	22.04	1.075	83.9	23.19	101.58	1	1160.42	600.96
65	0	1	4	6.25	11.5	15.75	17.5	50.25	59.25	16	8	11	0.59	0.515	1.05	1.075	1.1	20.08	26.25	1	0.895	15.32
66	0	1	7.5	21.5	7	11.25	11.5	19	105.5	29.25	15.25	11	1.02	0.515	0.07	1.075	0.025	5.52	51.63	1	0.895	15.32
75	0	1	10.5	13.5	12.5	8	10	20	23.75	17	16	6.25	1.38	0.515	1.24	1.075	0.025	5.97	0.26	1	0.895	1.18
27	1	2	9.5	24	7.5	14.5	57.5	30.5	112.5	95	18	17.5	1.55	0.665	0.02	5.17	15.94	8.32	30.54	8.24	21.12	2.21
28	1	2	10.25	29	12.25	13.75	33.5	28	81.5	15.5	18.75	6	1.66	0.665	0.25	4.96	0.95	6.91	17.54	0.405	23.76	2.21
29	1	2	10.5	175.5	22.5	15.75	154.5	43	184	188.5	43.5	21.25	1.69	38.28	1.38	5.52	54	15.06	56.95	20.83	105.39	2.21
30	1	2	11.75	24	15	19.25	13.5	37	133	39.5	18.25	18.75	1.86	0.665	0.56	6.47	0.95	11.88	38.39	0.405	22	2.21
31	1	2	11	13.5	12	11	26	28.75	94	23.25	17.5	4.75	1.76	0.665	0.22	4.14	0.95	7.34	23.03	0.405	19.35	2.21
32	1	2	25.75	23.5	52.25	11.5	34	15	104	31.25	37.25	16.25	3.64	0.665	4.47	4.3	0.95	0.425	27.16	0.405	85.38	2.21
33	1	2	8.5	23.5	19.5	11.75	28.25	24.5	98.5	37.5	19.75	8.5	1.42	0.665	1.05	4.37	0.95	4.87	24.91	0.405	27.24	2.21
34	1	2	10	28.25	16.5	11	24.75	26	163.5	30.5	22.75	19.5	1.62	0.665	0.73	4.14	0.95	5.76	49.59	0.405	37.55	2.21
35	1	2	11.75	40.5	13.5	11.25	14.75	24.5	58.5	22.5	11.75	13.75	1.86	0.665	0.39	4.22	0.95	4.87	4.29	0.405	0.28	2.21
36	1	2	5.5	21.75	10.5	11.25	20.5	27	34	20.25	13.5	17.5	0.99	0.665	0.04	4.22	0.95	6.34	1.165	0.405	4.64	2.21
37	1	2	11.75	12.75	6	4.5	10	14.5	18.5	45.5	17	15.5	1.86	0.665	0.02	1.8	0.95	0.425	1.165	0.81	17.57	2.21
38	1	2	10.25	15.5	8.5	11	10	23.25	76.25	71.5	13	19	1.66	0.665	0.02	4.14	0.95	4.12	15.06	4.91	2.66	2.21
60	1	2	16.25	40.5	17.75	16	38	38	114	18.25	16.5	14	2.04	4.69	2.15	1.075	7.39	14.27	56.08	1	0.895	23.99
67	1	2	6.5	24	13	5.25	17	20.75	35.75	37.5	16	8	0.9	0.515	1.33	1.075	0.96	6.31	10.63	1	0.895	6.49
68	1	2	10.75	16.5	9.75	1	13	28	26.25	8.75	17	10	1.41	0.515	0.71	1.075	0.025	9.62	0.26	1	0.895	12.4
69	1	2	13	20	41.5	5	20.5	21.5	100.5	7.25	13	9.75	1.67	0.515	5.77	1.075	1.96	6.65	49	1	0.895	11.66
70	1	2	13.75	11.5	7.5	16	16	20	28.75	15	16.5	13	1.76	0.515	0.21	1.075	0.68	5.97	3.52	1	0.895	21.11
71	1	2	20.5	13	10	15.25	17.75	19.5	32.25	22.25	18.5	8.75	2.52	0.515	0.76	1.075	1.17	5.75	7.56	1	0.895	8.72
72	1	2	17	91	11.5	9.25	71.75	21.75	27.5	6	33	11.25	2.13	26.23	1.05	1.075	18.75	6.76	0.52	1	140.22	16.04
73	1	2	11	41.5	14.25	8	14.5	15	37.5	13.25	24.75	12.5	1.44	5.24	1.56	1.075	0.29	3.74	12.01	1	56.37	19.67
74	1	2	6	31.25	17.25	10	17.25	22.5	88.75	20.5	16.25	10	0.84	0.515	2.07	1.075	1.03	7.1	42.74	1	0.895	12.4
76	1	2	10.5	16.5	16.25	49.25	22.5	17	85	29.25	18.75	14	1.38	0.515	1.9	6.66	2.55	4.63	40.72	1	0.895	23.99
77	1	2	8.75	37.75	9	10.75	27.5		21.75	8.25	18.75	4.25	1.17	3.1	0.56	1.075	4.07	2692.69	0.26	1	0.895	0.59
78	1	2	11.25	26.25	5	10.75	8.25	31.5	26	3.5	5	12.75	1.47	0.515	0.0035	1.075	0.025	11.24	0.26	1	0.895	20.39

Table A 11.5: Cumulative data of asthma control categories and FeNO, chemoattractants - RANTES and eotaxin, serum leukotrienes as well as IgE

ID	Group 1	Group 2	FENO 1	FENO 2	FENO avg	FENO cat	Eotaxin (FU)	RANTES (FU)	Eotaxin (pg/ml)	RANTES (pg/ml)	LTB4 (pg/ml)	LTC4 (pg/ml)	LTE4 (pg/ml)	IgE (kIU/L)
1	0	0	49	48	48.5	1	138.75	3955	61.75	2880.98	1282.71	58.743	10651.8	83.65
3	0	0	50	54	52	1	70.5	5386	0.67	5546.4	1102.9	215.401	4986.86	2000
5	0	0	17	10	13.5	0	160.5	5476	86.87	5804.34	1313.7	149.655	3952.88	57.7
8	0	0	15	14	14.5	0	152	4712.5	77.3	4026.93	1450.12	432.214	841.113	1111.5
10	0	0	22	22	22	0	94	5834	0.67	7016.06	1102.9	200.911	1889.52	84.85
11	0	0	59	56	57.5	1	38.75	4496.25	0.67	3653.28	1252.02	120.435	8542.66	566
12	0	0	50	50	50	1	152	6661	77.3	11804.25	2281.88	383.636	991.911	2000
15	0	0	30	33	31.5	1	101.5	5299	7.26	5311.75	2110.7	212.325	4352.79	230.5
18	0	0	82	77	79.5	1	133.75	5913.5	55.62	7334.16	2915.91	165.521	3128.21	357.5
19	0	0	38	37	37.5	1	26.5	4772.75	0.67	4139.2	1187.25	84.583	7855.54	153.5
20	0	0	18	22	20	0	35	5672	0.67	6427.04	1208.69	170.161	5243.43	83.5
21	0	0	5	11	8	0	39.25	5347.75	0.67	5441.53	1671.7	225.453	15352.3	99.05
22	0	0	60	63	61.5	1	81.25	5475.25	0.67	5802.12	1937.41	368.031	4690.79	660.5
23	0	0	45	45	45	1	158.5	6149	84.64	8412.93	1127.97	150.588	10437.8	96.7
24	0	0	146	149	147.5	1	52.25	5225.75	0.67	5124.47	992.43	183.004	12897.4	249
25	0	0	18	17	17.5	0	85.25	5126.25	0.67	4883.93	1440.84	181.436	2316.94	389.5
41	0	0	44	60	52	1	81.25	5237.5	8.27	5499.74	195.49	78.184	2812.37	401.5
42	0	0	106	108	107	1	60.25	5196.25	0.27	5374.65		86.201	1914.66	363.5
43	0	0	62	62	62	1	36.5	5622.5	0.27	6908.35	139.44	129.226	2294.83	379.5
44	0	0	27	31	29	1	108	5695.5	45.83	7236.73		136.012	2318.26	122.5
45	0	0	27	29	28	1	105	5187	41.8	5347.17	202.44	135.353	3188.63	176
46	0	0	66	69	67.5	1	59.5	4855	0.27	4479.88	434.27	118.676	2989.75	595
48	0	0	92	100	96	1	174.5	6009	130.67	8969.42	264.67	262.89	18737	34.3
49	0	0	71	84	77.5	1	156.25	5072	108	5021.76	395.96	168.277	2150.28	650
50	0	0	27	31	29	1	66.25	5511.25	0.27	6450.16	10.39	108.622	3576.91	409
54	0	0	130	129	129.5	1	83.5	5640	11.66	6984.95	247.42	159.848	1538.78	85.95
59	0	0	67	69	68	1	166.75	4896.5	121.08	4576.98	468.89	154.051	3922.89	382.5
61	0	0	50	60	55	1	42.5	5683.5	0.27	7181.12	312.94	118.564	1735.87	215.5
63	0	0	73	71	72	1	58.25	4626.25	0.27	3992.07	281.91	166.249	3504.25	539.5
64	0	0	15	14	14.5	0	60.25	4984.5	0.27	4792.76	501	94.535	609.962	43.4
2	0	1	60	55	57.5	1	66.75	7227.25	0.67	19125.39	8976.73	481.898	11892.2	227
4	0	1	27	31	29	1	75.5	6763	0.67	2269.05	2573.65	289.864	775.35	2000
6	0	1	34	37	35.5	1	71	5846.25	0.67	7063.75	1322.61	108.413	2669.05	183
7	0	1	33	36	34.5	1	76	6251.25	0.67	8957.42	1549.31	139.369	787.962	69.15
9	0	1	108	113	110.5	1	57.25	6513.75	0.67	10634.67	1872.82	250.751	921.971	277
13	0	1	108	105	106.5	1	49	5129.25	0.67	4890.97	1408.56	152.087	6097.31	155.5
14	0	1	131	163	147	1	47.75	5857.25	0.67	7106.97	1578.25	218.671	484.296	232
16	0	1	139	170	154.5	1	128	5885.75	48.34	7220.77	5796.43	291.882	3177	380.25
17	0	1	65	90	77.5	1	100	6218.5	3.48	8777.3	3447.69	413.474	1527.89	54.9
26	0	1	18	18	18	0	124.75	6428	44.08	10036.19	1182.98	325.684	432.379	125
47	0	1	194	224	209	1	64.25	5088.25	0.27	5065.99	361.29	107.317	6381.68	48.3
51	0	1	140	152	146	1	77.75	4290	2.72	3394.52	181.57	114.183	2274.39	1417.5
52	0	1	55	56	55.5	1	123	7358.5	65.61	48978.97	11180.9	311.041	1401.26	198
53	0	1	20	20	20	0	68.5	5122.75	0.27	5161.76		186.691	1285.93	2000
55	0	1	20	20	20	0	93.5	6554.5	26.03	14219.32	490.41	207.826	643.254	128.5
56	0	1	23	19	21	0	72	5855.5	0.27	8047.53	518.7	218.024	739.387	85.9
57	0	1	61	84	72.5	1	76.5	5147.25	0.54	5231.35	763.05	108.72	3163.98	71.35
58	0	1	69	98	83.5	1	72.25	5523	0.27	6496.33	550.72	96.924	3356.02	59.9
62	0	1	300	300	300	1			333.83	4580.54	278.46	635.244	3836.41	377
65	0	1	190	194	192	1	57.5	5506.25	0.27	6430.66	402.91	208.676	2030.79	42.75
66	0	1	80	86	83	1	30	5795	0.27	7724.93	427.29	173.168	1410.43	15.85
75	0	1	54	55	54.5	1	66.25	4330	0.27	3459.33	525.79	128.466	1050.17	412.5
27	1	2	84	86	85	1	73.25	6075.5	0.67	8052.02	1165.95	130.035	1331.36	247.5
28	1	2	111	117	114	1	151	7486	76.16	25557.61	1032.91	236.487	6153.74	241
29	1	2	61	65	63	1	233.5	6440.5	161.78	10120.08	2257.72	205.73	2804.77	1267
30	1	2	37	38	37.5	1	242.5	5738	170.46	6658.07	1098.74	261.992	1595.66	114
31	1	2	38	39	38.5	1	29	5686.5	0.67	6476.8	1322.61	193.802	9661.04	69.45
32	1	2	290	290	290	1	150	5035	75.01	4676.24	1004.52	512.77	7676.7	306
33	1	2	44	87	65.5	1	53.5	6675.5	0.67	11930.52	2048.11	206.485	1203.78	151
34	1	2	44	53	48.5	1	101.5	6163.75	7.26	8488.31	1161.71	142.863	16180.6	89.45
35	1	2	50	45	47.5	1	31.5	7147.25	0.67	17680.75	2814.49	140.57	2041.42	226.5
36	1	2	65	65	65	1	161	7018.75	87.42	15716.38	1032.91	133.297	585.743	472.5
37	1	2	53	59	56	1	42	5642.5	0.67	6327.46	1204.39	136.712	7291.33	31.8
38	1	2	158	166	162	1	41.5	5206	0.67	5075.5	1073.9	246.205	3930.52	920.5
60	1	2	59	60	59.5	1	153	6005.25	103.92	8944.92	4206.99	624.861	1191.02	457
67	1	2	21	20	20.5	0	80	5378	6.34	5959.38	1140.58	195.962	769.65	33.95
68	1	2	72	70	71	1	59.75	4980.25	0.27	4782.01	504.53	104.216	3835.44	33.85
69	1	2	63	64	63.5	1	46.75	5530	0.27	6524.07	354.37	232.55	848.09	185.5
70	1	2	120	127	123.5	1	61.5	4424	0.27	3617.88	529.35	96.826	769.662	62.1
71	1	2	11	16	13.5	0	34	5315.5	0.27	5748.19	1221.63	273.995	1859.14	508
72	1	2	11	11	11	0	120.25	5223.5	62.03	5456.82	3005.33	352.313	843.292	160
73	1	2	26	26	26	1	51.75	5348.75	0.27	5859.14	2967.78	337.138	545.709	2000
74	1	2	53	65	59	1	76	7225	0.27	35915.36	344	89.063	186.076	65.15
76	1	2	68	57	62.5	1	63.75	6686	0.27	16308.65	835.27	196.136	803.242	136.5
77	1	2	34	36	35	1	92	6957	23.93	22831.79	572.18	134.479	1656.41	160.5
78	1	2	8	9	8.5	0	50.5	6203.5	0.27	10407.65	1304.81	117.453	346.355	35.9

Table A 11.6: Cumulative data of asthma control categories and biomarkers of airway remodelling and angiogenesis used

ID	Group 1	Group 2	VEGF (FU)	CRP (FU)	SAAP (FU)	TGF β1 (FU)	TGF β2 (FU)	TGF β3 (FU)	VEGF (pg/ml)	CRP (pg/ml)	SAAP (pg/ml)	TGF β1 (pg/ml)	TGF β2 (pg/ml)	TGF β3 (pg/ml)
1	0	0	210	1401.25	142.25	3234	86	8.5	76.42	8609.28	5146.15	39426.1	2066.62	4.84
3	0	0	71.5	625.75	87.75	4725.75	60.25	6.5	19.81	4049.02	2980.19	51503.5	1535.5	4.84
5	0	0	69	1092.5	2014.5	3732.25	169.5	5.5	18.5	6852.49	72494.33	43433.6	3593.24	4.84
8	0	0	80.5	4309.5	3740.75	3745.5	94	8.25	24.3	26100.27	150531.44	43540.1	2223.82	4.84
10	0	0	124.25	578.75	208.5	3927.25	85.5	8.25	43.4	3747.02	7647.06	45002.7	2056.68	4.84
11	0	0	99	12965.5	9004.75	2747	43	7	32.76	854898.64	932555.1	23453	1149.73	4.84
12	0	0	110.25	4651.75	6889.25	6919.75	140.75	9	37.59	28537.32	414881.81	71148.9	3091.57	4.84
15	0	0	106.5	5628.5	1455.75	3685	106.25	8.5	36	36293.59	51524.35	43053.8	2458.89	4.84
18	0	0	250	3731.25	4170.75	3331	67.25	7	91.15	22238.49	174523.01	40207.6	1684.3	4.84
19	0	0	116.25	2979.5	628	4343.5	121	7.5	40.1	17595.78	22451.87	48370.8	2734.16	4.84
20	0	0	201	5606.25	983.5	5610.75	100	8.5	73.07	36101.25	34762.07	59004.1	2339.76	4.84
21	0	0	62.5	2136	1188.5	4590.75	111	7.75	14.88	12728.45	41956.11	50391.6	2548.4	4.84
22	0	0	222.5	573.25	323.75	4492.75	123.75	8.5	81.05	3711.3	11825.12	49588.4	2784.64	4.84
23	0	0	242	8193.75	2349.25	4862.75	127.75	9.25	88.22	66832.38	85853.94	52639.2	2857.64	4.84
24	0	0	46.5	3360.25	2613.75	4823.25	65.5	8	1.37	19901.58	96919.06	52310.9	1647.45	4.84
25	0	0	85	2847	876.75	4053.25	119.5	7.5	26.43	16812.01	31051.84	46018.8	2706.52	4.84
41	0	0	113.25	676.25	202.25	6761	169.75	8.75	70.24	5274.38	8671.46	84551	3261.55	4.46
42	0	0	59.75	80.75	177.75	1021.75	42	7.5	22.82	558.97	7563.44	24845.6	968.21	4.46
43	0	0	176.25	8809	1587	4124.75	83.25	8.5	110.65	93400.94	64128.54	57635.6	1783.58	4.46
44	0	0	195.75	1927.75	802.75	3759	87.25	7.25	122.22	15649.31	33050.51	54090.8	1857.25	4.46
45	0	0	62	3364.75	1788.5	4917	93.5	7.5	25.85	28647.26	72434.19	65375.9	1970.98	4.46
46	0	0	215.75	1944.5	2142	4100.25	101.25	7.5	133.82	15793.66	87510.01	57398.1	2109.85	4.46
48	0	0	663.5	17535.75	17669.25	6134.75	140.25	7.25	379.62	316561.11	729713.86	77771.3	2779.65	4.46
49	0	0	40	226.25	137	3858.5	56.25	8	1.225	1706.85	5658.24	55055.1	1263.98	4.46
50	0	0	36.25	1168	492.25	4798	123	7.5	1.225	9259.95	20799.19	64203	2488.72	4.46
54	0	0	169	2483	342.75	4799.5	80.75	7.5	106.27	20520.51	14705.09	64217.7	1737.17	4.46
59	0	0	151.75	3286.75	287	5435.25	71	6.5	95.63	27907.79	12361.25	70551.2	1553.14	4.46
61	0	0	501.5	924.25	202.75	3586	72.5	6	290.46	7271.06	8693.83	52412.3	1581.78	4.46
63	0	0	144.5	2494	921.5	4009.5	91.5	9	91.05	20618.86	37691.58	56518.3	1934.76	4.46
64	0	0	37.5	323.5	98.25	1334.5	28.5	7.75	1.225	2473.5	3738.46	28851.2	665.46	4.46
2	0	1	110.5	2398.75	222.5	4845	169.25	8.5	37.7	14217.66	8163.91	52491.6	3588.96	4.84
4	0	1	115.75	954.5	166	4316	122.25	7.5	39.9	6049.53	6054.97	48147.1	2757.14	4.84
6	0	1	374.75	2239	443.25	5027.25	115.75	7.25	136.35	13310.1	16038.76	54013.5	2637.08	4.84
7	0	1	323.75	3732.25	2999.25	5118.5	178	7.5	117.92	22244.92	113973.99	54781.5	3737.99	4.84
9	0	1	520	4309.5	1285.5	4407.75	93.75	8	189.18	26100.27	45400.91	48894.2	2218.95	4.84
13	0	1	144.75	12334.75	8619.25	4743.25	107.75	7.5	51.6	427449.32	782981.36	51648.2	2487.25	4.84
14	0	1	137.25	222	433.75	2095.25	54	7	48.63	1116.5	15706.49	30001	1399.21	4.84
16	0	1	259.5	1369.5	1157.75	3424	34.25	7.75	94.62	8430.31	40870.02	40956	940.02	4.84
17	0	1	199.5	1076.5	792.25	2533	53	9	72.51	6760.12	28126.49	33704.2	1377.07	4.84
26	0	1	156.75	1219.75	360	4820.5	63	8	56.28	7581.67	13112.33	32700.8	1594.41	4.84
47	0	1	131.5	2767.75	428.25	3578.25	53.5	6	82.64	23090.42	18216.8	52337	1208.34	4.46
51	0	1	42	2431	298.75	1808.25	46.75	6.5	1.225	20056.59	12859.69	34344.9	1068.99	4.46
52	0	1	204.25	11296.5	2059.75	4107.25	97	7.5	127.18	135497	83938.36	57465.9	2033.98	4.46
53	0	1	82.75	1415	376.5	5115.5	138.75	7	46.85	11304.38	16101.45	67344.2	2754.65	4.46
55	0	1	162.25	671.5	419	5191	102.75	7.75	102.15	5236.38	17840.7	68097.1	2136.48	4.46
56	0	1	332.25	196.5	429	5180.5	112.5	8	198.65	1472.47	18247.26	67992.3	2307.67	4.46
57	0	1	273	266.5	242.75	4491.25	97.25	9.75	166.09	2024.02	10458.12	61198.9	2038.46	4.46
58	0	1	298.25	305.5	423	5291	117.25	8	180.04	2331.5	18003.43	69098.4	2389.98	4.46
62	0	1							66.51	99316.56	40360.54	73249.8	3164.95	4.46
65	0	1	170	451	172.25	7244.25	117.25	6.5	106.88	3481.44	7311.28	90046.6	2389.98	4.46
66	0	1	178.25	365.75	51	6833.5	109.75	6.75	111.85	2807.06	1082.32	85359.4	2259.7	4.46
75	0	1	59	460	111.75	2814.75	78.75	6.75	21.73	3552.75	4423.2	44841.1	1699.82	4.46
27	1	2	596.5	21156.5	15653	4552.25	106	8	217.43	854898.64	6142094.4	50075.7	2454.16	4.84
28	1	2	228	5224.25	755.25	4318.5	87.5	7.5	83.08	32921.53	26847.63	48167.5	2096.33	4.84
29	1	2	541	14495.5	7055	4993.5	54.75	8.25	196.9	854898.64	438189.31	33427.7	1415.75	4.84
30	1	2	257.25	1199.75	83.5	5621.25	201.5	7.75	93.8	7467.64	2804.48	59095.8	4130.88	4.84
31	1	2	148.5	16037.5	10607.75	4701	118.5	7	53.07	854898.64	3071047.3	79658.2	3481.52	4.84
32	1	2	54.5	16902.5	7367	4496.75	103.75	7	9.75	854898.64	486843.24	24314.4	1513.93	4.84
33	1	2	555	18909.5	11893.75	5511.75	171.75	7.5	202.06	854898.64	6142094.4	58143	3631.7	4.84
34	1	2	313.5	3297.5	4085.25	3940.5	112.25	7.5	114.22	19515.61	169564.06	45109.4	2571.82	4.84
35	1	2	185.5	10086.5	10423	5050.75	176.75	9	67.26	114599.94	2461267.4	54210.9	3716.79	4.84
36	1	2	207.75	4711.75	3900	5892	209.25	7	75.59	28977.77	159147.7	61487.7	4258.32	4.84
37	1	2	65.5	2710	334	1441.5	99	6	16.59	16010.39	12190.07	24077.3	2320.54	4.84
38	1	2	97.75	2524	3574.5	3696	144.25	7.5	32.21	14934.77	141847.45	43142.2	3153.74	4.84
60	1	2	206.5	8364	690.25	4804	65.5	7.5	128.49	86894.97	28643.51	64262	1446.99	4.46
67	1	2	101.5	8630.5	6110.25	5352.75	84.25	6.5	61.75	90758.67	364856.93	69719	1802.07	4.46
68	1	2	44.25	683.75	89.5	2533.25	68.75	7.5	1.225	5334.39	3282.75	42006.1	1509.94	4.46
69	1	2	268.25	96.75	47	7457	114	8	163.45	685.62	817.27	92552.5	2333.74	4.46
70	1	2	76	1292.25	184.75	2094.25	65.75	8.25	40.82	10284.6	7882.46	37446.5	1451.85	4.46
71	1	2	112	6405.5	988.5	4347.75	60	6.75	69.36	61171.99	40311.62	59801.3	1338.9	4.46
72	1	2	112.25	1003.5	437.5	3399.75	124.5	7.75	69.53	7914.71	18592.11	50600.5	2514.33	4.46
73	1	2	83.5	182.75	69.5	5095.5	115.5	6.75	47.49	1364.14	2192.59	67145.2	2359.73	4.46
74	1	2	393.5	255	314.5	3043.75	77.5	5	231.91	1933.39	13523.89	47112.2	1676.37	4.46
76	1	2	898.5	10719.75	13912	6393	105	7.25	514.36	124762.51	729713.86	80525.5	2176.27	4.46
77	1	2	306.25	5686	246.75	3050.25	43.25	7	184.43	52755.56	10632.04	47176.3	994.97	4.46
78	1	2	75.75	70.5	44.25	4792	53.5	7.5	40.59	477.71	625.38	64143.9	1208.34	4.46

APPENDIX 12: CORRELATION MATRIX OF ALL VARIABLES MEASURED

A correlation matrix with the Rho (r) values of all variables measured in this thesis is reported in the Table A12.1. It represents the all the variables measured in the vertical columns numbered 1 - 113 correlated with all the variables measured in the horizontal rows label B - DI. The legend of table 12.1 is included in text box 1 and 2 as there was no space for it on the pages below.

Text box 1: Legend of the abbreviation of all the variables measured and included in table A12.1

ID: identification, Group 1: Group 1 analysis based on ACQ, Group 2: Group 2 analysis based on ACQ, SBP: systolic blood pressure, DBP: diabolic blood pressure, Abd Cirm: abdominal circumference, BMI: body mass index, ACQ: asthma control questionnaire, AST: Physicians Asthma Rating, sd: standard dose, ACT tot: Asthma control test as actual score, ACT cat: Asthma control test as a categorical variable less than or greater than 19, hd: high dose, LABA: long acting Beta 2 agonist, SABA: short acting β2 agonist, SAMA: short acting anti-muscarinic antagonist, PPI: proton pump inhibitor, H2 receptor blocker: Histamine 2 receptor blocker, Pre: pre bronchodilator, FVC: forced vital capacity, FEV_1: forced expiratory volume in one second, FEV_1/FVC: percentage of the FVC expired in one second, PEFR: peak expiratory flow, FEF: forced expiratory flow related to a specific portion of the FVC, FEF25-75: forced expiratory flow during the middle half of the FVC, FVC in: Forced inspiratory vital capacity, FIF 50: Forced inspiratory flow at 50% or mid inspiration, Post: post bronchodilator, %D: % change post bronchodilator, vt actual: R5: total airway resistance, R20: central airway resistance, R5-R20: resistance of the small airways, R35: airway resistance at 35Hz, zbei5: respiratory impedance at 5 Hz, Hz: Hertz, X5: peripheral capacitive reactance, X20: reactance at 20Hz, X35: reactance at 35Hz, AX: reactance area, res freq: resonant frequency, Post: post bronchodilator, %: percentage of predicted, %D: % change post bronchodilator, IL: interleukin, IFNγ: interferon gamma, TNFα: tumour necrosis factor alpha, FU: fluorescent intensity units, pg/ml: picograms per millilitre, FENO 1: fractional exhaled nitric oxide initial test, FENO 2: fractional exhaled nitric oxide 2nd test, FENO avg: fractional exhaled nitric oxide average, FENO cat: fractional exhaled nitric oxide as categorical value less then or greater then 25, RANTES: Regulated on activation normal T expressed and secreted, LT: Leukotriene, IgE: Immunoglobulin E, FU: fluorescent intensity units, kIU/L: kilo international units per litre, pg/ml: picograms per millilitre, VEGF: vascular endothelial growth factor, CRP: C-reactive protein, SAAP: serum amyloid associated protein, TGFβ: transforming growth factor beta,

Text box 2: Legend of the all the variables in the correlation matrix with the numbers representing the vertical columns of the table and the alphabets representing the horizontal rows of the variables included in table 12.1

SBP	2	B	ACT tot	40	AN	SAAP (pg/ml)	77	BY
DBP	3	C	IL-2 (FU)	41	AO	TGF b1 (pg/ml)	78	BZ
Abd Cirm	4	D	IL-4 (FU)	42	AP	TGF b2 (pg/ml)	79	CA
Age	5	E	IL-5 (FU)	43	AQ	TGF b3 (pg/ml)	80	CB
Height	6	F	IL-6 (FU)	44	AR	Pre vt actual	81	CC
Weight	7	G	IL-8 (FU)	45	AS	Pre R5	82	CD
BMI	8	H	IL-12 (FU)	46	AT	Pre R20	83	CE
Pre FVC	9	I	IL-13 (FU)	47	AU	Pre R5-R20	84	CF
Pre FEV1	10	J	Eotaxin (FU)	48	AV	Pre R35	85	CG
Pre FEV1/FVC	11	K	IFN l (FU)	49	AW	Pre zbei5	86	CH
Pre PEFR	12	L	RANTES (FU)	50	AX	Pre X5	87	CI
Pre FEF 25	13	M	TNF a (FU)	51	AY	Pre X20	88	CJ
Pre FEF 50	14	N	VEGF (FU)	52	AZ	Pre X35	89	CK
Pre FEF 75	15	O	IL-1b (FU)	53	BA	Pre AX	90	CL
Pre FEF 25-75	16	P	CRP (FU)	54	BB	Pre res_freq	91	CM
Pre FVCin	17	Q	SAAP (FU)	55	BC	Post vt actual	92	CN
Pre FIF 50	18	R	TGF b1 (FU)	56	BD	vt %D	93	CO
Post FVC	19	S	TGF b2 (FU)	57	BE	Post R5	94	CP
FVC%D	20	T	TGF b3 (FU)	58	BF	R5 %D	95	CQ
Post FEV1	21	U	IgE (kIU/L)	59	BG	Post R20	96	CR
FEV1%D	22	V	LTB4 (pg/ml)	60	BH	R20 %D	97	CS
Post FEV1/FVC	23	W	LTC4 (pg/ml)	61	BI	Post R5-R20	98	CT
Post PEFR	24	X	LTE4 (pg/ml)	62	BJ	R5-R20 %D	99	CU
PEFR %D	25	Y	IL-2 (pg/ml)	63	BK	Post R35	100	CV
Post FEF 25	26	Z	IL-4 (pg/ml)	64	BL	R35 %D	101	CW
FEF 25 %D	27	AA	IL-5 (pg/ml)	65	BM	Post zbei5	102	CX
Post FEF 50	28	AB	IL-6 (pg/ml)	66	BN	zbei5 %D	103	CY
FEF 50 %D	29	AC	IL-8 (pg/ml)	67	BO	Post X5	104	CZ
Post FEF 75	30	AD	IL-12 (pg/ml)	68	BP	X5 %D	105	DA
FEF 75 %D	31	AE	IL-13 (pg/ml)	69	BQ	Post X20	106	DB
Post FEF 25-75	32	AF	Eotaxin (pg/ml)	70	BR	X20 %D	107	DC
FEF 25-75 %D	33	AG	IFN l (pg/ml)	71	BS	Post X35	108	DD
Post FVCin	34	AH	RANTES (pg/ml)	71	BT	X35 %D	109	DE
FVCin %D	35	AI	TNF a (pg/ml)	73	BU	Post res_freq	110	DF
Post FIF 50	36	AJA	VEGF (pg/ml)	74	BV	Res freq %D	111	DG
FIF 50 %D	37	AK	IL-1b (pg/ml)	75	BW	Post AX	112	DH
ACQ score	38	AL	CRP (pg/ml)	76	BX	AX %D	113	DI
FENO avg	39	AM						

Table A12.1: Rho values of correlation matrix of all variables measured

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1		SBP	DBP	Abd Cirm	Age	Height	Weight	BMI	Pre FVC	Pre FEV1	Pre FEV1/FVC	Pre PEFR	Pre FEF 25	Pre FEF 50	Pre FEF 75	Pre FEF 25-75	Pre FVCin	Pre FIF 50	Post FVC
2	SBP		0.67	0.46	0.38	-0.09	0.30	0.39	0.06	0.01	-0.05	0.01	-0.03	-0.08	-0.06	-0.08	0.01	0.10	0.10
3	DBP	0.67		0.36	0.30	-0.22	0.25	0.37	0.20	0.01	-0.19	-0.07	-0.13	-0.14	-0.14	-0.16	0.16	0.09	0.24
4	Abd Cirm	0.46	0.36		0.47	0.20	0.72	0.68	0.12	-0.24	-0.36	-0.24	-0.32	-0.34	-0.41	-0.37	0.13	-0.08	0.19
5	Age	0.38	0.30	0.47		0.04	0.25	0.25	0.30	-0.04	-0.41	-0.17	-0.32	-0.37	-0.37	-0.37	0.24	-0.20	0.33
6	Height	-0.09	-0.22	0.20	0.04		0.44	-0.11	0.12	0.08	0.02	0.18	0.13	0.14	0.07	0.13	0.15	0.51	0.06
7	Weight	0.30	0.25	0.72	0.25	0.44		0.80	0.06	-0.11	-0.14	-0.04	-0.11	-0.10	-0.23	-0.13	0.08	0.04	0.11
8	BMI	0.39	0.37	0.68	0.25	-0.11	0.80		-0.02	-0.18	-0.14	-0.13	-0.21	-0.19	-0.28	-0.22	-0.02	-0.22	0.07
9	Pre FVC	0.06	0.20	0.12	0.30	0.12	0.06	-0.02		0.57	-0.08	0.43	0.20	0.14	0.12	0.14	0.96	0.34	0.92
10	Pre FEV1	0.01	0.01	-0.24	-0.04	0.08	-0.11	-0.18	0.57		0.70	0.86	0.86	0.83	0.78	0.83	0.52	0.34	0.43
11	Pre FEV1/FVC	-0.05	-0.19	-0.36	-0.41	0.02	-0.14	-0.14	-0.08	0.70		0.63	0.91	0.94	0.88	0.93	-0.08	0.18	-0.19
12	Pre PEFR	0.01	-0.07	-0.24	-0.17	0.18	-0.04	-0.13	0.43	0.86	0.63		0.86	0.79	0.63	0.78	0.42	0.46	0.35
13	Pre FEF 25	-0.03	-0.13	-0.32	-0.32	0.13	-0.11	-0.21	0.20	0.86	0.91	0.86		0.97	0.87	0.95	0.18	0.28	0.06
14	Pre FEF 50	-0.08	-0.14	-0.34	-0.37	0.14	-0.10	-0.19	0.14	0.83	0.94	0.79	0.97		0.92	0.99	0.12	0.30	0.00
15	Pre FEF 75	-0.06	-0.14	-0.41	-0.37	0.07	-0.23	-0.28	0.12	0.78	0.88	0.69	0.87	0.92		0.95	0.10	0.32	0.01
16	Pre FEF 25-75	-0.08	-0.16	-0.37	-0.37	0.13	-0.13	-0.22	0.14	0.83	0.93	0.78	0.95	0.99	0.95		0.12	0.31	0.01
17	Pre FVCin	0.01	0.16	0.13	0.24	0.15	0.08	-0.02	0.96	0.52	-0.08	0.42	0.18	0.12	0.10	0.12		0.33	0.92
18	Pre FIF 50	0.10	0.09	-0.08	-0.20	0.51	0.04	-0.22	0.34	0.34	0.18	0.46	0.28	0.30	0.32	0.31	0.33		0.35
19	Post FVC	0.10	0.24	0.19	0.33	0.06	0.11	0.07	0.92	0.43	-0.19	0.35	0.06	0.00	0.01	0.01	0.92	0.35	
20	FVC%D	0.12	0.07	0.17	0.10	-0.12	0.08	0.18	-0.10	-0.39	-0.37	-0.27	-0.43	-0.43	-0.35	-0.40	-0.03	-0.03	0.20
21	Post FEV1	0.02	0.03	-0.26	-0.03	0.09	-0.12	-0.18	0.54	0.92	0.66	0.82	0.80	0.77	0.73	0.77	0.43	0.32	0.43
22	FEV1%D	-0.05	0.04	0.13	-0.02	-0.04	0.06	0.11	-0.13	-0.53	-0.51	-0.45	-0.53	-0.53	-0.44	-0.51	-0.11	-0.19	0.02
23	Post FEV1/FVC	-0.09	-0.19	-0.36	-0.46	0.02	-0.15	-0.15	-0.15	0.58	0.92	0.59	0.82	0.85	0.79	0.83	-0.17	0.10	-0.30
24	Post PEFR	-0.02	-0.08	-0.25	-0.20	0.14	-0.03	-0.09	0.36	0.78	0.68	0.93	0.81	0.74	0.64	0.72	0.34	0.35	0.27
25	PEFR %D	-0.11	-0.01	0.08	-0.07	-0.19	0.03	0.16	-0.22	-0.41	-0.28	-0.43	-0.38	-0.37	-0.28	-0.37	-0.22	-0.38	-0.15
26	Post FEF 25	-0.07	-0.14	-0.31	-0.31	0.11	-0.15	-0.22	0.15	0.76	0.83	0.79	0.90	0.88	0.79	0.86	0.11	0.21	0.01
27	FEF 25 %D	-0.10	-0.05	0.09	-0.05	-0.02	0.03	0.09	-0.04	-0.32	-0.32	-0.28	-0.36	-0.34	-0.27	-0.33	-0.04	-0.19	-0.01
28	Post FEF 50	-0.08	-0.14	-0.34	-0.39	0.15	-0.12	-0.21	0.13	0.76	0.88	0.76	0.92	0.93	0.86	0.92	0.10	0.26	-0.01
29	FEF 50 %D	-0.10	-0.03	0.01	-0.08	0.05	-0.04	-0.08	0.03	-0.18	-0.23	-0.11	-0.16	-0.22	-0.18	-0.21	0.02	-0.10	0.02
30	Post FEF 75	-0.13	-0.20	-0.47	-0.46	0.13	-0.26	-0.35	0.04	0.68	0.83	0.65	0.83	0.86	0.88	0.88	0.02	0.27	-0.09
31	FEF 75 %D	-0.13	-0.13	-0.19	-0.29	0.11	-0.07	-0.18	-0.10	0.02	0.12	0.09	0.15	0.13	0.03	0.12	-0.12	-0.04	-0.19
32	Post FEF 25-75	-0.09	-0.17	-0.37	-0.40	0.18	-0.15	-0.26	0.10	0.74	0.86	0.73	0.89	0.91	0.87	0.91	0.07	0.27	-0.04
33	FEF 25-75 %D	-0.10	-0.07	0.04	-0.16	0.12	0.01	-0.06	-0.03	-0.19	-0.16	-0.09	-0.12	-0.17	-0.18	-0.19	-0.04	-0.06	-0.07
34	Post FVCin	0.06	0.23	0.20	0.37	0.07	0.11	0.07	0.90	0.37	-0.24	0.28	0.02	-0.04	-0.06	-0.04	0.92	0.30	0.95
35	FVCin %D	0.09	0.09	0.20	0.23	-0.29	0.11	0.34	-0.18	-0.42	-0.40	-0.38	-0.47	-0.49	-0.46	-0.49	-0.21	-0.38	0.03
36	Post FIF 50	-0.03	-0.06	-0.16	-0.27	0.51	0.00	-0.26	0.29	0.30	0.20	0.44	0.27	0.31	0.33	0.31	0.36	0.92	0.32
37	FIF 50 %D	-0.26	-0.35	-0.15	-0.15	-0.12	-0.08	0.01	-0.27	-0.18	0.01	-0.14	-0.09	-0.06	-0.04	-0.05	-0.25	-0.38	-0.23
38	ACQ score	0.00	0.00	0.01	-0.06	-0.21	-0.10	0.04	-0.27	-0.54	-0.41	-0.52	-0.51	-0.50	-0.45	-0.49	-0.24	-0.26	-0.18
39	FENO avg	-0.22	-0.20	-0.24	0.04	0.07	-0.25	-0.30	0.06	0.10	0.01	0.04	0.01	0.02	0.10	0.05	0.01	-0.09	0.05
40	ACT tot	0.02	0.00	0.00	0.03	0.14	0.00	-0.08	0.15	0.23	0.20	0.23	0.23	0.22	0.19	0.21	0.12	0.22	0.04
41	IL-2 (FU)	0.09	0.10	0.21	0.21	-0.11	0.13	0.19	0.08	-0.30	-0.41	-0.30	-0.38	-0.39	-0.37	-0.39	0.12	-0.13	0.23
42	IL-4 (FU)	-0.06	-0.10	-0.06	0.04	0.13	0.09	0.01	0.05	-0.15	-0.22	-0.16	-0.21	-0.22	-0.20	-0.21	0.08	0.03	0.06
43	IL-5 (FU)	-0.15	-0.20	0.01	0.19	-0.11	-0.09	-0.05	0.06	-0.01	-0.05	-0.04	-0.08	-0.11	-0.13	-0.10	0.04	-0.21	0.04
44	IL-6 (FU)	-0.05	-0.06	0.10	0.16	-0.15	0.08	0.14	-0.05	-0.19	-0.25	-0.19	-0.30	-0.32	-0.35	-0.32	0.01	-0.18	0.07
45	IL-8 (FU)	0.00	-0.09	0.04	0.38	-0.03	-0.06	-0.03	0.10	-0.12	-0.31	-0.15	-0.22	-0.27	-0.19	-0.24	0.09	-0.02	0.15
46	IL-12 (FU)	-0.17	-0.13	-0.25	0.10	-0.08	-0.36	-0.35	0.03	-0.03	-0.13	-0.03	-0.10	-0.16	-0.11	-0.14	0.01	-0.04	0.03
47	IL-13 (FU)	0.00	0.05	-0.02	0.00	-0.20	-0.08	-0.01	-0.05	0.03	0.01	-0.03	-0.02	-0.05	-0.07	-0.06	-0.07	-0.13	-0.07
48	Eotaxin (FU)	0.15	0.06	0.11	0.32	0.11	0.19	0.13	0.00	-0.13	-0.24	-0.09	-0.19	-0.23	-0.17	-0.23	0.03	0.05	0.09
49	IFN I (FU)	-0.05	0.04	0.12	0.13	-0.07	0.24	0.25	0.04	-0.04	-0.12	-0.02	-0.12	-0.15	-0.18	-0.15	0.06	-0.22	0.12
50	RANTES (FU)	0.02	-0.15	-0.16	0.01	-0.18	-0.19	-0.14	-0.04	-0.12	-0.18	-0.15	-0.17	-0.19	-0.09	-0.16	0.02	-0.02	0.04
51	TNF a (FU)	-0.02	-0.10	-0.18	-0.09	0.03	-0.09	-0.09	0.02	-0.02	-0.01	-0.06	0.00	0.00	0.07	0.02	0.06	0.06	0.06
52	VEGF (FU)	0.01	-0.07	-0.11	0.23	-0.22	-0.27	-0.18	0.02	-0.10	-0.23	-0.10	-0.22	-0.27	-0.21	-0.27	0.01	-0.13	0.07
53	IL-1b (FU)	-0.08	-0.12	0.09	0.02	0.16	0.23	0.18	0.11	0.07	0.06	0.20	0.09	0.09	0.06	0.09	0.16	0.23	0.14

Table A12.1: continued from above

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1		SBP	DBP	Abd Cirm	Age	Height	Weight	BMI	Pre FVC	Pre FEV1	Pre FEV1/FVC	Pre PEFR	Pre FEF 25	Pre FEF 50	Pre FEF 75	Pre FEF 25-75	Pre FVCin	Pre FIF 50	Post FVC
54	CRP (FU)	-0.03	-0.11	0.21	0.03	-0.17	0.06	0.13	-0.04	-0.20	-0.28	-0.22	-0.30	-0.35	-0.33	-0.34	-0.03	-0.27	0.02
55	SAAP (FU)	0.05	-0.04	0.09	0.12	-0.29	-0.06	0.06	-0.02	-0.12	-0.20	-0.20	-0.20	-0.26	-0.25	-0.25	-0.04	-0.38	0.00
56	TGF b1 (FU)	-0.05	-0.09	0.06	-0.13	-0.04	-0.07	-0.08	-0.05	-0.19	-0.14	-0.19	-0.15	-0.17	-0.16	-0.19	-0.03	0.02	-0.09
57	TGF b2 (FU)	0.12	0.08	0.15	-0.05	-0.17	0.07	0.19	-0.04	-0.15	-0.14	-0.14	-0.14	-0.17	-0.17	-0.17	-0.01	-0.05	-0.01
58	TGF b3 (FU)	-0.17	-0.18	-0.26	-0.24	0.05	-0.16	-0.19	-0.07	0.07	0.20	0.15	0.21	0.21	0.17	0.18	-0.02	0.17	-0.02
59	IgE (kIU/L)	-0.10	-0.08	0.23	0.04	0.12	0.12	0.03	0.03	-0.11	-0.12	-0.28	-0.17	-0.13	-0.06	-0.09	0.04	-0.05	0.04
60	LTB4 (pg/ml)	-0.01	0.04	0.03	-0.02	-0.24	-0.05	0.06	0.18	0.01	-0.15	-0.04	-0.12	-0.14	-0.02	-0.09	0.25	-0.07	0.27
61	LTC4 (pg/ml)	-0.28	-0.19	-0.13	-0.22	0.02	-0.15	-0.26	-0.02	-0.13	-0.11	-0.18	-0.13	-0.12	-0.06	-0.11	0.10	-0.02	0.02
62	LTE4 (pg/ml)	0.07	0.03	0.14	0.07	0.12	0.22	0.16	0.03	0.10	0.08	0.11	0.09	0.07	0.04	0.08	0.02	-0.08	0.06
63	IL-2 (pg/ml)	0.00	0.03	0.12	0.01	-0.01	0.12	0.08	0.08	-0.19	-0.31	-0.23	-0.28	-0.27	-0.18	-0.23	0.16	-0.12	0.20
64	IL-4 (pg/ml)	-0.03	-0.01	0.06	0.06	0.15	0.19	0.12	-0.05	-0.25	-0.21	-0.19	-0.24	-0.25	-0.29	-0.28	-0.03	-0.01	-0.03
65	IL-5 (pg/ml)	-0.07	-0.14	-0.12	-0.01	-0.12	-0.09	-0.05	-0.03	0.04	-0.01	-0.04	-0.02	-0.02	0.05	0.02	-0.03	-0.18	-0.04
66	IL-6 (pg/ml)	0.01	-0.03	0.10	0.14	-0.19	0.09	0.20	-0.08	-0.21	-0.20	-0.22	-0.28	-0.28	-0.31	-0.29	-0.02	-0.24	0.01
67	IL-8 (pg/ml)	0.04	-0.02	0.11	0.33	0.03	0.04	0.05	0.04	-0.15	-0.26	-0.15	-0.20	-0.23	-0.22	-0.24	0.02	-0.02	0.08
68	IL-12 (pg/ml)	-0.09	-0.07	-0.16	0.14	-0.02	-0.26	-0.27	-0.03	-0.11	-0.16	-0.05	-0.15	-0.20	-0.20	-0.21	-0.05	-0.05	-0.05
69	IL-13 (pg/ml)	-0.07	-0.04	0.09	0.01	0.07	0.08	0.07	-0.07	-0.15	-0.05	-0.10	-0.09	-0.11	-0.15	-0.13	-0.08	0.02	-0.09
70	Eotaxin (pg/ml)	0.01	-0.07	0.06	0.19	0.11	0.11	0.04	-0.04	-0.12	-0.18	-0.11	-0.17	-0.18	-0.08	-0.16	0.03	-0.02	0.07
71	IFN I (pg/ml)	-0.01	0.10	0.00	0.06	-0.12	0.12	0.12	0.03	0.07	0.00	0.03	0.03	0.00	0.02	0.01	0.08	-0.26	0.10
72	RANTES (pg/ml)	0.04	-0.16	-0.20	0.03	-0.18	-0.22	-0.15	-0.09	-0.17	-0.15	-0.15	-0.17	-0.20	-0.11	-0.19	-0.04	-0.04	-0.02
73	TNF a (pg/ml)	0.00	0.06	-0.02	-0.08	0.03	-0.02	-0.02	0.03	-0.03	0.07	0.08	0.09	0.06	-0.04	0.02	0.06	0.11	0.03
74	VEGF (pg/ml)	0.00	-0.07	-0.06	0.24	-0.18	-0.25	-0.17	0.02	-0.14	-0.25	-0.11	-0.25	-0.30	-0.26	-0.31	0.01	-0.10	0.06
75	IL-1b (pg/ml)	-0.11	-0.12	0.01	0.02	0.09	0.16	0.09	0.10	0.07	0.03	0.14	0.05	0.05	0.05	0.06	0.17	0.11	0.15
76	CRP (pg/ml)	-0.01	-0.11	0.25	0.09	-0.11	0.11	0.14	-0.04	-0.23	-0.29	-0.22	-0.31	-0.36	-0.36	-0.36	-0.03	-0.27	0.03
77	SAAP (pg/ml)	0.07	-0.02	0.11	0.12	-0.28	-0.06	0.06	-0.03	-0.14	-0.22	-0.22	-0.21	-0.27	-0.26	-0.27	-0.05	-0.37	0.00
78	TGF b1 (pg/ml)	0.00	-0.01	0.13	-0.07	-0.09	-0.07	-0.03	-0.10	-0.19	-0.08	-0.15	-0.12	-0.14	-0.17	-0.18	-0.07	0.03	-0.12
79	TGF b2 (pg/ml)	0.15	0.07	0.12	-0.06	-0.18	0.04	0.15	-0.04	-0.11	-0.11	-0.10	-0.11	-0.13	-0.13	-0.13	-0.01	-0.04	-0.01
80	TGF b3 (pg/ml)	-0.03	-0.06	-0.14	-0.03	-0.08	-0.11	-0.10	0.06	0.12	0.00	0.00	0.02	0.04	0.13	0.09	0.06	-0.09	0.05
81	Pre vt actual	-0.21	-0.04	0.01	0.17	0.42	-0.01	-0.29	0.40	0.10	-0.16	0.03	-0.04	-0.02	-0.02	-0.03	0.41	0.26	0.41
82	Pre R5	-0.09	0.06	0.11	-0.17	-0.18	-0.08	0.03	-0.18	-0.53	-0.43	-0.57	-0.52	-0.49	-0.42	-0.49	-0.21	-0.24	-0.14
83	Pre R20	-0.13	0.03	-0.05	-0.46	-0.13	-0.09	-0.02	-0.28	-0.40	-0.17	-0.38	-0.27	-0.22	-0.20	-0.24	-0.28	-0.08	-0.26
84	Pre R5-R20	0.09	0.04	0.32	0.24	-0.14	0.06	0.19	0.10	-0.42	-0.57	-0.41	-0.56	-0.59	-0.51	-0.57	0.09	-0.29	0.19
85	Pre R35	-0.18	0.03	-0.09	-0.56	-0.23	-0.13	0.01	-0.33	-0.33	-0.04	-0.30	-0.16	-0.10	-0.10	-0.12	-0.32	-0.11	-0.33
86	Pre zbei5	-0.08	0.05	0.16	-0.12	-0.16	-0.05	0.06	-0.17	-0.54	-0.46	-0.58	-0.55	-0.51	-0.44	-0.51	-0.20	-0.26	-0.13
87	Pre X5	0.10	0.16	0.17	0.31	-0.09	0.09	0.15	0.13	-0.06	-0.19	-0.12	-0.17	-0.19	-0.21	-0.22	0.07	-0.30	0.16
88	Pre X20	0.02	-0.13	-0.22	-0.19	0.15	-0.05	-0.17	-0.03	0.49	0.60	0.49	0.60	0.61	0.59	0.62	-0.02	0.27	-0.11
89	Pre X35	0.08	0.03	0.05	0.07	0.21	0.11	-0.01	0.13	0.49	0.42	0.46	0.49	0.50	0.46	0.50	0.11	0.21	0.07
90	Pre AX	-0.03	0.11	0.19	0.12	-0.26	-0.04	0.13	-0.03	-0.55	-0.60	-0.56	-0.63	-0.66	-0.63	-0.66	-0.06	-0.39	0.03
91	Pre res_freq	-0.04	0.07	0.12	0.12	-0.14	-0.02	0.05	-0.05	-0.53	-0.60	-0.57	-0.62	-0.64	-0.61	-0.65	-0.07	-0.31	-0.01
92	Post vt actual	-0.17	-0.03	0.00	0.18	0.42	-0.07	-0.33	0.47	0.27	-0.04	0.17	0.12	0.11	0.10	0.10	0.47	0.30	0.42
93	vt %D	0.02	0.04	-0.05	0.08	-0.05	-0.18	-0.17	0.15	0.34	0.20	0.24	0.28	0.24	0.23	0.24	0.13	-0.01	0.06
94	Post R5	0.09	0.17	0.24	0.07	-0.25	0.00	0.15	-0.18	-0.57	-0.52	-0.63	-0.60	-0.57	-0.55	-0.57	-0.19	-0.32	-0.11
95	R5 %D	0.25	0.06	0.15	0.42	-0.01	0.07	0.05	0.13	0.15	-0.01	0.10	0.05	0.02	-0.07	0.02	0.14	-0.03	0.16
96	Post R20	-0.07	0.07	0.06	-0.25	-0.15	-0.03	0.03	-0.22	-0.45	-0.29	-0.47	-0.37	-0.33	-0.32	-0.33	-0.22	-0.21	-0.18
97	R20 %D	0.13	0.01	0.20	0.34	0.01	0.04	0.00	0.21	-0.03	-0.24	-0.07	-0.15	-0.19	-0.23	-0.17	0.23	-0.10	0.25
98	Post R5-R20	0.29	0.17	0.36	0.48	-0.24	0.02	0.23	0.06	-0.25	-0.39	-0.29	-0.41	-0.44	-0.41	-0.43	0.03	-0.28	0.13
99	R5-R20 %D	0.32	0.17	0.14	0.31	-0.06	0.09	0.14	-0.04	0.07	0.08	0.01	0.04	0.05	-0.01	0.03	-0.05	-0.01	-0.03
100	Post R35	-0.16	-0.01	-0.06	-0.46	-0.15	-0.06	0.01	-0.26	-0.36	-0.11	-0.32	-0.20	-0.15	-0.17	-0.16	-0.25	-0.15	-0.24
101	R35 %D	0.00	-0.09	0.03	0.13	0.13	0.10	-0.02	0.09	0.02	-0.06	0.03	0.00	-0.03	-0.10	-0.03	0.12	-0.05	0.13
102	Post zbei5	0.09	0.16	0.25	0.06	-0.24	-0.01	0.13	-0.19	-0.58	-0.52	-0.63	-0.59	-0.57	-0.55	-0.58	-0.21	-0.32	-0.13
103	zbei5 %D	0.21	0.04	0.08	0.35	-0.02	0.02	-0.02	0.16	0.21	0.04	0.17	0.13	0.09	0.00	0.09	0.17	0.02	0.17
104	Post X5	0.19	0.23	0.21	0.36	-0.12	0.06	0.13	0.12	0.02	-0.10	-0.04	-0.09	-0.11	-0.13	-0.13	0.07	-0.24	0.12
105	X5 %D	0.20	0.14	0.02	0.04	0.04	0.01	-0.04	0.08	0.17	0.09	0.17	0.15	0.14	0.11	0.14	0.10	0.23	0.01
106	Post X20	-0.21	-0.23	-0.30	-0.27	0.15	-0.04	-0.16	0.04	0.50	0.61	0.50	0.60	0.63	0.61	0.63	0.01	0.18	-0.05
107	X20 %D	0.08	0.07	-0.04	0.03	-0.02	-0.11	-0.13	0.01	0.11	0.14	0.08	0.11	0.13	0.17	0.14	0.05	0.22	0.04
108	Post X35	-0.05	0.06	0.00	0.08	0.16	0.04	-0.05	0.15	0.39	0.33	0.33	0.41	0.42	0.37	0.40	0.10	0.16	0.06
109	X35 %D	-0.07	-0.02	-0.20	-0.13	0.14	-0.02	-0.07	0.14	0.25	0.27	0.25	0.30	0.32	0.26	0.31	0.11	0.32	0.03
110	Post res_freq	0.16	0.20	0.27	0.42	-0.28	0.01	0.18	-0.05	-0.49	-0.65	-0.55	-0.65	-0.69	-0.66	-0.69	-0.05	-0.37	0.02
111	Res freq %D	0.34	0.18	0.25	0.40	-0.14	0.06	0.14	0.00	-0.07	-0.20	-0.07	-0.16	-0.18	-0.15	-0.16	0.02	-0.09	0.06
112	Post AX	0.09	0.16	0.22	0.30	-0.47	-0.07	0.21	-0.12	-0.50	-0.53	-0.57	-0.60	-0.62	-0.59	-0.62	-0.14	-0.48	-0.03
113	AX %D	0.34	0.16	0.19	0.45	-0.14	0.03	0.08	0.11	0.16	0.01	0.12	0.07	0.04	0.03	0.05	0.10	-0.02	0.13

Table A12.1: continued form above

	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM
1	FVC %D	Post FEV1	FEV1%D	Post FEV1/FVC	Post PEFR	PEFR %D	Post FEF 25	FEF 25 %D	Post FEF 50	FEF 50 %D	Post FEF 75	FEF 75 %D	Post FEF 25-75	FEF 25-75 %D	Post FVCin	FVCin %D	Post FIF 50	FIF 50 %D	ACQ score	FENO avg
2	0.12	0.02	-0.05	-0.09	-0.02	-0.11	-0.07	-0.10	-0.08	-0.10	-0.13	-0.13	-0.09	-0.10	0.06	0.09	-0.03	-0.26	0.00	-0.22
3	0.07	0.03	0.04	-0.19	-0.08	-0.01	-0.14	-0.05	-0.14	-0.03	-0.20	-0.13	-0.17	-0.07	0.23	0.09	-0.06	-0.35	0.00	-0.20
4	0.17	-0.26	0.13	-0.36	-0.25	0.08	-0.31	0.09	-0.34	0.01	-0.47	-0.19	-0.37	0.04	0.20	0.20	-0.16	-0.15	0.01	-0.24
5	0.10	-0.03	-0.02	-0.46	-0.20	-0.07	-0.31	-0.05	-0.39	-0.08	-0.46	-0.29	-0.40	-0.16	0.37	0.23	-0.27	-0.15	-0.06	0.04
6	-0.12	0.09	-0.04	0.02	0.14	-0.19	0.11	-0.02	0.15	0.05	0.13	0.11	0.18	0.12	0.07	-0.29	0.51	-0.12	-0.21	0.07
7	0.08	-0.12	0.06	-0.15	-0.03	0.03	-0.15	0.03	-0.12	-0.04	-0.26	-0.07	-0.15	0.01	0.11	0.11	0.00	-0.08	-0.10	-0.25
8	0.18	-0.18	0.11	-0.15	-0.09	0.16	-0.22	0.09	-0.21	-0.08	-0.35	-0.18	-0.26	-0.06	0.07	0.34	-0.26	0.01	0.04	-0.30
9	-0.10	0.54	-0.13	-0.15	0.36	-0.22	0.15	-0.04	0.13	0.03	0.04	-0.10	0.10	-0.03	0.90	-0.18	0.29	-0.27	-0.27	0.06
10	-0.39	0.92	-0.53	0.58	0.78	-0.41	0.76	-0.32	0.76	-0.18	0.68	0.02	0.74	-0.19	0.37	-0.42	0.30	-0.18	-0.54	0.10
11	-0.37	0.66	-0.51	0.92	0.68	-0.28	0.83	-0.32	0.89	-0.23	0.83	0.12	0.86	-0.16	-0.24	-0.40	0.20	0.01	-0.41	0.01
12	-0.27	0.82	-0.45	0.59	0.93	-0.43	0.79	-0.28	0.76	-0.11	0.65	0.09	0.73	-0.09	0.28	-0.38	0.44	-0.14	-0.52	0.04
13	-0.43	0.80	-0.53	0.82	0.81	-0.38	0.90	-0.36	0.92	-0.16	0.83	0.15	0.89	-0.12	0.02	-0.47	0.27	-0.09	-0.51	0.01
14	-0.43	0.77	-0.53	0.85	0.74	-0.37	0.88	-0.34	0.93	-0.22	0.86	0.13	0.91	-0.17	-0.04	-0.49	0.31	-0.06	-0.50	0.02
15	-0.35	0.73	-0.44	0.79	0.64	-0.28	0.79	-0.27	0.86	-0.18	0.88	0.03	0.87	-0.18	-0.06	-0.46	0.33	-0.04	-0.45	0.10
16	-0.40	0.77	-0.51	0.83	0.72	-0.37	0.86	-0.33	0.92	-0.21	0.88	0.12	0.91	-0.19	-0.04	-0.49	0.31	-0.05	-0.49	0.05
17	-0.03	0.49	-0.11	-0.17	0.34	-0.22	0.11	-0.04	0.10	0.02	0.02	-0.12	0.07	-0.04	0.92	-0.21	0.36	-0.25	-0.24	0.01
18	-0.03	0.32	-0.19	0.10	0.35	-0.38	0.21	-0.19	0.26	-0.10	0.27	-0.04	0.27	-0.06	0.30	-0.38	0.92	-0.38	-0.26	-0.09
19	0.20	0.43	0.02	-0.30	0.27	-0.15	0.01	-0.01	-0.01	0.02	-0.09	-0.19	-0.04	-0.07	0.95	0.03	0.32	-0.23	-0.18	0.05
20		-0.26	0.56	-0.41	-0.25	0.28	-0.38	0.23	-0.38	0.14	-0.37	-0.18	-0.38	-0.01	0.20	0.66	0.06	0.24	0.20	0.01
21	-0.26		-0.25	0.64	0.83	-0.22	0.83	-0.08	0.83	0.08	0.74	0.21	0.80	0.05	0.38	-0.30	0.32	-0.09	-0.53	0.14
22	0.56	-0.25		-0.24	-0.27	0.72	-0.27	0.79	-0.28	0.75	-0.21	0.37	-0.25	0.62	0.05	0.48	-0.08	0.35	0.35	0.05
23	-0.41	0.64	-0.24		0.67	-0.04	0.88	-0.03	0.92	0.08	0.87	0.37	0.91	0.15	-0.33	-0.36	0.13	0.09	-0.34	0.02
24	-0.25	0.83	-0.27	0.67		-0.16	0.84	-0.11	0.79	0.06	0.69	0.25	0.76	0.09	0.22	-0.33	0.34	-0.07	-0.48	0.04
25	0.28	-0.22	0.72	-0.04	-0.16		-0.16	0.62	-0.17	0.54	-0.12	0.28	-0.16	0.45	-0.11	0.35	-0.28	0.33	0.37	0.03
26	-0.38	0.83	-0.27	0.88	0.84	-0.16		-0.05	0.94	0.10	0.86	0.36	0.92	0.14	-0.04	-0.41	0.24	0.02	-0.46	0.05
27	0.23	-0.08	0.79	-0.03	-0.11	0.62	-0.05		-0.03	0.68	-0.02	0.44	-0.06	0.66	-0.01	0.24	-0.10	0.35	0.27	0.16
28	-0.38	0.83	-0.28	0.92	0.79	-0.17	0.94	-0.09		0.11	0.93	0.38	0.99	0.14	-0.05	-0.43	0.29	0.01	-0.49	0.05
29	0.14	0.08	0.75	0.08	0.06	0.54	0.10	0.68	0.11		0.14	0.68	0.13	0.91	0.04	0.09	-0.04	0.19	0.06	0.00
30	-0.37	0.74	-0.21	0.87	0.69	-0.12	0.86	-0.02	0.93	0.14		0.46	0.97	0.19	-0.14	-0.49	0.30	0.03	-0.43	0.07
31	-0.18	0.21	0.37	0.37	0.25	0.28	0.36	0.44	0.38	0.68	0.46		0.43	0.76	-0.17	-0.22	-0.02	0.13	-0.07	-0.07
32	-0.38	0.80	-0.25	0.91	0.76	-0.16	0.92	-0.06	0.93	0.13	0.97	0.43		0.17	-0.08	-0.47	0.30	0.00	-0.50	0.04
33	-0.01	0.05	0.62	0.15	0.09	0.45	0.14	0.66	0.14	0.91	0.19	0.76	0.17		-0.05	0.01	-0.03	0.13	0.01	-0.07
34	0.20	0.38	0.05	-0.33	0.22	-0.11	-0.04	-0.01	-0.05	0.04	-0.14	-0.17	-0.08	-0.05		0.07	0.26	-0.24	-0.19	0.01
35	0.66	-0.30	0.48	-0.36	-0.33	0.35	-0.41	0.24	-0.43	0.09	-0.49	-0.22	-0.47	0.01	0.07		-0.30	0.30	0.28	0.08
36	0.06	0.32	-0.08	0.13	0.34	-0.28	0.24	-0.10	0.29	-0.04	0.30	-0.02	0.30	-0.03	0.26	-0.30		-0.02	-0.27	-0.07
37	0.24	-0.09	0.35	0.09	-0.07	0.33	0.02	0.35	0.01	0.19	0.03	0.13	0.00	0.13	-0.24	0.30	-0.02		0.14	0.08
38	0.20	-0.53	0.35	-0.34	-0.48	0.37	-0.46	0.27	-0.49	0.06	-0.43	-0.07	-0.50	0.01	-0.19	0.28	-0.27	0.14		0.09
39	0.01	0.14	0.05	0.02	0.04	0.03	0.05	0.16	0.05	0.00	0.07	-0.07	0.04	-0.07	0.01	0.08	-0.07	0.08	0.09	
40	-0.17	0.17	-0.35	0.12	0.17	-0.34	0.18	-0.25	0.19	-0.13	0.12	-0.08	0.18	-0.11	-0.02	-0.36	0.21	-0.19	-0.65	-0.24
41	0.31	-0.33	0.22	-0.43	-0.31	0.21	-0.40	0.04	-0.42	0.05	-0.41	-0.19	-0.42	-0.05	0.26	0.25	-0.16	-0.05	0.35	-0.09
42	0.09	-0.12	0.08	-0.23	-0.14	0.01	-0.20	0.00	-0.22	0.02	-0.18	-0.07	-0.20	0.01	0.10	0.08	-0.02	-0.07	0.08	0.03
43	0.06	-0.01	-0.07	-0.11	-0.06	0.00	-0.11	-0.01	-0.12	-0.07	-0.12	-0.03	-0.13	-0.09	0.08	0.17	-0.19	0.14	0.11	0.26
44	0.27	-0.27	0.00	-0.38	-0.21	0.06	-0.35	-0.05	-0.42	-0.21	-0.39	-0.25	-0.43	-0.20	0.12	0.30	-0.18	0.07	0.21	0.03
45	0.16	-0.18	-0.09	-0.41	-0.22	-0.12	-0.30	-0.14	-0.34	-0.11	-0.27	-0.23	-0.33	-0.11	0.18	0.07	-0.05	-0.06	0.08	0.13
46	0.04	0.01	0.09	-0.12	-0.03	-0.02	-0.05	0.02	-0.14	0.08	-0.08	-0.04	-0.14	0.01	0.07	0.07	-0.04	0.01	0.13	0.31
47	-0.09	-0.06	-0.10	0.00	-0.07	-0.05	-0.09	-0.04	-0.08	-0.02	-0.10	-0.06	-0.11	-0.05	-0.07	0.02	-0.21	-0.13	0.06	0.18
48	0.23	-0.12	0.07	-0.28	-0.13	0.08	-0.23	-0.16	-0.26	-0.04	-0.25	-0.24	-0.25	-0.10	0.18	0.23	0.09	0.06	-0.07	-0.04
49	0.13	-0.10	-0.02	-0.21	-0.03	0.04	-0.18	-0.15	-0.19	-0.06	-0.23	-0.16	-0.21	-0.12	0.12	0.24	-0.18	0.13	0.12	0.02
50	0.21	-0.16	0.17	-0.24	-0.24	0.03	-0.23	0.06	-0.25	-0.01	-0.16	-0.22	-0.23	-0.11	0.07	0.12	0.02	0.16	0.34	-0.05
51	0.00	-0.05	0.04	-0.05	-0.09	0.08	-0.06	-0.09	-0.03	0.05	0.06	0.05	0.01	0.01	0.05	-0.08	0.09	0.05	0.15	-0.02
52	0.17	-0.09	0.07	-0.27	-0.12	-0.02	-0.18	0.03	-0.29	-0.03	-0.27	-0.23	-0.30	-0.10	0.11	0.17	-0.16	-0.03	0.20	0.18
53	-0.13	0.00	-0.14	-0.02	0.14	-0.20	0.02	-0.12	0.02	-0.06	0.04	-0.03	0.02	0.00	0.10	-0.18	0.20	-0.06	0.02	-0.35

Table A12.1: continued from above

	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM
1	FVC %D	Post FEV1	FEV1%D	Post FEV1/FVC	Post PEFR	PEFR %D	Post FEF 25	FEF 25 %D	Post FEF 50	FEF 50 %D	Post FEF 75	FEF 75 %D	Post FEF 25-75	FEF 25-75 %D	Post FVCin	FVCin %D	Post FIF 50	FIF 50 %D	ACQ score	FENO avg
54	0.18	-0.21	0.15	-0.27	-0.19	0.19	-0.26	0.14	-0.33	0.08	-0.30	-0.07	-0.33	0.11	0.00	0.29	-0.25	0.11	0.21	0.09
55	0.05	-0.08	0.11	-0.16	-0.13	0.20	-0.12	0.10	-0.22	0.09	-0.19	0.01	-0.21	0.10	0.00	0.27	-0.37	0.12	0.10	0.08
56	0.00	-0.18	0.07	-0.12	-0.18	-0.05	-0.16	-0.02	-0.15	0.02	-0.11	0.03	-0.15	0.12	-0.01	-0.05	0.00	-0.02	0.11	-0.10
57	0.02	-0.18	0.06	-0.14	-0.14	-0.07	-0.19	-0.04	-0.18	-0.03	-0.11	0.03	-0.17	0.07	0.02	0.02	-0.13	-0.08	0.08	-0.21
58	0.04	0.04	-0.13	0.13	0.12	-0.14	0.09	-0.30	0.16	-0.10	0.15	-0.04	0.17	-0.11	0.01	-0.02	0.23	0.00	-0.17	-0.06
59	0.12	-0.17	0.06	-0.16	-0.31	0.06	-0.22	0.01	-0.16	-0.07	-0.11	-0.10	-0.13	-0.11	0.08	0.10	-0.05	-0.04	-0.15	0.07
60	0.16	-0.07	0.03	-0.22	-0.07	0.08	-0.22	-0.09	-0.19	0.01	-0.10	-0.20	-0.16	-0.11	0.28	0.14	-0.04	0.04	0.18	-0.26
61	0.01	-0.20	0.01	-0.15	-0.21	0.08	-0.22	-0.12	-0.18	-0.05	-0.08	-0.14	-0.15	-0.07	0.08	-0.04	0.06	0.11	0.16	-0.01
62	0.10	0.19	0.11	0.10	0.17	0.04	0.09	0.14	0.17	0.21	0.13	0.20	0.16	0.23	0.04	0.13	-0.08	0.07	-0.33	0.22
63	0.32	-0.19	0.28	-0.33	-0.23	0.24	-0.32	0.10	-0.29	0.14	-0.15	0.00	-0.23	0.07	0.23	0.17	-0.07	0.12	0.20	-0.13
64	0.10	-0.20	0.10	-0.19	-0.16	0.06	-0.19	0.03	-0.22	0.05	-0.26	-0.05	-0.23	0.08	0.00	0.09	-0.07	-0.14	0.07	0.14
65	0.01	0.00	0.00	-0.05	-0.05	0.03	-0.07	0.06	-0.06	-0.06	0.04	-0.02	-0.03	-0.11	-0.02	0.13	-0.12	0.26	0.13	0.05
66	0.16	-0.26	-0.02	-0.29	-0.24	0.04	-0.30	-0.07	-0.36	-0.18	-0.38	-0.24	-0.40	-0.14	0.07	0.25	-0.23	0.06	0.23	-0.06
67	0.14	-0.20	-0.06	-0.33	-0.21	-0.09	-0.25	-0.12	-0.29	-0.10	-0.29	-0.20	-0.31	-0.07	0.12	0.05	-0.06	-0.09	0.09	0.14
68	0.00	-0.05	0.09	-0.11	-0.05	-0.04	-0.06	0.06	-0.16	0.11	-0.15	0.01	-0.18	0.08	-0.02	0.02	-0.08	-0.03	0.13	0.37
69	-0.09	-0.21	-0.05	-0.04	-0.11	0.07	-0.10	-0.04	-0.11	-0.03	-0.18	-0.01	-0.15	0.03	-0.08	-0.13	-0.04	-0.17	0.11	0.11
70	0.30	-0.09	0.14	-0.22	-0.13	0.09	-0.21	-0.05	-0.21	-0.01	-0.12	-0.19	-0.17	-0.01	0.13	0.23	0.05	0.14	-0.06	0.00
71	0.07	0.02	-0.01	-0.07	0.02	0.02	-0.06	-0.14	-0.05	-0.03	0.01	-0.05	-0.04	-0.09	0.12	0.09	-0.20	0.17	0.05	0.07
72	0.24	-0.12	0.21	-0.17	-0.18	0.10	-0.16	0.09	-0.20	0.06	-0.14	-0.16	-0.18	-0.05	0.02	0.17	0.02	0.19	0.33	-0.05
73	0.01	0.03	-0.03	0.08	0.09	-0.04	0.12	-0.12	0.10	0.03	0.02	0.12	0.07	0.10	0.06	-0.06	0.12	-0.09	-0.11	-0.02
74	0.14	-0.12	0.06	-0.28	-0.13	-0.02	-0.19	0.01	-0.30	-0.01	-0.32	-0.22	-0.33	-0.05	0.10	0.14	-0.15	-0.10	0.20	0.18
75	-0.08	0.01	-0.08	-0.05	0.08	-0.16	-0.03	-0.08	-0.02	-0.03	0.05	-0.01	0.00	0.01	0.13	-0.11	0.12	0.04	0.03	-0.24
76	0.21	-0.22	0.17	-0.29	-0.19	0.17	-0.27	0.15	-0.33	0.08	-0.32	-0.05	-0.34	0.12	0.01	0.30	-0.25	0.10	0.19	0.12
77	0.07	-0.10	0.13	-0.17	-0.14	0.20	-0.13	0.11	-0.23	0.10	-0.20	0.02	-0.23	0.11	0.00	0.29	-0.37	0.11	0.11	0.09
78	0.07	-0.15	0.06	-0.05	-0.14	-0.07	-0.11	-0.04	-0.10	0.04	-0.12	0.05	-0.12	0.16	-0.05	-0.01	-0.02	-0.08	0.04	-0.07
79	0.05	-0.14	0.05	-0.11	-0.10	-0.10	-0.17	-0.03	-0.14	-0.03	-0.07	0.11	-0.13	0.07	0.02	0.00	-0.11	-0.04	0.04	-0.21
80	-0.03	0.06	-0.02	-0.04	-0.01	-0.01	-0.03	0.01	-0.01	-0.03	0.12	0.02	0.04	-0.08	0.05	0.02	-0.05	0.13	0.01	-0.04
81	0.12	0.16	0.05	-0.16	-0.02	-0.14	-0.01	-0.02	-0.02	0.06	-0.03	-0.04	0.00	0.01	0.45	0.01	0.31	-0.07	-0.17	0.17
82	0.21	-0.51	0.42	-0.32	-0.55	0.40	-0.43	0.46	-0.45	0.15	-0.36	-0.02	-0.44	0.19	-0.14	0.24	-0.19	0.18	0.40	0.02
83	0.08	-0.41	0.28	-0.09	-0.37	0.27	-0.21	0.31	-0.21	0.03	-0.12	0.03	-0.20	0.17	-0.28	0.03	-0.05	0.12	0.27	-0.13
84	0.33	-0.35	0.47	-0.46	-0.37	0.39	-0.42	0.47	-0.49	0.27	-0.50	-0.09	-0.50	0.20	0.20	0.43	-0.22	0.21	0.36	0.13
85	0.05	-0.35	0.22	0.05	-0.27	0.28	-0.10	0.29	-0.10	0.07	-0.01	0.12	-0.09	0.16	-0.34	0.01	-0.08	0.14	0.27	-0.15
86	0.22	-0.52	0.42	-0.35	-0.56	0.40	-0.45	0.47	-0.47	0.14	-0.40	-0.05	-0.47	0.17	-0.13	0.27	-0.21	0.19	0.41	0.05
87	0.01	-0.02	0.07	-0.17	-0.14	-0.02	-0.18	0.19	-0.18	0.01	-0.21	-0.10	-0.19	0.08	0.14	0.35	-0.36	-0.04	0.20	0.33
88	-0.32	0.39	-0.58	0.44	0.46	-0.38	0.45	-0.53	0.50	-0.35	0.51	0.01	0.51	-0.30	-0.15	-0.46	0.24	-0.16	-0.35	0.07
89	-0.20	0.39	-0.50	0.27	0.39	-0.43	0.37	-0.39	0.39	-0.31	0.33	-0.13	0.38	-0.31	0.02	-0.29	0.16	-0.19	-0.48	0.08
90	0.24	-0.50	0.48	-0.46	-0.52	0.37	-0.51	0.50	-0.56	0.25	-0.56	-0.02	-0.59	0.22	0.06	0.40	-0.33	0.23	0.43	0.06
91	0.18	-0.46	0.51	-0.45	-0.53	0.36	-0.49	0.47	-0.54	0.30	-0.51	0.07	-0.54	0.29	0.04	0.34	-0.26	0.19	0.43	-0.04
92	-0.02	0.29	-0.08	-0.05	0.10	-0.24	0.12	-0.05	0.10	-0.02	0.06	-0.10	0.10	-0.04	0.44	-0.11	0.36	-0.06	-0.26	0.11
93	-0.22	0.27	-0.20	0.17	0.21	-0.12	0.24	-0.06	0.22	-0.10	0.18	-0.08	0.19	-0.08	0.01	-0.20	-0.03	-0.04	-0.12	0.01
94	0.23	-0.59	0.25	-0.49	-0.64	0.22	-0.55	0.26	-0.60	-0.07	-0.53	-0.15	-0.60	-0.04	-0.11	0.31	-0.32	0.08	0.37	-0.02
95	-0.03	0.07	-0.33	-0.14	0.02	-0.42	-0.04	-0.37	-0.08	-0.33	-0.15	-0.21	-0.09	-0.34	0.15	0.07	-0.11	-0.14	-0.13	-0.05
96	0.14	-0.45	0.27	-0.23	-0.45	0.24	-0.32	0.25	-0.33	0.04	-0.23	0.07	-0.31	0.12	-0.17	0.14	-0.20	0.08	0.30	-0.13
97	0.11	-0.03	0.02	-0.27	-0.08	-0.11	-0.15	-0.11	-0.18	0.01	-0.20	0.04	-0.17	-0.02	0.29	0.12	-0.15	-0.08	0.03	-0.06
98	0.20	-0.26	0.08	-0.41	-0.31	0.04	-0.37	0.14	-0.45	-0.12	-0.52	-0.38	-0.43	-0.19	0.11	0.34	-0.30	0.04	0.22	0.14
99	-0.02	-0.03	-0.37	-0.08	-0.08	-0.35	-0.10	-0.33	-0.10	-0.43	-0.18	-0.43	-0.14	-0.50	-0.07	0.05	-0.08	-0.10	-0.06	0.06
100	0.09	-0.37	0.21	-0.05	-0.31	0.21	-0.15	0.22	-0.16	0.04	-0.08	0.11	-0.15	0.12	-0.24	0.07	-0.12	0.12	0.29	-0.09
101	0.00	0.02	-0.02	-0.07	0.00	-0.14	0.02	-0.12	-0.02	0.03	-0.05	0.09	-0.02	0.05	0.15	0.07	-0.05	-0.06	0.00	-0.01
102	0.21	-0.60	0.24	-0.48	-0.64	0.21	-0.54	0.26	-0.59	-0.06	-0.54	-0.16	-0.60	-0.03	-0.13	0.30	-0.34	0.04	0.37	0.00
103	-0.08	0.14	-0.34	-0.07	0.10	-0.43	0.05	-0.39	0.01	-0.26	-0.06	-0.13	0.00	-0.27	0.16	-0.01	-0.07	-0.23	-0.20	-0.07
104	-0.07	0.06	-0.04	-0.08	-0.05	-0.12	-0.10	0.11	-0.09	-0.03	-0.14	-0.09	-0.11	0.05	0.09	0.25	-0.34	-0.13	0.08	0.29
105	-0.12	0.14	-0.18	0.06	0.18	-0.21	0.14	-0.22	0.11	-0.06	0.07	-0.01	0.12	-0.08	0.01	-0.14	0.17	-0.29	-0.27	-0.24
106	-0.23	0.49	-0.29	0.55	0.53	-0.13	0.58	-0.18	0.61	-0.07	0.62	0.20	0.61	-0.02	-0.08	-0.40	0.24	0.08	-0.32	0.14
107	0.04	0.06	-0.24	0.04	0.01	-0.21	0.05	-0.23	0.06	-0.26	0.03	-0.14	0.06	-0.21	0.02	-0.15	0.20	-0.11	-0.14	0.00
108	-0.19	0.37	-0.23	0.31	0.33	-0.13	0.41	-0.08	0.40	-0.01	0.34	0.07	0.39	-0.02	0.03	-0.31	0.16	-0.09	-0.44	0.12
109	-0.12	0.34	0.02	0.35	0.32	0.04	0.39	0.04	0.40	0.19	0.35	0.28	0.39	0.12	0.03	-0.26	0.32	-0.10	-0.29	-0.16
110	0.17	-0.51	0.22	-0.62	-0.57	0.13	-0.62	0.14	-0.69	-0.01	-0.69	-0.22	-0.70	-0.06	0.07	0.43	-0.41	0.01	0.38	-0.06
111	0.09	-0.16	-0.21	-0.32	-0.15	-0.23	-0.29	-0.29	-0.28	-0.30	-0.29	-0.32	-0.27	-0.35	0.06	0.17	-0.17	-0.16	0.03	-0.04
112	0.26	-0.51	0.25	-0.49	-0.55	0.25	-0.55	0.20	-0.61	0.00	-0.62	-0.20	-0.64	-0.06	-0.01	0.49	-0.51	0.01	0.34	0.03
113	-0.02	0.07	-0.35	-0.13	0.04	-0.34	-0.03	-0.38	-0.08	-0.35	-0.13	-0.32	-0.09	-0.38	0.11	0.04	-0.16	-0.30	-0.14	0.02

Table A12.1: continued from above

	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF
1	FENO avg	ACT tot	IL-2 (FU)	IL-4 (FU)	IL-5 (FU)	IL-6 (FU)	IL-8 (FU)	IL-12 (FU)	IL-13 (FU)	Eotaxin (FU)	IFN I (FU)	RANTES (FU)	TNF a (FU)	VEGF (FU)	IL-1b (FU)	CRP (FU)	SAAP (FU)	TGF b1 (FU)	TGF b2 (FU)	TGF b3 (FU)
2	-0.22	0.02	0.09	-0.06	-0.15	-0.05	0.00	-0.17	0.00	0.15	-0.05	0.02	-0.02	0.01	-0.08	-0.03	0.05	-0.05	0.12	-0.17
3	-0.20	0.00	0.10	-0.10	-0.20	-0.06	-0.09	-0.13	0.05	0.06	0.04	-0.15	-0.10	-0.07	-0.12	-0.11	-0.04	-0.09	0.08	-0.18
4	-0.24	0.00	0.21	-0.06	0.01	0.10	0.04	-0.25	-0.02	0.11	0.12	-0.16	-0.18	-0.11	0.09	0.21	0.09	0.06	0.15	-0.26
5	0.04	0.03	0.21	0.04	0.19	0.16	0.38	0.10	0.00	0.32	0.13	0.01	-0.09	0.23	0.02	0.09	0.12	-0.13	-0.05	-0.24
6	0.07	0.14	-0.11	0.13	-0.11	-0.15	-0.03	-0.08	-0.20	0.11	-0.07	-0.18	0.03	-0.22	0.16	-0.17	-0.29	-0.04	-0.17	0.05
7	-0.25	0.00	0.13	0.09	-0.09	0.08	-0.06	-0.36	-0.08	0.19	0.24	-0.19	-0.09	-0.27	0.23	0.06	-0.06	-0.07	0.07	-0.16
8	-0.30	-0.08	0.19	0.01	-0.05	0.14	-0.03	-0.35	-0.01	0.13	0.25	-0.14	-0.09	-0.18	0.18	0.13	0.06	-0.08	0.19	-0.19
9	0.06	0.15	0.08	0.05	0.06	-0.05	0.10	0.03	-0.05	0.00	0.04	-0.04	0.02	0.02	0.11	-0.04	-0.02	-0.05	-0.04	-0.07
10	0.10	0.23	-0.30	-0.15	-0.01	-0.19	-0.12	-0.03	0.03	-0.13	-0.04	-0.12	-0.02	-0.10	0.07	-0.20	-0.12	-0.19	-0.15	0.07
11	0.01	0.20	-0.41	-0.22	-0.05	-0.25	-0.31	-0.13	0.01	-0.24	-0.12	-0.18	-0.01	-0.23	0.06	-0.28	-0.20	-0.14	-0.14	0.20
12	0.04	0.23	-0.30	-0.16	-0.04	-0.19	-0.15	-0.03	-0.03	-0.09	-0.02	-0.15	-0.06	-0.10	0.20	-0.22	-0.20	-0.19	-0.14	0.15
13	0.01	0.23	-0.38	-0.21	-0.08	-0.30	-0.22	-0.10	-0.02	-0.19	-0.12	-0.17	0.00	-0.22	0.09	-0.30	-0.20	-0.15	-0.14	0.21
14	0.02	0.22	-0.39	-0.22	-0.11	-0.32	-0.27	-0.16	-0.05	-0.23	-0.15	-0.19	0.00	-0.27	0.09	-0.35	-0.26	-0.17	-0.17	0.21
15	0.10	0.19	-0.37	-0.20	-0.13	-0.35	-0.19	-0.11	-0.07	-0.17	-0.18	-0.09	0.07	-0.21	0.06	-0.33	-0.25	-0.16	-0.17	0.17
16	0.05	0.21	-0.39	-0.21	-0.10	-0.32	-0.24	-0.14	-0.06	-0.23	-0.15	-0.16	0.02	-0.27	0.09	-0.34	-0.25	-0.19	-0.17	0.18
17	0.01	0.12	0.12	0.08	0.04	0.01	0.09	0.01	-0.07	0.03	0.06	0.02	0.06	0.01	0.16	-0.03	-0.04	-0.03	-0.01	-0.02
18	-0.09	0.22	-0.13	0.03	-0.21	-0.18	-0.02	-0.04	-0.13	0.05	-0.22	-0.02	0.06	-0.13	0.23	-0.27	-0.38	0.02	-0.05	0.17
19	0.05	0.04	0.23	0.06	0.04	0.07	0.15	0.03	-0.07	0.09	0.12	0.04	0.06	0.07	0.14	0.02	0.00	-0.09	-0.01	-0.02
20	0.01	-0.17	0.31	0.09	0.06	0.27	0.16	0.04	-0.09	0.23	0.13	0.21	0.00	0.17	-0.13	0.18	0.05	0.00	0.02	0.04
21	0.14	0.17	-0.33	-0.12	-0.01	-0.27	-0.18	0.01	-0.06	-0.12	-0.10	-0.16	-0.05	-0.09	0.00	-0.21	-0.08	-0.18	-0.18	0.04
22	0.05	-0.35	0.22	0.08	-0.07	0.00	-0.09	0.09	-0.10	0.07	-0.02	0.17	0.04	0.07	-0.14	0.15	0.11	0.07	0.06	-0.13
23	0.02	0.12	-0.43	-0.23	-0.11	-0.38	-0.41	-0.12	0.00	-0.28	-0.21	-0.24	-0.05	-0.27	-0.02	-0.27	-0.16	-0.12	-0.14	0.13
24	0.04	0.17	-0.31	-0.14	-0.06	-0.21	-0.22	-0.03	-0.07	-0.13	-0.03	-0.24	-0.09	-0.12	0.14	-0.19	-0.13	-0.18	-0.14	0.12
25	0.03	-0.34	0.21	0.01	0.00	0.06	-0.12	-0.02	-0.05	0.08	0.04	0.03	0.08	-0.02	-0.20	0.19	0.20	-0.05	-0.07	-0.14
26	0.05	0.18	-0.40	-0.20	-0.11	-0.35	-0.30	-0.05	-0.09	-0.23	-0.18	-0.23	-0.06	-0.18	0.02	-0.26	-0.12	-0.16	-0.19	0.09
27	0.16	-0.25	0.04	0.00	-0.01	-0.05	-0.14	0.02	-0.04	-0.16	-0.15	0.06	-0.09	0.03	-0.12	0.14	0.10	-0.02	-0.04	-0.30
28	0.05	0.19	-0.42	-0.22	-0.12	-0.42	-0.34	-0.14	-0.08	-0.26	-0.19	-0.25	-0.03	-0.29	0.02	-0.33	-0.22	-0.15	-0.18	0.16
29	0.00	-0.13	0.05	0.02	-0.07	-0.21	-0.11	0.08	-0.02	-0.04	-0.06	-0.01	0.05	-0.03	-0.06	0.08	0.09	0.02	-0.03	-0.10
30	0.07	0.12	-0.41	-0.18	-0.12	-0.39	-0.27	-0.08	-0.10	-0.25	-0.23	-0.16	0.06	-0.27	0.04	-0.30	-0.19	-0.11	-0.11	0.15
31	-0.07	-0.08	-0.19	-0.07	-0.03	-0.25	-0.23	-0.04	-0.06	-0.24	-0.16	-0.22	0.05	-0.23	-0.03	-0.07	0.01	0.03	0.09	-0.04
32	0.04	0.18	-0.42	-0.20	-0.13	-0.43	-0.33	-0.14	-0.11	-0.25	-0.21	-0.23	0.01	-0.30	0.02	-0.33	-0.21	-0.15	-0.17	0.17
33	-0.07	-0.11	-0.05	0.01	-0.09	-0.20	-0.11	0.01	-0.05	-0.10	-0.12	-0.11	0.01	-0.10	0.00	0.11	0.10	0.12	0.07	-0.11
34	0.01	-0.02	0.26	0.10	0.08	0.12	0.18	0.07	-0.07	0.18	0.12	0.07	0.05	0.11	0.10	0.00	0.00	-0.01	0.02	0.01
35	0.08	-0.36	0.25	0.08	0.17	0.30	0.07	0.07	0.02	0.23	0.24	0.12	-0.08	0.17	-0.18	0.29	0.27	-0.05	0.02	-0.02
36	-0.07	0.21	-0.16	-0.02	-0.19	-0.18	-0.05	-0.04	-0.21	0.09	-0.18	0.02	0.09	-0.16	0.20	-0.25	-0.37	0.00	-0.13	0.23
37	0.08	-0.19	-0.05	-0.07	0.14	0.07	-0.06	0.01	-0.13	0.06	0.13	0.16	0.05	-0.03	-0.06	0.11	0.12	-0.02	-0.08	0.00
38	0.09	-0.65	0.35	0.08	0.11	0.21	0.08	0.13	0.06	-0.07	0.12	0.34	0.15	0.20	0.02	0.21	0.10	0.11	0.08	-0.17
39		-0.24	-0.09	0.03	0.26	0.03	0.13	0.31	0.18	-0.04	0.02	-0.05	-0.02	0.18	-0.35	0.09	0.08	-0.10	-0.21	-0.06
40	-0.24		-0.33	-0.06	-0.14	-0.27	-0.12	-0.10	-0.06	-0.06	-0.12	-0.32	-0.20	-0.18	-0.01	-0.15	-0.17	-0.10	-0.12	0.08
41	-0.09	-0.33		0.27	-0.11	0.46	0.27	0.16	0.15	0.21	0.32	0.33	0.17	0.27	0.12	0.01	0.05	0.02	0.17	0.04
42	0.03	-0.06	0.27		0.14	0.31	0.16	0.35	-0.01	0.30	0.25	0.24	0.16	0.29	0.15	0.11	0.23	0.23	0.17	-0.03
43	0.26	-0.14	-0.11	0.14		0.21	0.15	0.37	0.18	0.21	0.15	0.09	0.02	0.25	-0.07	0.37	0.23	0.23	0.05	0.00
44	0.03	-0.27	0.46	0.31	0.21		0.16	0.32	0.22	0.25	0.52	0.32	-0.10	0.40	0.18	0.48	0.37	0.15	0.19	-0.03
45	0.13	-0.12	0.27	0.16	0.15	0.16		0.14	-0.06	0.35	-0.04	0.25	0.09	0.26	0.12	0.04	0.00	0.08	-0.01	-0.08
46	0.31	-0.10	0.16	0.35	0.37	0.32	0.14		0.52	0.16	0.24	0.34	0.05	0.72	0.04	0.20	0.25	0.34	0.23	0.21
47	0.18	-0.06	0.15	-0.01	0.18	0.22	-0.06	0.52		0.01	0.19	0.16	0.06	0.40	-0.03	0.34	0.33	0.09	0.27	0.22
48	-0.04	-0.06	0.21	0.30	0.21	0.25	0.35	0.16	0.01		0.31	0.18	0.22	0.16	0.07	0.11	0.09	0.05	0.03	0.13
49	0.02	-0.12	0.32	0.25	0.15	0.52	-0.04	0.24	0.19	0.31		0.21	0.09	0.31	0.20	0.33	0.28	0.06	0.14	0.08
50	-0.05	-0.32	0.33	0.24	0.09	0.32	0.25	0.34	0.16	0.18	0.21		0.29	0.59	0.07	0.19	0.22	0.33	0.21	0.07
51	-0.02	-0.20	0.17	0.16	0.02	-0.10	0.09	0.05	0.06	0.22	0.09	0.29		0.05	0.21	-0.07	0.08	0.08	0.19	0.20
52	0.18	-0.18	0.27	0.29	0.25	0.40	0.26	0.72	0.40	0.16	0.31	0.53	0.05		-0.03	0.23	0.26	0.32	0.19	0.13
53	-0.35	-0.01	0.12	0.15	-0.07	0.18	0.12	0.04	-0.03	0.07	0.20	0.07	0.21	-0.03		0.03	-0.07	0.01	0.18	0.17
54	0.09	-0.15	0.01	0.11	0.37	0.48	0.04	0.20	0.34	0.11	0.33	0.19	-0.07	0.23	0.03		0.82	0.16	0.13	0.01

Table A12.1: continued from above

	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX	AY	AZ	BA	BB	BC	BD	BE	BF
1	FENO avg	ACT tot	IL-2 (FU)	IL-4 (FU)	IL-5 (FU)	IL-6 (FU)	IL-8 (FU)	IL-12 (FU)	IL-13 (FU)	Eotaxin (FU)	IFN I (FU)	RANTES (FU)	TNF a (FU)	VEGF (FU)	IL-1b (FU)	CRP (FU)	SAAP (FU)	TGF b1 (FU)	TGF b2 (FU)	TGF b3 (FU)
53	-0.35	-0.01	0.12	0.15	-0.07	0.18	0.12	0.04	-0.03	0.07	0.20	0.07	0.21	-0.03		0.03	-0.07	0.01	0.18	0.17
54	0.09	-0.15	0.01	0.11	0.37	0.46	0.04	0.20	0.34	0.11	0.33	0.19	-0.07	0.23	0.03		0.82	0.16	0.13	0.01
55	0.08	-0.17	0.05	0.23	0.23	0.37	0.00	0.25	0.33	0.09	0.28	0.22	0.08	0.26	-0.07	0.82		0.14	0.24	0.07
56	-0.10	-0.10	0.02	0.23	0.23	0.15	0.08	0.34	0.09	0.05	0.06	0.33	0.08	0.32	0.01	0.16	0.14		0.56	0.12
57	-0.21	-0.12	0.17	0.17	0.05	0.19	-0.01	0.23	0.27	0.03	0.14	0.21	0.19	0.19	0.18	0.13	0.24	0.56		0.15
58	-0.06	0.08	0.04	-0.03	0.00	-0.03	-0.08	0.21	0.22	0.13	0.08	0.07	0.20	0.13	0.17	0.01	0.07	0.12	0.15	
59	0.07	0.13	0.13	0.09	0.13	0.13	-0.02	-0.07	0.06	0.10	0.00	-0.14	-0.11	-0.18	-0.11	0.12	0.11	-0.02	-0.01	0.06
60	-0.26	-0.18	0.42	0.12	0.05	0.29	0.01	0.15	0.21	0.09	0.30	0.34	0.22	0.09	0.26	0.24	0.28	-0.08	0.14	0.18
61	-0.01	-0.15	0.20	0.23	0.09	0.32	-0.06	0.20	0.13	0.24	0.27	0.33	0.17	0.07	0.24	0.17	0.19	0.26	0.17	0.14
62	0.22	0.14	-0.17	-0.09	0.07	0.07	0.01	0.10	0.25	-0.02	0.13	-0.28	-0.14	-0.10	0.02	0.31	0.24	-0.09	0.10	0.06
63	-0.13	-0.19	0.46	0.12	0.13	0.42	0.01	0.15	0.16	0.20	0.37	0.29	0.19	0.10	0.19	0.36	0.30	-0.03	0.22	0.11
64	0.14	-0.02	0.13	0.74	0.01	0.16	0.14	0.04	-0.22	0.19	0.14	-0.03	0.05	0.14	0.04	-0.08	-0.03	0.16	-0.03	-0.20
65	0.05	-0.15	0.05	0.17	0.48	0.25	-0.05	0.47	0.46	0.15	0.26	0.29	0.10	0.23	0.06	0.42	0.44	0.11	0.25	0.22
66	-0.06	-0.23	0.34	0.21	0.21	0.75	0.10	0.25	0.25	0.14	0.36	0.33	0.05	0.45	0.16	0.40	0.33	0.16	0.16	-0.10
67	0.14	-0.12	0.24	0.08	0.06	0.05	0.88	-0.07	-0.23	0.29	-0.08	0.11	0.05	0.18	0.03	-0.14	-0.21	0.03	-0.14	-0.17
68	0.37	-0.04	0.07	0.34	0.33	0.24	0.14	0.89	0.42	0.11	0.19	0.23	0.03	0.72	-0.03	0.09	0.13	0.38	0.17	0.05
69	0.11	-0.05	0.01	-0.21	-0.09	-0.12	0.22	-0.18	-0.09	-0.05	-0.12	-0.20	0.09	0.00	-0.08	-0.23	-0.32	-0.05	-0.21	-0.13
70	0.00	-0.08	0.12	0.22	0.26	0.29	0.29	0.18	0.07	0.79	0.30	0.22	0.23	0.11	0.11	0.40	0.32	0.09	0.09	0.13
71	0.07	-0.20	0.27	0.22	0.13	0.41	0.05	0.33	0.26	0.32	0.80	0.26	0.18	0.29	0.19	0.24	0.32	0.09	0.26	0.12
72	-0.05	-0.29	0.27	0.30	0.15	0.31	0.19	0.35	0.04	0.18	0.19	0.93	0.23	0.61	0.00	0.18	0.23	0.36	0.15	0.06
73	-0.02	0.03	0.00	0.07	-0.15	-0.14	0.05	-0.23	-0.34	-0.11	-0.11	-0.09	0.31	-0.04	0.07	-0.32	-0.19	0.15	-0.03	0.02
74	0.18	-0.16	0.24	0.27	0.22	0.36	0.27	0.60	0.27	0.11	0.27	0.50	0.03	0.34	-0.06	0.16	0.17	0.35	0.11	0.04
75	-0.24	-0.07	0.19	0.18	0.04	0.30	0.12	0.22	0.16	0.14	0.31	0.21	0.23	0.07	0.89	0.21	0.15	-0.03	0.23	0.23
76	0.12	-0.14	-0.01	0.10	0.37	0.46	0.04	0.17	0.30	0.11	0.32	0.15	-0.10	0.21	0.01	0.39	0.80	0.15	0.09	-0.01
77	0.09	-0.18	0.05	0.23	0.23	0.36	-0.01	0.24	0.32	0.09	0.27	0.20	0.08	0.25	-0.09	0.82	1.00	0.14	0.23	0.06
78	-0.07	-0.04	-0.02	0.09	0.12	0.04	0.13	0.10	-0.09	-0.08	-0.05	0.18	-0.09	0.25	-0.08	0.00	-0.01	0.81	0.43	-0.02
79	-0.21	-0.10	0.15	0.13	0.06	0.20	0.00	0.25	0.32	0.01	0.14	0.25	0.17	0.21	0.15	0.17	0.27	0.52	0.37	0.19
80	-0.04	-0.05	0.11	0.05	0.15	0.21	-0.05	0.35	0.44	0.09	0.19	0.25	0.13	0.09	0.12	0.35	0.40	-0.03	0.25	0.23
81	0.17	0.13	0.17	0.12	0.07	-0.11	0.04	0.05	-0.21	0.20	-0.02	-0.12	-0.05	0.02	-0.17	-0.16	-0.21	-0.13	-0.30	0.08
82	0.02	-0.06	0.07	-0.15	-0.05	0.04	-0.03	-0.08	-0.07	-0.14	-0.08	0.03	-0.07	-0.04	-0.22	0.19	0.04	0.11	-0.02	-0.24
83	-0.13	0.00	-0.02	-0.07	-0.18	-0.09	-0.15	-0.12	-0.04	-0.23	-0.15	0.08	0.02	-0.10	-0.07	-0.03	-0.10	0.11	0.00	-0.11
84	0.13	-0.16	0.31	-0.09	0.07	0.20	-0.03	-0.01	-0.04	0.03	0.17	-0.03	-0.03	0.11	-0.19	0.33	0.23	-0.04	-0.04	-0.24
85	-0.15	-0.05	-0.06	-0.15	-0.17	-0.13	-0.20	-0.24	-0.05	-0.33	-0.19	-0.04	-0.04	-0.23	-0.13	-0.06	-0.14	0.07	0.00	-0.13
86	0.05	-0.07	0.09	-0.13	-0.01	0.05	-0.02	-0.09	-0.06	-0.13	-0.08	0.00	-0.09	-0.04	-0.24	0.21	0.05	0.10	-0.02	-0.27
87	0.33	-0.21	-0.02	0.00	0.16	0.08	0.19	0.07	0.19	-0.06	-0.01	-0.10	-0.19	0.03	-0.04	0.28	0.18	-0.07	-0.06	-0.21
88	0.07	0.15	-0.17	-0.03	0.01	-0.15	0.04	-0.02	0.04	-0.04	-0.01	-0.14	0.08	-0.10	0.15	-0.21	-0.17	-0.13	-0.05	0.24
89	0.08	0.29	-0.19	0.03	-0.09	-0.25	0.10	-0.07	0.05	-0.01	-0.01	-0.23	-0.05	-0.05	0.10	-0.35	-0.22	-0.20	-0.09	0.11
90	0.06	-0.16	0.21	-0.09	0.04	0.16	-0.03	0.07	0.06	-0.08	0.04	0.01	-0.06	0.08	-0.20	0.28	0.23	0.10	0.04	-0.23
91	-0.04	-0.21	0.14	-0.02	0.02	0.15	-0.03	0.08	0.00	0.01	0.01	0.17	-0.01	0.12	-0.17	0.29	0.26	0.21	0.10	-0.16
92	0.11	0.25	0.05	0.04	-0.03	-0.19	0.08	0.00	-0.24	0.10	-0.19	-0.07	-0.18	-0.03	-0.19	-0.18	-0.19	-0.11	-0.31	0.04
93	0.01	0.13	-0.11	-0.20	-0.24	-0.09	0.15	-0.07	0.00	-0.23	-0.25	0.05	-0.27	-0.04	-0.08	-0.03	0.02	-0.07	-0.14	-0.15
94	-0.02	-0.11	0.17	-0.06	-0.07	0.11	0.08	-0.13	-0.09	-0.10	-0.08	0.02	-0.09	-0.03	-0.21	0.18	0.16	0.03	-0.04	-0.22
95	-0.05	-0.02	0.13	0.10	-0.07	0.07	0.17	-0.01	-0.04	0.04	-0.02	0.06	-0.10	0.01	0.00	-0.02	0.18	-0.08	0.02	0.03
96	-0.13	-0.11	0.04	-0.05	-0.17	0.00	-0.07	-0.15	-0.15	-0.20	-0.07	0.07	0.00	-0.09	-0.07	0.09	0.09	0.11	-0.03	-0.09
97	-0.06	-0.11	0.23	0.10	-0.01	0.14	0.05	0.00	-0.14	0.04	0.15	0.04	0.06	0.01	-0.02	0.11	0.27	0.08	0.07	-0.05
98	0.14	-0.03	0.27	-0.06	0.05	0.15	0.12	0.04	0.06	0.05	0.11	0.00	-0.19	0.22	-0.21	0.23	0.23	-0.17	-0.05	-0.22
99	0.06	0.05	0.08	0.06	0.00	0.00	0.24	0.00	0.15	0.05	-0.05	0.06	-0.13	0.08	-0.10	-0.08	-0.01	-0.15	0.01	0.01
100	-0.09	-0.12	0.02	-0.05	-0.16	-0.01	-0.12	-0.16	-0.11	-0.24	-0.07	0.05	-0.02	-0.17	-0.03	0.08	0.04	0.06	-0.06	-0.03
101	-0.01	-0.12	0.16	0.14	-0.10	0.22	0.01	0.11	-0.05	0.10	0.18	0.12	0.14	0.09	0.21	0.18	0.26	0.06	-0.06	0.14
102	0.00	-0.07	0.17	-0.08	-0.07	0.10	0.08	-0.12	-0.05	-0.11	-0.10	0.01	-0.12	-0.01	-0.23	0.19	0.16	0.01	-0.05	-0.21
103	-0.07	0.04	0.13	0.08	-0.09	0.02	0.15	0.02	0.01	0.02	-0.02	0.07	-0.09	0.04	0.01	-0.03	0.15	-0.10	-0.01	0.11
104	0.29	-0.10	-0.11	-0.07	0.12	-0.02	0.10	0.01	0.16	-0.17	-0.09	-0.19	-0.32	-0.04	-0.05	0.23	0.15	-0.11	-0.01	-0.24
105	-0.24	0.27	-0.11	-0.04	-0.20	-0.19	-0.10	-0.04	0.03	0.09	-0.06	0.00	-0.17	0.03	-0.06	-0.08	-0.08	-0.16	-0.09	0.24
106	0.14	0.05	-0.19	-0.07	0.07	-0.12	-0.03	0.09	0.07	-0.11	-0.02	-0.11	0.10	-0.02	0.21	-0.18	-0.22	-0.03	-0.08	0.20
107	0.00	0.12	-0.06	0.05	0.03	-0.08	0.26	0.02	0.07	-0.03	-0.27	-0.02	-0.12	0.00	0.00	0.00	-0.08	-0.01	-0.23	-0.02
108	0.12	0.32	-0.12	-0.02	-0.04	-0.33	0.12	-0.03	-0.01	-0.05	-0.12	-0.27	0.00	-0.05	0.06	-0.44	-0.36	-0.17	-0.14	0.11
109	-0.16	0.26	-0.23	0.03	0.02	-0.35	-0.19	0.00	-0.13	-0.04	-0.23	-0.21	0.05	-0.16	-0.06	-0.34	-0.23	-0.10	0.01	0.23
110	-0.06	-0.18	0.26	-0.01	0.00	0.23	0.13	-0.01	0.00	0.06	0.13	0.12	-0.10	0.13	-0.20	0.30	0.35	0.01	0.08	-0.19
111	-0.04	-0.01	0.19	-0.09	-0.03	0.02	0.19	-0.18	-0.02	0.05	0.04	0.02	-0.15	-0.01	-0.13	0.01	0.04	-0.23	-0.03	-0.06
112	0.03	-0.10	0.28	-0.10	0.04	0.18	0.15	-0.04	0.03	0.00	0.02	-0.03	-0.17	0.11	-0.23	0.25	0.24	-0.07	-0.04	-0.13
113	0.02	0.06	0.16	-0.06	-0.01	0.02	0.27	-0.01	0.09	0.15	-0.01	0.08	-0.14	0.14	-0.07	0.03	0.09	-0.18	0.00	-0.01

Table A12.1: continued from above

	BG	BH	BI	BJ	BK	BL	BM	BN	BO	BP	BQ	BR	BS	BT	BU	BV	BW	BX	BY	BZ
1	IgE (kIU/L)	LTB4 (pg/ml)	LTC4 (pg/ml)	LTE4 (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-12 (pg/ml)	IL-13 (pg/ml)	Eotaxin (pg/ml)	IFN I (pg/ml)	RANTES (pg/ml)	TNF a (pg/ml)	VEGF (pg/ml)	IL-1b (pg/ml)	CRP (pg/ml)	SAAP (pg/ml)	TGF b1 (pg/ml)
2	-0.10	-0.01	-0.28	0.07	0.00	-0.03	-0.07	0.01	0.04	-0.09	-0.07	0.01	-0.01	0.04	0.00	0.00	-0.11	-0.01	0.07	0.00
3	-0.08	0.04	-0.19	0.03	0.03	-0.01	-0.14	-0.03	-0.02	-0.07	-0.04	-0.07	0.10	-0.16	0.06	-0.07	-0.12	-0.11	-0.02	-0.01
4	0.23	0.03	-0.13	0.14	0.12	0.06	-0.12	0.10	0.11	-0.16	0.09	0.06	0.00	-0.20	-0.02	-0.06	0.01	0.25	0.11	0.13
5	0.04	-0.02	-0.22	0.07	0.01	0.06	-0.01	0.14	0.33	0.14	0.01	0.19	0.06	0.03	-0.08	0.24	0.02	0.09	0.12	-0.07
6	0.12	-0.24	0.02	0.12	-0.01	0.15	-0.12	-0.19	0.03	-0.02	0.07	0.11	-0.12	-0.18	0.03	-0.18	0.09	-0.11	-0.28	-0.09
7	0.12	-0.05	-0.15	0.22	0.12	0.19	-0.09	0.09	0.04	-0.26	0.08	0.11	0.12	-0.22	-0.02	-0.25	0.16	0.11	-0.06	-0.07
8	0.03	0.06	-0.26	0.16	0.08	0.12	-0.05	0.20	0.05	-0.27	0.07	0.04	0.12	-0.15	-0.02	-0.17	0.09	0.14	0.06	-0.03
9	0.03	0.18	-0.02	0.03	0.08	-0.05	-0.03	-0.08	0.04	-0.03	-0.07	-0.04	0.03	-0.09	0.03	0.02	0.10	-0.04	-0.03	-0.10
10	-0.11	0.01	-0.13	0.10	-0.19	-0.25	0.04	-0.21	-0.15	-0.11	-0.15	-0.12	0.07	-0.17	-0.03	-0.14	0.07	-0.23	-0.14	-0.19
11	-0.12	-0.15	-0.11	0.08	-0.31	-0.21	-0.01	-0.20	-0.26	-0.16	-0.05	-0.18	0.00	-0.15	0.07	-0.25	0.03	-0.29	-0.22	-0.08
12	-0.28	-0.04	-0.18	0.11	-0.23	-0.19	-0.04	-0.22	-0.15	-0.05	-0.10	-0.11	0.03	-0.15	0.08	-0.11	0.14	-0.22	-0.22	-0.15
13	-0.17	-0.12	-0.13	0.09	-0.28	-0.24	-0.02	-0.28	-0.20	-0.15	-0.09	-0.17	0.03	-0.17	0.09	-0.25	0.05	-0.31	-0.21	-0.12
14	-0.13	-0.14	-0.12	0.07	-0.27	-0.25	-0.02	-0.28	-0.23	-0.20	-0.11	-0.18	0.00	-0.20	0.06	-0.30	0.05	-0.36	-0.27	-0.14
15	-0.06	-0.02	-0.06	0.04	-0.18	-0.29	0.05	-0.31	-0.22	-0.20	-0.15	-0.08	0.02	-0.11	-0.04	-0.26	0.05	-0.36	-0.26	-0.17
16	-0.09	-0.09	-0.11	0.08	-0.23	-0.28	0.02	-0.29	-0.24	-0.21	-0.13	-0.16	0.01	-0.19	0.02	-0.31	0.06	-0.36	-0.27	-0.18
17	0.04	0.25	0.10	0.02	0.16	-0.03	-0.03	-0.02	0.02	-0.05	-0.08	0.03	0.08	-0.04	0.06	0.01	0.17	-0.03	-0.05	-0.07
18	-0.05	-0.07	-0.02	-0.08	-0.12	-0.01	-0.18	-0.24	-0.02	-0.05	0.02	-0.02	-0.26	-0.04	0.11	-0.10	0.11	-0.27	-0.37	0.03
19	0.04	0.27	0.02	0.06	0.20	-0.03	-0.04	0.01	0.08	-0.05	-0.09	0.07	0.10	-0.02	0.03	0.06	0.15	0.03	0.00	-0.12
20	0.12	0.16	0.01	0.10	0.32	0.10	0.01	0.16	0.14	0.00	-0.09	0.30	0.07	0.24	0.01	0.14	-0.08	0.21	0.07	0.07
21	-0.17	-0.07	-0.20	0.19	-0.19	-0.20	0.00	-0.26	-0.20	-0.05	-0.21	-0.09	0.02	-0.12	0.03	-0.12	0.01	-0.22	-0.10	-0.15
22	0.06	0.03	0.01	0.11	0.28	0.10	0.00	-0.02	-0.06	0.09	-0.05	0.14	-0.01	0.21	-0.03	0.06	-0.08	0.17	0.13	0.06
23	-0.16	-0.22	-0.15	0.10	-0.33	-0.19	-0.05	-0.29	-0.33	-0.11	-0.04	-0.22	-0.07	-0.17	0.08	-0.28	-0.05	-0.29	-0.17	-0.05
24	-0.31	-0.07	-0.21	0.17	-0.23	-0.16	-0.05	-0.24	-0.21	-0.05	-0.11	-0.13	0.02	-0.18	0.09	-0.13	0.08	-0.19	-0.14	-0.14
25	0.06	0.08	0.08	0.04	0.24	0.06	0.03	0.04	-0.09	-0.04	0.07	0.09	0.02	0.10	-0.04	-0.02	-0.16	0.17	0.20	-0.07
26	-0.22	-0.22	-0.22	0.09	-0.32	-0.19	-0.07	-0.30	-0.25	-0.06	-0.10	-0.21	-0.06	-0.16	0.12	-0.19	-0.03	-0.27	-0.13	-0.11
27	0.01	-0.09	-0.12	0.14	0.10	0.03	0.06	-0.07	-0.12	0.06	-0.04	-0.05	-0.14	0.09	-0.12	0.01	-0.08	0.15	0.11	-0.04
28	-0.16	-0.19	-0.18	0.17	-0.29	-0.22	-0.06	-0.36	-0.29	-0.16	-0.11	-0.21	-0.05	-0.20	0.10	-0.30	-0.02	-0.33	-0.23	-0.10
29	-0.07	0.01	-0.05	0.21	0.14	0.05	-0.06	-0.18	-0.10	0.11	-0.03	-0.01	-0.03	0.06	0.03	-0.01	-0.03	0.08	0.10	0.04
30	-0.11	-0.10	-0.08	0.13	-0.15	-0.26	0.04	-0.38	-0.29	-0.15	-0.18	-0.12	0.01	-0.14	0.02	-0.32	0.05	-0.32	-0.20	-0.12
31	-0.10	-0.20	-0.14	0.20	0.00	-0.05	-0.02	-0.24	-0.20	0.01	-0.01	-0.13	-0.05	-0.16	0.12	-0.22	-0.01	-0.05	0.02	0.05
32	-0.13	-0.16	-0.15	0.16	-0.23	-0.23	-0.03	-0.40	-0.31	-0.18	-0.15	-0.17	-0.04	-0.18	0.07	-0.33	0.00	-0.34	-0.23	-0.12
33	-0.11	-0.11	-0.07	0.23	0.07	0.08	-0.11	-0.14	-0.07	0.08	0.03	-0.01	-0.09	-0.05	0.10	-0.05	0.01	0.12	0.11	0.16
34	0.08	0.28	0.08	0.04	0.23	0.00	-0.02	0.07	0.12	-0.02	-0.08	0.13	0.12	0.02	0.06	0.10	0.13	0.01	0.00	-0.05
35	0.10	0.14	-0.04	0.13	0.17	0.09	0.13	0.25	0.05	0.02	-0.13	0.23	0.09	0.17	-0.06	0.14	-0.11	0.30	0.29	-0.01
36	-0.05	-0.04	0.06	-0.08	-0.07	-0.07	-0.12	-0.23	-0.06	-0.08	-0.04	0.05	-0.20	0.02	0.12	-0.15	0.12	-0.25	-0.37	-0.02
37	-0.04	0.04	0.11	0.07	0.12	-0.14	0.26	0.06	-0.09	-0.03	-0.17	0.14	0.17	0.19	-0.09	-0.10	0.04	0.10	0.11	-0.08
38	-0.15	0.18	0.16	-0.33	0.20	0.07	0.13	0.23	0.09	0.13	0.11	-0.06	0.05	0.33	-0.11	0.20	0.03	0.19	0.11	0.04
39	0.07	-0.26	-0.01	0.22	-0.13	0.14	0.05	-0.06	0.14	0.37	0.11	0.00	0.07	-0.05	-0.02	0.18	-0.24	0.12	0.09	-0.07
40	0.13	-0.18	-0.15	0.14	-0.19	-0.02	-0.15	-0.23	-0.12	-0.04	-0.05	-0.08	-0.20	-0.29	0.03	-0.16	-0.07	-0.14	-0.18	-0.04
41	0.13	0.42	0.20	-0.17	0.46	0.13	0.05	0.34	0.24	0.07	0.01	0.12	0.27	0.27	0.00	0.24	0.19	-0.01	0.05	-0.02
42	0.09	0.12	0.23	-0.09	0.12	0.74	0.17	0.21	0.08	0.34	-0.21	0.22	0.22	0.30	0.07	0.27	0.18	0.10	0.23	0.09
43	0.13	0.05	0.09	0.07	0.13	0.01	0.48	0.21	0.06	0.33	-0.09	0.28	0.13	0.15	-0.15	0.22	0.04	0.37	0.23	0.12
44	0.13	0.29	0.32	0.07	0.42	0.16	0.25	0.75	0.05	0.24	-0.12	0.29	0.41	0.31	-0.14	0.36	0.30	0.46	0.36	0.04
45	-0.02	0.01	-0.06	0.01	0.01	0.14	-0.05	0.10	0.88	0.14	0.22	0.29	0.05	0.19	0.05	0.27	0.12	0.04	-0.01	0.13
46	-0.07	0.15	0.20	0.10	0.15	0.04	0.47	0.25	-0.07	0.89	-0.18	0.18	0.33	0.35	-0.23	0.60	0.22	0.17	0.24	0.10
47	0.06	0.21	0.13	0.25	0.16	-0.22	0.46	0.25	-0.23	0.42	-0.09	0.07	0.26	0.04	-0.34	0.27	0.16	0.30	0.32	-0.09
48	0.10	0.09	0.24	-0.02	0.20	0.19	0.15	0.14	0.29	0.11	-0.05	0.73	0.32	0.18	-0.11	0.11	0.14	0.11	0.09	-0.08
49	0.00	0.30	0.27	0.13	0.37	0.14	0.26	0.36	-0.08	0.19	-0.12	0.30	0.80	0.19	-0.11	0.27	0.31	0.32	0.27	-0.05
50	-0.14	0.34	0.33	-0.28	0.29	-0.03	0.29	0.33	0.11	0.23	-0.20	0.22	0.26	0.93	-0.09	0.50	0.21	0.15	0.20	0.18
51	-0.11	0.22	0.17	-0.14	0.19	0.05	0.10	0.05	0.05	0.03	0.09	0.23	0.18	0.23	0.31	0.03	0.23	-0.10	0.08	-0.09
52	-0.18	0.09	0.07	-0.10	0.10	0.14	0.23	0.45	0.18	0.72	0.00	0.11	0.29	0.61	-0.04	0.34	0.07	0.21	0.25	0.25
53	-0.11	0.26	0.24	0.02	0.19	0.04	0.06	0.16	0.03	-0.03	-0.08	0.11	0.19	0.00	0.07	-0.06	0.89	0.01	-0.09	-0.08

Table A12.1: continued from above

	BG	BH	BI	BJ	BK	BL	BM	BN	BO	BP	BQ	BR	BS	BT	BU	BV	BW	BX	BY	BZ
1	IgE (kIU/L)	LTB4 (pg/ml)	LTC4 (pg/ml)	LTE4 (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-12 (pg/ml)	IL-13 (pg/ml)	Eotaxin (pg/ml)	IFN I (pg/ml)	RANTES (pg/ml)	TNF a (pg/ml)	VEGF (pg/ml)	IL-1b (pg/ml)	CRP (pg/ml)	SAAP (pg/ml)	TGF b1 (pg/ml)
54	0.12	0.24	0.17	0.31	0.36	-0.08	0.42	0.40	-0.14	0.09	-0.23	0.40	0.24	0.18	-0.32	0.16	0.21	0.99	0.82	0.00
55	0.11	0.28	0.19	0.24	0.30	-0.03	0.44	0.33	-0.21	0.13	-0.32	0.32	0.32	0.23	-0.19	0.17	0.15	0.80	1.00	-0.01
56	-0.02	-0.08	0.26	-0.09	-0.03	0.16	0.11	0.16	0.03	0.38	-0.05	0.09	0.09	0.36	0.15	0.35	-0.03	0.15	0.14	0.81
57	-0.01	0.14	0.17	0.10	0.22	-0.03	0.25	0.16	-0.14	0.17	-0.21	0.09	0.26	0.15	-0.03	0.11	0.23	0.09	0.23	0.43
58	0.06	0.18	0.14	0.06	0.11	-0.20	0.22	-0.10	-0.17	0.05	-0.13	0.13	0.12	0.06	0.02	0.04	0.23	-0.01	0.06	-0.02
59		0.14	0.27	-0.02	0.29	0.05	0.12	0.05	-0.04	-0.11	0.04	0.15	0.05	-0.23	-0.17	-0.20	-0.01	0.14	0.12	-0.07
60	0.14		0.47	-0.05	0.73	-0.34	0.52	0.19	-0.28	-0.16	-0.44	0.25	0.42	0.24	-0.35	-0.06	0.48	0.16	0.26	-0.31
61	0.27	0.47		-0.09	0.49	0.05	0.21	0.30	-0.15	0.10	-0.25	0.38	0.40	0.22	-0.09	0.01	0.43	0.16	0.18	0.11
62	-0.02	-0.05	-0.09		0.12	-0.19	0.22	-0.02	-0.14	0.05	-0.26	0.19	0.17	-0.28	-0.22	-0.20	0.15	0.31	0.24	-0.13
63	0.29	0.73	0.49	0.12		-0.25	0.51	0.39	-0.18	-0.08	-0.39	0.46	0.54	0.16	-0.38	-0.07	0.49	0.34	0.29	-0.27
64	0.05	-0.34	0.05	-0.19	-0.25		-0.43	0.18	0.37	0.31	0.33	-0.01	-0.04	0.08	0.50	0.30	-0.13	-0.01	-0.01	0.33
65	0.12	0.52	0.21	0.22	0.51	-0.43		0.13	-0.42	0.13	-0.60	0.34	0.43	0.21	-0.65	-0.04	0.36	0.34	0.41	-0.32
66	0.05	0.19	0.30	-0.02	0.39	0.18	0.13		0.12	0.28	0.05	0.23	0.25	0.30	0.01	0.43	0.28	0.39	0.32	0.12
67	-0.04	-0.28	-0.15	-0.14	-0.18	0.37	-0.42	0.12		0.13	0.55	0.15	-0.10	0.06	0.33	0.30	-0.09	-0.08	-0.19	0.27
68	-0.11	-0.16	0.10	0.05	-0.08	0.31	0.13	0.28	0.13		0.11	0.06	0.19	0.26	0.07	0.70	0.05	0.11	0.14	0.31
69	0.04	-0.44	-0.25	-0.26	-0.39	0.33	-0.60	0.05	0.55	0.11		-0.25	-0.25	-0.19	0.49	0.21	-0.30	-0.17	-0.29	0.23
70	0.15	0.25	0.38	0.19	0.46	-0.01	0.34	0.23	0.15	0.06	-0.25		0.42	0.16	-0.27	-0.01	0.34	0.40	0.32	-0.08
71	0.05	0.42	0.40	0.17	0.54	-0.04	0.43	0.25	-0.10	0.19	-0.25	0.42		0.16	-0.21	0.15	0.42	0.23	0.31	-0.11
72	-0.23	0.24	0.22	-0.28	0.16	0.08	0.21	0.30	0.06	0.26	-0.19	0.16	0.16		0.00	0.56	0.06	0.13	0.20	0.24
73	-0.17	-0.35	-0.09	-0.22	-0.38	0.50	-0.65	0.01	0.33	0.07	0.49	-0.27	-0.21	0.00		0.17	-0.17	-0.25	-0.17	0.46
74	-0.20	-0.06	0.01	-0.20	-0.07	0.30	-0.04	0.43	0.30	0.70	0.21	-0.01	0.15	0.56	0.17		-0.08	0.16	0.17	0.39
75	-0.01	0.48	0.43	0.15	0.49	-0.13	0.36	0.28	-0.09	0.05	-0.30	0.34	0.42	0.06	-0.17	-0.08		0.19	0.12	-0.22
76	0.14	0.16	0.16	0.31	0.34	-0.01	0.34	0.39	-0.08	0.11	-0.17	0.40	0.23	0.13	-0.25	0.16	0.19		0.80	0.04
77	0.12	0.26	0.18	0.24	0.29	-0.01	0.41	0.32	-0.19	0.14	-0.29	0.32	0.31	0.20	-0.17	0.17	0.12	0.80		0.01
78	-0.07	-0.31	0.11	-0.13	-0.27	0.33	-0.32	0.12	0.27	0.31	0.23	-0.08	-0.11	0.24	0.46	0.39	-0.22	0.04	0.01	
79	-0.01	0.18	0.18	0.15	0.31	-0.12	0.33	0.19	-0.15	0.17	-0.28	0.15	0.30	0.15	-0.10	0.09	0.28	0.15	0.27	0.38
80	0.11	0.62	0.27	0.32	0.61	-0.59	0.86	0.08	-0.48	-0.03	-0.71	0.35	0.42	0.11	-0.71	-0.19	0.46	0.26	0.37	-0.47
81	0.22	-0.02	0.11	-0.11	0.10	0.09	-0.08	-0.17	0.09	0.03	-0.04	0.13	-0.02	-0.13	0.07	0.03	-0.10	-0.12	-0.19	-0.08
82	0.23	-0.09	0.09	-0.14	0.13	0.02	-0.11	0.07	0.05	-0.03	0.20	-0.06	-0.14	-0.02	-0.04	-0.01	-0.18	0.22	0.06	0.12
83	0.12	-0.05	0.14	-0.22	0.06	0.07	-0.17	0.00	-0.02	-0.07	0.16	-0.16	-0.16	0.03	0.08	-0.07	-0.08	0.00	-0.09	0.12
84	0.17	0.04	-0.01	-0.03	0.25	0.03	-0.01	0.22	0.04	0.06	0.17	0.06	0.00	-0.04	-0.05	0.15	-0.14	0.35	0.26	-0.04
85	0.10	-0.05	0.08	-0.24	0.00	0.02	-0.18	-0.06	-0.05	-0.20	0.20	-0.25	-0.20	-0.08	0.09	-0.20	-0.16	-0.05	-0.13	0.11
86	0.24	-0.10	0.07	-0.13	0.13	0.04	-0.10	0.08	0.06	-0.02	0.20	-0.06	-0.15	-0.05	-0.05	-0.01	-0.20	0.23	0.07	0.11
87	-0.07	-0.03	-0.08	0.27	-0.04	0.07	0.03	0.04	0.14	0.11	-0.04	0.04	0.06	-0.08	-0.11	0.03	0.02	0.32	0.20	-0.03
88	-0.12	-0.02	-0.08	0.09	-0.13	-0.13	0.12	-0.17	-0.01	-0.10	-0.01	-0.07	0.10	-0.14	-0.01	-0.15	0.13	-0.24	-0.19	-0.15
89	-0.02	-0.12	-0.20	0.12	-0.29	-0.01	0.00	-0.30	0.07	-0.07	-0.03	-0.13	0.08	-0.27	0.03	-0.08	0.00	-0.36	-0.23	-0.16
90	0.11	0.01	0.06	-0.05	0.14	0.04	-0.03	0.19	0.00	0.14	0.14	-0.05	-0.04	-0.01	-0.01	0.11	-0.12	0.30	0.25	0.09
91	0.08	0.00	0.12	-0.12	0.18	0.07	-0.04	0.21	0.02	0.14	0.07	0.06	-0.07	0.17	-0.03	0.16	-0.09	0.32	0.28	0.17
92	0.18	-0.12	0.02	-0.15	-0.05	0.02	-0.11	-0.24	0.12	-0.04	-0.02	0.07	-0.13	-0.09	0.02	-0.03	-0.15	-0.15	-0.18	-0.06
93	-0.04	-0.13	-0.14	-0.04	-0.21	-0.17	-0.11	-0.11	0.08	-0.11	0.04	-0.15	-0.11	0.02	-0.11	-0.06	-0.10	-0.04	0.01	-0.03
94	0.31	-0.03	0.03	-0.19	0.11	0.05	-0.08	0.12	0.12	-0.08	0.16	-0.10	-0.11	-0.05	-0.05	-0.01	-0.17	0.21	0.18	0.04
95	-0.04	0.07	-0.12	0.02	-0.16	-0.06	0.08	-0.03	0.03	-0.08	-0.20	-0.03	0.03	0.03	-0.08	-0.03	0.01	-0.03	0.17	-0.08
96	0.22	0.03	0.16	-0.18	0.14	0.04	-0.08	0.07	-0.01	-0.13	0.10	-0.14	-0.04	-0.01	0.03	-0.09	-0.04	0.12	0.10	0.06
97	0.13	0.16	0.05	-0.04	0.16	-0.01	0.12	0.09	-0.06	-0.04	-0.12	0.01	0.20	-0.01	-0.02	-0.02	0.02	0.12	0.27	-0.01
98	0.04	0.00	-0.19	0.02	-0.03	-0.04	0.02	0.11	0.12	0.08	0.06	0.05	-0.02	0.00	-0.14	0.22	-0.17	0.22	0.23	-0.07
99	-0.07	-0.03	-0.24	0.08	-0.24	-0.03	0.05	-0.10	0.16	-0.02	-0.08	0.00	-0.06	0.06	-0.10	0.05	-0.10	-0.10	-0.02	-0.06
100	0.17	0.05	0.21	-0.16	0.12	0.01	-0.06	0.06	-0.07	-0.16	0.04	-0.17	-0.03	-0.03	0.05	-0.19	0.01	0.10	0.05	0.01
101	0.08	0.10	0.19	0.01	0.18	0.06	0.04	0.23	-0.06	0.10	-0.13	0.08	0.25	0.06	0.04	0.06	0.28	0.21	0.26	-0.06
102	0.31	-0.06	0.01	-0.19	0.09	0.06	-0.09	0.12	0.13	-0.06	0.17	-0.13	-0.14	-0.06	-0.07	0.01	-0.20	0.22	0.18	0.04
103	-0.05	0.06	-0.14	0.01	-0.16	-0.07	0.07	-0.05	0.02	-0.05	-0.19	-0.05	0.01	0.04	-0.09	0.01	0.02	-0.05	0.14	-0.10
104	-0.08	-0.11	-0.15	0.31	-0.15	0.01	0.00	-0.03	0.04	0.05	-0.14	-0.08	-0.04	-0.17	-0.13	-0.03	-0.02	0.25	0.16	0.01
105	0.05	-0.03	-0.19	-0.07	-0.11	-0.17	0.06	-0.17	-0.14	-0.12	-0.17	0.02	-0.13	-0.02	-0.25	-0.01	-0.06	-0.11	-0.08	-0.18
106	-0.13	-0.12	-0.12	0.07	-0.10	-0.11	0.10	-0.05	-0.04	0.01	0.03	-0.09	0.10	-0.09	0.07	-0.05	0.18	-0.19	-0.24	-0.08
107	0.05	-0.10	-0.09	0.02	-0.21	0.03	-0.02	-0.03	0.18	0.01	0.09	-0.05	-0.18	0.00	0.07	0.00	-0.05	0.00	-0.08	0.06
108	0.03	-0.33	-0.31	-0.05	-0.33	0.09	-0.17	-0.33	0.18	0.03	0.15	-0.26	-0.07	-0.27	0.21	-0.03	-0.08	-0.43	-0.37	-0.06
109	0.02	-0.20	-0.27	-0.04	-0.22	-0.03	-0.01	-0.31	-0.20	-0.03	-0.06	-0.18	-0.23	-0.14	0.04	-0.17	-0.14	-0.37	-0.23	-0.10
110	0.10	0.12	0.00	-0.08	0.10	0.03	0.02	0.18	0.12	0.04	0.05	0.05	0.00	0.09	-0.12	0.16	-0.14	0.30	0.37	0.05
111	0.04	0.18	-0.18	0.01	0.02	-0.17	0.12	-0.09	0.10	-0.20	-0.09	0.00	0.01	-0.01	-0.20	-0.04	-0.13	-0.01	0.05	-0.16
112	0.17	0.00	-0.12	-0.07	0.01	0.05	-0.07	0.15	0.16	0.02	0.18	-0.03	-0.09	-0.04	-0.07	0.15	-0.26	0.26	0.26	0.03
113	-0.01	0.04	-0.22	0.03	-0.12	-0.15	0.06	-0.04	0.15	-0.05	-0.09	0.07	0.01	0.06	-0.18	0.10	-0.07	0.00	0.08	-0.11

Table A12.1: continued from above

	CA	CB	CC	CD	CE	CF	CG	CH	CI	CJ	CK	CL	CM	CN	CO	CP	CQ	CR	CS	CT
1	TGF b2 (pg/ml)	TGF b3 (pg/ml)	Pre vt actual	Pre R5	Pre R20	Pre R5-R20	Pre R35	Pre abei5	Pre X5	Pre X20	Pre X35	Pre AX	Pre res_freq	Post vt actual	vt %D	Post R5	R5 %D	Post R20	R20 %D	Post R5-R20
2	0.15	-0.03	-0.21	-0.09	-0.13	0.09	-0.18	-0.08	0.10	0.02	0.08	-0.03	-0.04	-0.17	0.02	0.09	0.25	-0.07	0.13	0.29
3	0.07	-0.06	-0.04	0.06	0.03	0.04	0.03	0.05	0.16	-0.13	0.03	0.11	0.07	-0.03	0.04	0.17	0.06	0.07	0.01	0.17
4	0.12	-0.14	0.01	0.11	-0.05	0.32	-0.09	0.16	0.17	-0.22	0.05	0.19	0.12	0.00	-0.05	0.24	0.15	0.06	0.20	0.36
5	-0.06	-0.03	0.17	-0.17	-0.46	0.24	-0.56	-0.12	0.31	-0.19	0.07	0.12	0.12	0.18	0.08	0.07	0.42	-0.25	0.34	0.48
6	-0.18	-0.08	0.42	-0.18	-0.13	-0.14	-0.23	-0.16	-0.09	0.15	0.21	-0.26	-0.14	0.42	-0.05	-0.25	-0.01	-0.15	0.01	-0.24
7	0.04	-0.11	-0.01	-0.08	-0.09	0.06	-0.13	-0.05	0.09	-0.05	0.11	-0.04	-0.02	-0.07	-0.18	0.00	0.07	-0.03	0.04	0.02
8	0.15	-0.10	-0.29	0.03	-0.02	0.19	0.01	0.06	0.15	-0.17	-0.01	0.13	0.05	-0.33	-0.17	0.15	0.05	0.03	0.00	0.23
9	-0.04	0.06	0.40	-0.18	-0.28	0.10	-0.33	-0.17	0.13	-0.03	0.13	-0.03	-0.05	0.47	0.15	-0.18	0.13	-0.22	0.21	0.06
10	-0.11	0.12	0.10	-0.53	-0.40	-0.42	-0.33	-0.54	-0.06	0.43	0.43	-0.55	-0.53	0.27	0.34	-0.57	0.15	-0.45	-0.03	-0.25
11	-0.11	0.00	-0.16	-0.43	-0.17	-0.57	-0.04	-0.46	-0.13	0.60	0.42	-0.60	-0.60	-0.04	0.20	-0.52	-0.01	-0.29	-0.24	-0.33
12	-0.10	0.00	0.03	-0.57	-0.38	-0.41	-0.30	-0.58	-0.12	0.43	0.46	-0.56	-0.57	0.17	0.24	-0.63	0.10	-0.47	-0.07	-0.29
13	-0.11	0.02	-0.04	-0.52	-0.27	-0.56	-0.16	-0.55	-0.17	0.60	0.43	-0.63	-0.62	0.12	0.28	-0.60	0.05	-0.37	-0.15	-0.41
14	-0.13	0.04	-0.02	-0.49	-0.22	-0.59	-0.10	-0.51	-0.19	0.61	0.50	-0.66	-0.64	0.11	0.24	-0.57	0.02	-0.33	-0.19	-0.44
15	-0.13	0.13	-0.02	-0.42	-0.20	-0.51	-0.10	-0.44	-0.21	0.59	0.46	-0.63	-0.61	0.10	0.23	-0.55	-0.07	-0.32	-0.23	-0.41
16	-0.13	0.09	-0.03	-0.49	-0.24	-0.57	-0.12	-0.51	-0.22	0.62	0.50	-0.66	-0.65	0.10	0.24	-0.57	0.02	-0.33	-0.17	-0.43
17	-0.01	0.06	0.41	-0.21	-0.28	0.09	-0.32	-0.20	0.07	-0.02	0.11	-0.06	-0.07	0.47	0.13	-0.19	0.14	-0.22	0.23	0.03
18	-0.04	-0.09	0.26	-0.24	-0.08	-0.29	-0.11	-0.26	-0.30	0.27	0.21	-0.39	-0.31	0.30	-0.01	-0.32	-0.03	-0.21	-0.10	-0.28
19	-0.01	0.05	0.41	-0.14	-0.26	0.19	-0.33	-0.13	0.16	-0.11	0.07	0.03	-0.01	0.42	0.06	-0.11	0.16	-0.18	0.25	0.13
20	0.05	-0.03	0.12	0.21	0.08	0.33	0.05	0.22	0.01	-0.32	-0.20	0.24	0.18	-0.02	-0.22	0.23	-0.03	0.14	0.11	0.20
21	-0.14	0.06	0.16	-0.51	-0.41	-0.35	-0.35	-0.52	-0.02	0.39	0.39	-0.50	-0.46	0.23	0.27	-0.59	0.07	-0.45	-0.03	-0.26
22	0.05	-0.02	0.05	0.42	0.28	0.47	0.22	0.42	0.07	-0.58	-0.50	0.48	0.51	-0.08	-0.20	0.25	-0.33	0.27	0.02	0.08
23	-0.11	-0.04	-0.16	-0.32	-0.09	-0.46	0.05	-0.35	-0.17	0.44	0.27	-0.46	-0.45	-0.05	0.17	-0.49	-0.14	-0.23	-0.27	-0.41
24	-0.10	-0.01	-0.02	-0.55	-0.37	-0.37	-0.27	-0.56	-0.14	0.46	0.39	-0.52	-0.53	0.10	0.21	-0.64	0.02	-0.45	-0.08	-0.31
25	-0.10	-0.01	-0.14	0.40	0.27	0.39	0.28	0.40	-0.02	-0.38	-0.43	0.37	0.36	-0.24	-0.12	0.22	-0.42	0.24	-0.11	0.04
26	-0.17	-0.03	-0.01	-0.43	-0.21	-0.42	-0.10	-0.45	-0.18	0.45	0.37	-0.51	-0.49	0.12	0.24	-0.55	-0.04	-0.32	-0.15	-0.37
27	-0.03	0.01	-0.02	0.46	0.31	0.47	0.29	0.47	0.19	-0.53	-0.39	0.50	0.47	-0.05	-0.06	0.26	-0.37	0.25	-0.11	0.14
28	-0.14	-0.01	-0.02	-0.45	-0.21	-0.49	-0.10	-0.47	-0.18	0.50	0.39	-0.56	-0.54	0.10	0.22	-0.60	-0.08	-0.33	-0.18	-0.45
29	-0.03	-0.03	0.06	0.15	0.09	0.27	0.07	0.14	0.01	-0.35	-0.31	0.25	0.30	-0.02	-0.10	-0.07	-0.33	0.04	0.01	-0.12
30	-0.07	0.12	-0.03	-0.36	-0.12	-0.50	-0.01	-0.40	-0.21	0.51	0.33	-0.56	-0.51	0.06	0.18	-0.53	-0.15	-0.23	-0.20	-0.52
31	0.11	0.02	-0.04	-0.02	0.09	-0.09	0.12	-0.05	-0.10	0.01	-0.13	-0.02	0.07	-0.10	-0.08	-0.15	-0.21	0.07	0.04	-0.38
32	-0.13	0.04	0.00	-0.44	-0.20	-0.50	-0.09	-0.47	-0.19	0.51	0.38	-0.53	-0.54	0.10	0.19	-0.60	-0.09	-0.31	-0.17	-0.49
33	0.07	-0.08	0.01	0.19	0.17	0.20	0.16	0.17	0.08	-0.30	-0.31	0.22	0.23	-0.04	-0.08	-0.04	-0.34	0.12	-0.02	-0.19
34	0.02	0.05	0.45	-0.14	-0.28	0.20	-0.34	-0.13	0.14	-0.15	0.02	0.06	0.04	0.44	0.01	-0.11	0.15	-0.17	0.23	0.11
35	0.00	0.02	0.01	0.24	0.03	0.43	0.01	0.27	0.35	-0.46	-0.23	0.40	0.34	-0.11	-0.20	0.31	0.07	0.14	0.12	0.34
36	-0.11	-0.05	0.31	-0.19	-0.05	-0.22	-0.08	-0.21	-0.36	0.24	0.16	-0.33	-0.26	0.36	-0.03	-0.32	-0.11	-0.20	-0.15	-0.30
37	-0.04	0.13	-0.07	0.18	0.12	0.21	0.14	0.19	-0.04	-0.16	-0.19	0.23	0.19	-0.06	-0.04	0.08	-0.14	0.08	-0.08	0.04
38	0.04	0.01	-0.17	0.40	0.27	0.36	0.27	0.41	0.20	-0.35	-0.48	0.43	0.43	-0.26	-0.12	0.37	-0.13	0.30	0.03	0.22
39	-0.21	-0.04	0.17	0.02	-0.13	0.13	-0.15	0.05	0.33	0.07	0.08	0.06	-0.04	0.11	0.01	-0.02	-0.05	-0.13	-0.06	0.14
40	-0.10	-0.05	0.13	-0.06	0.00	-0.16	-0.05	-0.07	-0.21	0.15	0.29	-0.16	-0.21	0.25	0.13	-0.11	-0.02	-0.11	-0.11	-0.03
41	0.15	0.11	0.17	0.07	-0.02	0.31	-0.06	0.09	-0.02	-0.17	-0.19	0.21	0.14	0.05	-0.11	0.17	0.13	0.04	0.23	0.27
42	0.13	0.05	0.12	-0.15	-0.07	-0.09	-0.15	-0.13	0.00	-0.03	0.03	-0.09	-0.02	0.04	-0.20	-0.06	0.10	-0.05	0.10	-0.06
43	0.06	0.15	0.07	-0.05	-0.18	0.07	-0.17	-0.01	0.16	0.01	-0.09	0.04	0.02	-0.03	-0.24	-0.07	-0.07	-0.17	-0.01	0.05
44	0.20	0.21	-0.11	0.04	-0.09	0.20	-0.13	0.05	0.08	-0.15	-0.25	0.16	0.15	-0.19	-0.09	0.11	0.07	0.00	0.14	0.15
45	0.00	-0.05	0.04	-0.03	-0.15	-0.03	-0.20	-0.02	0.13	0.04	0.10	-0.03	-0.03	0.08	0.15	0.08	0.17	-0.07	0.05	0.12
46	0.25	0.35	0.05	-0.08	-0.12	-0.01	-0.24	-0.09	0.07	-0.02	-0.07	0.07	0.08	0.00	-0.07	-0.13	-0.01	-0.15	0.00	0.04
47	0.32	0.44	-0.21	-0.07	-0.04	-0.04	-0.05	-0.06	0.13	0.04	0.05	0.06	0.00	-0.24	0.00	-0.09	-0.04	-0.15	-0.14	0.06
48	0.01	0.09	0.20	-0.14	-0.23	0.03	-0.33	-0.13	-0.06	-0.04	-0.01	-0.08	0.01	0.10	-0.23	-0.10	0.04	-0.20	0.04	0.05
49	0.14	0.19	-0.02	-0.08	-0.15	0.17	-0.19	-0.08	-0.01	-0.01	-0.01	0.04	0.01	-0.19	-0.25	-0.08	-0.02	-0.07	0.15	0.11
50	0.25	0.25	-0.12	0.03	0.08	-0.03	-0.04	0.00	-0.10	-0.14	-0.23	0.01	0.17	-0.07	0.05	0.02	0.06	0.07	0.04	0.00
51	0.17	0.13	-0.05	-0.07	0.02	-0.03	-0.04	-0.09	-0.19	0.08	-0.05	-0.06	-0.01	-0.18	-0.27	-0.09	-0.10	0.00	0.06	-0.19
52	0.21	0.09	0.02	-0.04	-0.10	0.11	-0.23	-0.04	0.03	-0.10	-0.05	0.08	0.12	-0.03	-0.04	-0.03	0.01	-0.09	0.01	0.22
53	0.15	0.12	-0.17	-0.22	-0.07	-0.19	-0.13	-0.24	-0.04	0.15	0.10	-0.20	-0.17	-0.19	-0.08	-0.21	0.00	-0.07	-0.02	-0.21

Table A12.1: continued from above

	CA	CB	CC	CD	CE	CF	CG	CH	CI	CJ	CK	CL	CM	CN	CO	CP	CQ	CR	CS	CT
1	TGF b2 (pg/ml)	TGF b3 (pg/ml)	Pre vt actual	Pre R5	Pre R20	Pre R5-R20	Pre R35	Pre abei5	Pre X5	Pre X20	Pre X35	Pre AX	Pre res_freq	Post vt actual	vt %D	Post R5	R5 %D	Post R20	R20 %D	Post R5-R20
53	0.15	0.12	-0.17	-0.22	-0.07	-0.19	-0.13	-0.24	-0.04	0.15	0.10	-0.20	-0.17	-0.19	-0.08	-0.21	0.00	-0.07	-0.02	-0.21
54	0.17	0.35	-0.16	0.19	-0.03	0.33	-0.06	0.21	0.28	-0.21	-0.35	0.28	0.29	-0.18	-0.03	0.18	-0.02	0.09	0.11	0.23
55	0.27	0.40	-0.21	0.04	-0.10	0.23	-0.14	0.05	0.18	-0.17	-0.22	0.23	0.26	-0.19	0.02	0.16	0.18	0.09	0.27	0.23
56	0.52	-0.03	-0.13	0.11	0.11	-0.04	0.07	0.10	-0.07	-0.13	-0.20	0.10	0.21	-0.11	-0.07	0.03	-0.08	0.11	0.08	-0.17
57	0.37	0.25	-0.30	-0.02	0.00	-0.04	0.00	-0.02	-0.06	-0.05	-0.09	0.04	0.10	-0.31	-0.14	-0.04	0.02	-0.03	0.07	-0.05
58	0.19	0.23	0.08	-0.24	-0.11	-0.24	-0.13	-0.27	-0.21	0.24	0.11	-0.23	-0.16	0.04	-0.15	-0.22	0.03	-0.09	-0.05	-0.22
59	-0.01	0.11	0.22	0.23	0.12	0.17	0.10	0.24	-0.07	-0.12	-0.02	0.11	0.08	0.18	-0.04	0.31	-0.04	0.22	0.13	0.04
60	0.18	0.62	-0.02	-0.09	-0.05	0.04	-0.05	-0.10	-0.03	-0.02	-0.12	0.01	0.00	-0.12	-0.13	-0.03	0.07	0.03	0.16	0.00
61	0.18	0.27	0.11	0.09	0.14	-0.01	0.08	0.07	-0.08	-0.08	-0.20	0.06	0.12	0.02	-0.14	0.03	-0.12	0.16	0.05	-0.13
62	0.15	0.32	-0.11	-0.14	-0.22	-0.03	-0.24	-0.13	0.27	0.09	0.12	-0.05	-0.12	-0.15	-0.04	-0.19	0.02	-0.18	-0.04	0.02
63	0.31	0.61	0.10	0.13	0.06	0.25	0.00	0.13	-0.04	-0.13	-0.29	0.14	0.18	-0.05	-0.21	0.11	-0.16	0.14	0.16	-0.03
64	-0.12	-0.59	0.09	0.02	0.07	0.03	0.02	0.04	0.07	-0.13	-0.01	0.04	0.07	0.02	-0.17	0.05	-0.06	0.04	-0.01	-0.04
65	0.33	0.86	-0.08	-0.11	-0.17	-0.01	-0.18	-0.10	0.03	0.12	0.00	-0.03	-0.04	-0.11	-0.11	-0.08	0.08	-0.08	0.12	0.02
66	0.19	0.08	-0.17	0.07	0.00	0.22	-0.06	0.08	0.04	-0.17	-0.30	0.19	0.21	-0.24	-0.11	0.12	-0.03	0.07	0.09	0.11
67	-0.15	-0.48	0.09	0.05	-0.02	0.04	-0.05	0.06	0.14	-0.01	0.07	0.00	0.02	0.12	0.08	0.12	0.03	-0.01	-0.06	0.12
68	0.17	-0.03	0.03	-0.03	-0.07	0.06	-0.20	-0.02	0.11	-0.10	-0.07	0.14	0.14	-0.04	-0.11	-0.08	-0.08	-0.13	-0.04	0.08
69	-0.28	-0.71	-0.04	0.20	0.16	0.17	0.20	0.20	-0.04	-0.01	-0.03	0.14	0.07	-0.02	0.04	0.16	-0.20	0.10	-0.12	0.06
70	0.15	0.35	0.13	-0.06	-0.16	0.06	-0.25	-0.06	0.04	-0.07	-0.13	-0.05	0.06	0.07	-0.15	-0.10	-0.03	-0.14	0.01	0.05
71	0.30	0.42	-0.02	-0.14	-0.16	0.00	-0.20	-0.15	0.06	0.10	0.08	-0.04	-0.07	-0.13	-0.11	-0.11	0.03	-0.04	0.20	-0.02
72	0.15	0.11	-0.13	-0.02	0.03	-0.04	-0.08	-0.05	-0.08	-0.14	-0.27	-0.01	0.17	-0.09	0.02	-0.05	0.03	-0.01	-0.01	0.00
73	-0.10	-0.71	0.07	-0.04	0.08	-0.05	0.09	-0.05	-0.11	-0.01	0.03	-0.01	-0.03	0.02	-0.11	-0.05	-0.08	0.03	-0.02	-0.14
74	0.09	-0.19	0.03	-0.01	-0.07	0.15	-0.20	-0.01	0.03	-0.15	-0.08	0.11	0.16	-0.03	-0.06	-0.01	-0.03	-0.09	-0.02	0.22
75	0.28	0.46	-0.10	-0.18	-0.08	-0.14	-0.16	-0.20	0.02	0.13	0.00	-0.12	-0.09	-0.15	-0.10	-0.17	0.01	-0.04	0.02	-0.17
76	0.15	0.26	-0.12	0.22	0.00	0.35	-0.05	0.23	0.32	-0.24	-0.36	0.30	0.32	-0.15	-0.04	0.21	-0.03	0.12	0.12	0.22
77	0.27	0.37	-0.19	0.06	-0.09	0.26	-0.13	0.07	0.20	-0.19	-0.23	0.25	0.28	-0.18	0.01	0.18	0.17	0.10	0.27	0.23
78	0.38	-0.47	-0.08	0.12	0.12	-0.04	0.11	0.11	-0.03	-0.15	-0.16	0.09	0.17	-0.06	-0.03	0.04	-0.08	0.06	-0.01	-0.07
79		0.36	-0.28	-0.03	-0.01	-0.05	-0.02	-0.03	-0.06	-0.03	-0.11	0.04	0.09	-0.30	-0.13	-0.03	0.04	-0.02	0.10	-0.04
80	0.36		-0.07	-0.12	-0.13	-0.07	-0.17	-0.12	0.00	0.11	-0.02	-0.06	-0.04	-0.09	-0.02	-0.07	0.13	-0.04	0.12	-0.02
81	-0.28	-0.07		-0.01	-0.14	0.17	-0.19	0.02	0.02	-0.07	0.09	0.04	0.02	0.84	-0.25	0.00	0.04	-0.05	0.16	0.10
82	-0.03	-0.12	-0.01		0.83	0.59	0.75	0.99	0.16	-0.67	-0.59	0.82	0.73	-0.03	-0.03	0.79	-0.46	0.73	-0.21	0.29
83	-0.01	-0.13	-0.14	0.83		0.13	0.92	0.77	-0.04	-0.44	-0.42	0.50	0.50	-0.12	-0.01	0.63	-0.48	0.80	-0.39	-0.09
84	-0.05	-0.07	0.17	0.59	0.13		0.10	0.64	0.20	-0.62	-0.48	0.80	0.61	0.03	-0.20	0.47	-0.23	0.19	0.19	0.63
85	-0.02	-0.17	-0.19	0.75	0.92	0.10		0.71	-0.07	-0.31	-0.32	0.42	0.34	-0.17	-0.01	0.57	-0.49	0.73	-0.40	-0.09
86	-0.03	-0.12	0.02	0.99	0.77	0.64	0.71		0.20	-0.68	-0.58	0.85	0.72	0.00	-0.03	0.80	-0.43	0.69	-0.17	0.35
87	-0.06	0.00	0.02	0.16	-0.04	0.20	-0.07	0.20		-0.26	-0.13	0.33	0.24	0.04	0.18	0.16	0.09	0.02	0.01	0.21
88	-0.03	0.11	-0.07	-0.67	-0.44	-0.62	-0.31	-0.68	-0.26		0.68	-0.84	-0.88	0.00	0.12	-0.55	0.23	-0.41	-0.02	-0.35
89	-0.11	-0.02	0.09	-0.59	-0.42	-0.48	-0.32	-0.58	-0.13	0.68		-0.71	-0.83	0.19	0.20	-0.38	0.37	-0.32	0.09	-0.12
90	0.04	-0.06	0.04	0.82	0.50	0.80	0.42	0.85	0.33	-0.84	-0.71		0.89	-0.06	-0.13	0.71	-0.25	0.50	0.04	0.48
91	0.09	-0.04	0.02	0.73	0.50	0.61	0.34	0.72	0.24	-0.88	-0.83	0.89		-0.05	-0.14	0.60	-0.25	0.49	0.00	0.28
92	-0.30	-0.09	0.84	-0.03	-0.12	0.03	-0.17	0.00	0.04	0.00	0.19	-0.06	-0.05		0.24	-0.01	0.18	-0.03	0.16	0.06
93	-0.13	-0.02	-0.25	-0.03	-0.01	-0.20	-0.01	-0.03	0.18	0.12	0.20	-0.13	-0.14	0.24		0.01	0.28	0.03	0.11	-0.01
94	-0.03	-0.07	0.00	0.79	0.63	0.47	0.57	0.80	0.16	-0.55	-0.38	0.71	0.60	-0.01	0.01		0.06	0.83	0.20	0.44
95	0.04	0.13	0.04	-0.46	-0.48	-0.23	-0.49	-0.43	0.09	0.23	0.37	-0.25	-0.25	0.18	0.28	0.06		-0.09	0.68	0.26
96	-0.02	-0.04	-0.05	0.73	0.80	0.19	0.73	0.69	0.02	-0.41	-0.32	0.50	0.49	-0.03	0.03	0.83	-0.09		0.13	-0.02
97	0.10	0.12	0.16	-0.21	-0.39	0.19	-0.40	-0.17	0.01	-0.02	0.09	0.04	0.00	0.16	0.11	0.20	0.68	0.13		0.14
98	-0.04	-0.02	0.10	0.29	-0.09	0.63	-0.09	0.35	0.21	-0.35	-0.12	0.48	0.28	0.06	-0.01	0.44	0.26	-0.02	0.14	
99	0.03	0.05	-0.03	-0.23	-0.20	-0.28	-0.18	-0.20	0.11	0.23	0.32	-0.22	-0.25	0.04	0.12	0.08	0.57	-0.22	-0.07	0.49
100	-0.04	-0.02	-0.09	0.65	0.81	0.08	0.78	0.61	-0.03	-0.32	-0.31	0.41	0.36	-0.10	0.00	0.68	-0.18	0.32	0.02	-0.17
101	-0.02	0.13	0.15	-0.19	-0.19	-0.03	-0.33	-0.19	0.07	-0.03	-0.06	0.00	0.08	0.13	0.04	0.10	0.46	0.22	0.67	-0.20
102	-0.05	-0.09	0.02	0.79	0.61	0.49	0.56	0.81	0.18	-0.53	-0.36	0.71	0.57	0.00	0.02	0.39	0.05	0.81	0.19	0.45
103	0.01	0.13	0.05	-0.53	-0.51	-0.29	-0.52	-0.52	0.03	0.30	0.39	-0.35	-0.31	0.17	0.28	-0.03	0.36	-0.13	0.67	0.15
104	-0.01	-0.02	-0.02	0.05	-0.15	0.10	-0.14	0.09	0.90	-0.16	-0.01	0.20	0.10	0.04	0.25	0.10	0.22	-0.05	0.07	0.24
105	-0.05	0.15	0.05	-0.32	-0.21	-0.29	-0.23	-0.34	-0.27	0.27	0.28	-0.40	-0.29	0.15	0.18	-0.13	0.35	-0.13	0.14	-0.02
106	-0.06	0.05	-0.07	-0.41	-0.15	-0.49	-0.10	-0.43	-0.19	0.69	0.39	-0.56	-0.57	-0.01	0.10	-0.53	-0.17	-0.25	-0.25	-0.56
107	-0.22	-0.05	0.10	-0.12	-0.09	-0.26	-0.02	-0.12	0.12	0.29	0.27	-0.25	-0.33	0.17	0.20	0.04	0.23	-0.04	0.03	0.03
108	-0.17	-0.22	0.16	-0.30	-0.13	-0.34	-0.07	-0.30	-0.16	0.42	0.69	-0.44	-0.55	0.23	0.15	-0.28	-0.02	-0.20	-0.16	-0.27
109	0.01	-0.02	0.12	-0.25	-0.12	-0.26	-0.08	-0.26	-0.51	0.10	0.07	-0.22	-0.16	0.08	-0.20	-0.30	-0.09	-0.21	-0.09	-0.29
110	0.07	0.01	-0.04	0.40	0.05	0.58	0.00	0.43	0.27	-0.61	-0.45	0.66	0.65	-0.09	-0.04	0.60	0.29	0.27	0.38	0.66
111	-0.03	0.11	-0.02	-0.25	-0.44	0.09	-0.34	-0.20	0.13	0.13	0.32	-0.15	-0.27	0.01	0.17	0.13	0.63	-0.17	0.49	0.49
112	-0.06	-0.09	0.02	0.54	0.20	0.60	0.20	0.58	0.27	-0.56	-0.40	0.68	0.54	-0.06	-0.03	0.74	0.18	0.41	0.28	0.67
113	0.00	0.11	0.02	-0.46	-0.57	-0.19	-0.52	-0.43	0.11	0.35	0.44	-0.38	-0.43	0.10	0.26	-0.05	0.75	-0.32	0.42	0.39

Table A12.1: continued from above

	CU	CV	CW	CX	CY	CZ	DA	DB	DC	DD	DE	DF	DG	DH	DI
1	R5-R20 %D	Post R35	R35 %D	Post abei5	abei5 %D	Post X5	X5 %D	Post X20	X20 %D	Post X35	X35 %D	Post res_freq	Res freq %D	Post AX	AX %D
2	0.32	-0.16	0.00	0.09	0.21	0.19	0.20	-0.21	0.08	-0.05	-0.07	0.16	0.34	0.09	0.34
3	0.17	-0.01	-0.09	0.16	0.04	0.23	0.14	-0.23	0.07	0.06	-0.02	0.20	0.18	0.16	0.16
4	0.14	-0.06	0.03	0.25	0.08	0.21	0.02	-0.30	-0.04	0.00	-0.20	0.27	0.25	0.22	0.19
5	0.31	-0.46	0.13	0.06	0.35	0.36	0.04	-0.27	0.03	0.08	-0.13	0.42	0.40	0.30	0.45
6	-0.06	-0.15	0.13	-0.24	-0.02	-0.12	0.04	0.15	-0.02	0.16	0.14	-0.28	-0.14	-0.47	-0.14
7	0.09	-0.06	0.10	-0.01	0.02	0.06	0.01	-0.04	-0.11	0.04	-0.02	0.01	0.06	-0.07	0.03
8	0.14	0.01	-0.02	0.13	-0.02	0.13	-0.04	-0.16	-0.13	-0.05	-0.07	0.18	0.14	0.21	0.08
9	-0.04	-0.26	0.09	-0.19	0.16	0.12	0.08	0.04	0.01	0.15	0.14	-0.05	0.00	-0.12	0.11
10	0.07	-0.36	0.02	-0.58	0.21	0.02	0.17	0.50	0.11	0.39	0.25	-0.49	-0.07	-0.50	0.16
11	0.08	-0.11	-0.06	-0.52	0.04	-0.10	0.09	0.61	0.14	0.33	0.27	-0.65	-0.20	-0.53	0.01
12	0.01	-0.32	0.03	-0.63	0.17	-0.04	0.17	0.50	0.08	0.33	0.25	-0.55	-0.07	-0.57	0.12
13	0.04	-0.20	0.00	-0.59	0.13	-0.09	0.15	0.60	0.11	0.41	0.30	-0.65	-0.16	-0.60	0.07
14	0.05	-0.15	-0.03	-0.57	0.09	-0.11	0.14	0.63	0.13	0.42	0.32	-0.69	-0.18	-0.62	0.04
15	-0.01	-0.17	-0.10	-0.55	0.00	-0.13	0.11	0.61	0.17	0.37	0.26	-0.66	-0.15	-0.59	0.03
16	0.03	-0.16	-0.03	-0.58	0.09	-0.13	0.14	0.63	0.14	0.40	0.31	-0.69	-0.16	-0.62	0.05
17	-0.05	-0.25	0.12	-0.21	0.17	0.07	0.10	0.01	0.05	0.10	0.11	-0.05	0.02	-0.14	0.10
18	-0.01	-0.15	-0.05	-0.32	0.02	-0.24	0.23	0.18	0.22	0.16	0.32	-0.37	-0.09	-0.48	-0.02
19	-0.03	-0.24	0.13	-0.13	0.17	0.12	0.01	-0.05	0.04	0.06	0.03	0.02	0.06	-0.03	0.13
20	-0.02	0.09	0.00	0.21	-0.08	-0.07	-0.12	-0.23	0.04	-0.19	-0.12	0.17	0.09	0.26	-0.02
21	-0.03	-0.37	0.02	-0.60	0.14	0.06	0.14	0.43	0.06	0.37	0.34	-0.51	-0.16	-0.51	0.07
22	-0.37	0.21	-0.02	0.24	-0.34	-0.04	-0.18	-0.29	-0.24	-0.23	0.02	0.22	-0.21	0.25	-0.35
23	-0.08	-0.05	-0.07	-0.48	-0.07	-0.08	0.06	0.55	0.04	0.31	0.35	-0.62	-0.32	-0.49	-0.13
24	-0.08	-0.31	0.00	-0.64	0.10	-0.05	0.18	0.53	0.01	0.33	0.32	-0.57	-0.15	-0.55	0.04
25	-0.35	0.21	-0.14	0.21	-0.43	-0.12	-0.21	-0.13	-0.21	-0.13	0.04	0.13	-0.23	0.25	-0.34
26	-0.10	-0.15	0.02	-0.54	0.05	-0.10	0.14	0.58	0.05	0.41	0.39	-0.62	-0.29	-0.55	-0.03
27	-0.33	0.22	-0.12	0.26	-0.39	0.11	-0.22	-0.18	-0.23	-0.08	0.04	0.14	-0.29	0.20	-0.38
28	-0.10	-0.16	-0.02	-0.59	0.01	-0.09	0.11	0.61	0.06	0.40	0.40	-0.63	-0.28	-0.61	-0.08
29	-0.49	0.04	0.03	-0.06	-0.26	-0.03	-0.06	-0.07	-0.26	-0.01	0.19	-0.01	-0.30	0.00	-0.35
30	-0.18	-0.08	-0.05	-0.54	-0.06	-0.14	0.07	0.62	0.09	0.34	0.35	-0.69	-0.29	-0.62	-0.13
31	-0.43	0.11	0.09	-0.16	-0.13	-0.09	-0.01	0.20	-0.14	0.07	0.28	-0.22	-0.32	-0.20	-0.32
32	-0.14	-0.15	-0.02	-0.60	0.00	-0.11	0.12	0.61	0.06	0.39	0.39	-0.70	-0.27	-0.64	-0.09
33	-0.50	0.12	0.05	-0.03	-0.27	0.05	-0.08	-0.02	-0.21	-0.02	0.12	-0.06	-0.35	-0.06	-0.38
34	-0.07	-0.24	0.15	-0.13	0.16	0.09	0.01	-0.08	0.02	0.03	0.03	0.07	0.06	-0.01	0.11
35	0.05	0.07	0.07	0.30	-0.01	0.25	-0.14	-0.40	-0.15	-0.31	-0.26	0.43	0.17	0.49	0.04
36	-0.08	-0.12	-0.05	-0.34	-0.07	-0.34	0.17	0.24	0.20	0.16	0.32	-0.41	-0.17	-0.51	-0.16
37	-0.10	0.12	-0.06	0.04	-0.23	-0.13	-0.29	0.08	-0.11	-0.09	-0.10	0.01	-0.16	0.01	-0.30
38	-0.06	0.29	0.00	0.37	-0.20	0.08	-0.27	-0.32	-0.14	-0.44	-0.29	0.38	0.03	0.34	-0.14
39	0.06	-0.09	-0.01	0.00	-0.07	0.29	-0.24	0.14	0.00	0.12	-0.16	-0.06	-0.04	0.03	0.02
40	0.05	-0.12	-0.12	-0.07	0.04	-0.10	0.27	0.05	0.12	0.32	0.26	-0.18	-0.01	-0.10	0.06
41	0.08	0.02	0.16	0.17	0.13	-0.11	-0.11	-0.19	-0.06	-0.12	-0.23	0.26	0.19	0.28	0.16
42	0.06	-0.05	0.14	-0.08	0.08	-0.07	-0.04	-0.07	0.05	-0.02	0.03	-0.01	-0.09	-0.10	-0.06
43	0.00	-0.16	-0.10	-0.07	-0.09	0.12	-0.20	0.07	0.03	-0.04	0.02	0.00	-0.03	0.04	-0.01
44	0.00	-0.01	0.22	0.10	0.02	-0.02	-0.19	-0.12	-0.08	-0.33	-0.35	0.23	0.02	0.18	0.02
45	0.24	-0.12	0.01	0.08	0.15	0.10	-0.10	-0.03	0.26	0.12	-0.19	0.13	0.19	0.15	0.27
46	0.00	-0.16	0.11	-0.12	0.02	0.01	-0.04	0.09	0.02	-0.03	0.00	-0.01	-0.18	-0.04	-0.01
47	0.15	-0.11	-0.05	-0.05	0.01	0.16	0.03	0.07	0.07	-0.01	-0.13	0.00	-0.02	0.03	0.09
48	0.05	-0.24	0.10	-0.11	0.02	-0.17	0.09	-0.11	-0.03	-0.05	-0.04	0.06	0.05	0.00	0.15
49	-0.05	-0.07	0.18	-0.10	-0.02	-0.09	-0.06	-0.02	-0.27	-0.12	-0.23	0.13	0.04	0.02	-0.01
50	0.06	0.05	0.12	0.01	0.07	-0.19	0.00	-0.11	-0.02	-0.27	-0.21	0.12	0.02	-0.03	0.08
51	-0.13	-0.02	0.14	-0.12	-0.09	-0.32	-0.17	0.10	-0.12	0.00	0.05	-0.10	-0.15	-0.17	-0.14
52	0.08	-0.17	0.09	-0.01	0.04	-0.04	0.03	-0.02	0.00	-0.05	-0.16	0.13	-0.01	0.11	0.14
53	-0.10	-0.03	0.21	-0.23	0.01	-0.05	-0.06	0.21	0.00	0.06	-0.06	-0.20	-0.13	-0.23	-0.07

Table A12.1: continued from above

	CU	CV	CW	CX	CY	CZ	DA	DB	DC	DD	DE	DF	DG	DH	DI
1	R5-R20 %D	Post R35	R35 %D	Post abei5	abei5 %D	Post X5	X5 %D	Post X20	X20 %D	Post X35	X35 %D	Post res_freq	Res freq %D	Post AX	AX %D
55	-0.01	0.04	0.26	0.16	0.15	0.15	-0.08	-0.22	-0.08	-0.36	-0.23	0.35	0.04	0.24	0.09
56	-0.15	0.06	0.06	0.01	-0.10	-0.11	-0.16	-0.03	-0.01	-0.17	-0.10	0.01	-0.23	-0.07	-0.18
57	0.01	-0.06	-0.06	-0.05	-0.01	-0.01	-0.09	-0.08	-0.23	-0.14	0.01	0.08	-0.03	-0.04	0.00
58	0.01	-0.03	0.14	-0.21	0.11	-0.24	0.24	0.20	-0.02	0.11	0.23	-0.19	-0.06	-0.19	-0.01
59	-0.07	0.17	0.08	0.31	-0.05	-0.08	0.05	-0.13	0.05	0.03	0.02	0.10	0.04	0.17	-0.01
60	-0.03	0.05	0.10	-0.06	0.06	-0.11	-0.03	-0.12	-0.10	-0.33	-0.20	0.12	0.18	0.00	0.04
61	-0.24	0.21	0.19	0.01	-0.14	-0.15	-0.19	-0.12	-0.09	-0.31	-0.27	0.00	-0.18	-0.12	-0.22
62	0.08	-0.16	0.01	-0.19	0.01	0.31	-0.07	0.07	0.02	-0.05	-0.04	-0.08	0.01	-0.07	0.03
63	-0.24	0.12	0.18	0.09	-0.16	-0.15	-0.11	-0.10	-0.21	-0.33	-0.22	0.10	0.02	0.01	-0.12
64	-0.03	0.01	0.06	0.06	-0.07	0.01	-0.17	-0.11	0.03	0.09	-0.03	0.03	-0.17	0.05	-0.15
65	0.05	-0.06	0.04	-0.09	0.07	0.00	0.06	0.10	-0.02	-0.17	-0.01	0.02	0.12	-0.07	0.06
66	-0.10	0.06	0.23	0.12	-0.05	-0.03	-0.17	-0.05	-0.03	-0.33	-0.31	0.18	-0.09	0.15	-0.04
67	0.16	-0.07	-0.06	0.13	0.02	0.04	-0.14	-0.04	0.18	0.18	-0.20	0.12	0.10	0.16	0.15
68	-0.02	-0.16	0.10	-0.06	-0.05	0.05	-0.12	0.01	0.01	0.03	-0.03	0.04	-0.20	0.02	-0.05
69	-0.08	0.04	-0.13	0.17	-0.19	-0.14	-0.17	0.03	0.09	0.15	-0.06	0.05	-0.09	0.18	-0.09
70	0.00	-0.17	0.08	-0.13	-0.05	-0.08	0.02	-0.09	-0.05	-0.26	-0.18	0.05	0.00	-0.03	0.07
71	-0.06	-0.03	0.25	-0.14	0.01	-0.04	-0.13	0.10	-0.18	-0.07	-0.23	0.00	0.01	-0.09	0.01
72	0.06	-0.03	0.06	-0.06	0.04	-0.17	-0.02	-0.09	0.00	-0.27	-0.14	0.09	-0.01	-0.04	0.06
73	-0.10	0.05	0.04	-0.07	-0.09	-0.13	-0.25	0.07	0.07	0.21	0.04	-0.12	-0.20	-0.07	-0.18
74	0.05	-0.19	0.06	0.01	0.01	-0.03	-0.01	-0.05	0.00	-0.03	-0.17	0.16	-0.04	0.15	0.10
75	-0.10	0.01	0.28	-0.20	0.02	-0.02	-0.06	0.18	-0.05	-0.08	-0.14	-0.14	-0.13	-0.26	-0.07
76	-0.10	0.10	0.21	0.22	-0.05	0.25	-0.11	-0.19	0.00	-0.43	-0.37	0.30	-0.01	0.26	0.00
77	-0.02	0.05	0.26	0.18	0.14	0.16	-0.08	-0.24	-0.08	-0.37	-0.23	0.37	0.05	0.26	0.08
78	-0.06	0.01	-0.06	0.04	-0.10	0.01	-0.18	-0.08	0.06	-0.06	-0.10	0.05	-0.16	0.03	-0.11
79	0.03	-0.04	-0.02	-0.05	0.01	-0.01	-0.05	-0.06	-0.22	-0.17	0.01	0.07	-0.03	-0.06	0.00
80	0.05	-0.02	0.13	-0.09	0.13	-0.02	0.15	0.05	-0.05	-0.22	-0.02	0.01	0.11	-0.09	0.11
81	-0.03	-0.09	0.15	0.02	0.05	-0.02	0.05	-0.07	0.10	0.16	0.12	-0.04	-0.02	0.02	0.02
82	-0.23	0.65	-0.19	0.79	-0.53	0.05	-0.32	-0.41	-0.12	-0.30	-0.25	0.40	-0.25	0.54	-0.46
83	-0.20	0.81	-0.19	0.61	-0.51	-0.15	-0.21	-0.15	-0.09	-0.13	-0.12	0.05	-0.44	0.20	-0.57
84	-0.28	0.08	-0.03	0.49	-0.29	0.10	-0.29	-0.49	-0.26	-0.34	-0.26	0.58	0.09	0.60	-0.19
85	-0.18	0.78	-0.33	0.56	-0.52	-0.14	-0.23	-0.10	-0.02	-0.07	-0.08	0.00	-0.34	0.20	-0.52
86	-0.20	0.61	-0.19	0.81	-0.52	0.09	-0.34	-0.43	-0.12	-0.30	-0.26	0.43	-0.20	0.58	-0.43
87	0.11	-0.03	0.07	0.18	0.03	0.30	-0.27	-0.19	0.12	-0.16	-0.51	0.27	0.13	0.27	0.11
88	0.23	-0.32	-0.03	-0.53	0.30	-0.16	0.27	0.69	0.29	0.42	0.10	-0.61	0.19	-0.56	0.35
89	0.32	-0.31	-0.06	-0.36	0.33	-0.01	0.28	0.33	0.27	0.69	0.07	-0.45	0.32	-0.40	0.44
90	-0.22	0.41	0.00	0.71	-0.35	0.20	-0.40	-0.56	-0.25	-0.44	-0.22	0.66	-0.15	0.68	-0.38
91	-0.25	0.36	0.08	0.57	-0.31	0.10	-0.29	-0.57	-0.33	-0.55	-0.16	0.65	-0.27	0.54	-0.43
92	0.04	-0.10	0.13	0.00	0.17	0.04	0.15	-0.01	0.17	0.23	0.08	-0.09	0.01	-0.06	0.10
93	0.12	0.00	0.04	0.02	0.28	0.25	0.18	0.10	0.20	0.15	-0.20	-0.04	0.17	-0.03	0.26
94	0.08	0.68	0.10	0.99	-0.03	0.10	-0.13	-0.53	0.04	-0.28	-0.30	0.60	0.13	0.74	-0.05
95	0.57	-0.18	0.46	0.05	0.96	0.22	0.35	-0.17	0.23	-0.02	-0.09	0.29	0.63	0.18	0.75
96	-0.22	0.92	0.22	0.81	-0.13	-0.05	-0.13	-0.25	-0.04	-0.20	-0.21	0.27	-0.17	0.41	-0.32
97	-0.07	0.02	0.67	0.19	0.67	0.07	0.14	-0.25	0.03	-0.16	-0.09	0.38	0.49	0.28	0.42
98	0.49	-0.17	-0.20	0.45	0.15	0.24	-0.02	-0.56	0.03	-0.27	-0.29	0.66	0.49	0.67	0.39
99		-0.28	-0.27	0.07	0.49	0.21	0.21	-0.17	0.35	0.03	-0.09	0.20	0.51	0.19	0.65
100	-0.28		0.23	0.67	-0.19	-0.09	-0.13	-0.10	-0.03	-0.14	-0.10	0.06	-0.29	0.26	-0.38
101	-0.27	0.23		0.10	0.50	0.03	0.12	0.02	-0.09	-0.11	-0.08	0.08	-0.01	0.03	0.12
102	0.07	0.67	0.10		-0.03	0.14	-0.09	-0.53	0.04	-0.25	-0.28	0.60	0.15	0.77	-0.01
103	0.49	-0.19	0.50	-0.03		0.19	0.51	-0.09	0.26	0.05	0.02	0.21	0.61	0.14	0.79
104	0.21	-0.09	0.03	0.14	0.19		-0.07	-0.16	0.21	-0.08	-0.38	0.24	0.27	0.27	0.29
105	0.21	-0.13	0.12	-0.09	0.51	-0.07		0.01	0.14	0.17	0.31	-0.04	0.36	-0.01	0.51
106	-0.17	-0.10	0.02	-0.53	-0.09	-0.16	0.01		0.11	0.54	0.23	-0.84	-0.40	-0.68	-0.17
107	0.35	-0.03	-0.09	0.04	0.26	0.21	0.14	0.11		0.13	-0.13	-0.09	0.33	0.05	0.44
108	0.03	-0.14	-0.11	-0.25	0.05	-0.08	0.17	0.54	0.13		0.37	-0.53	-0.15	-0.39	0.04
109	-0.09	-0.10	-0.08	-0.28	0.02	-0.38	0.31	0.23	-0.13	0.37		-0.31	-0.28	-0.23	-0.15
110	0.20	0.06	0.08	0.60	0.21	0.24	-0.04	-0.84	-0.09	-0.53	-0.31		0.48	0.81	0.23
111	0.51	-0.29	-0.01	0.15	0.61	0.27	0.36	-0.40	0.33	-0.15	-0.28	0.48		0.40	0.80
112	0.19	0.26	0.03	0.77	0.14	0.27	-0.01	-0.68	0.05	-0.39	-0.23	0.81	0.40		0.26
113	0.65	-0.38	0.12	-0.01	0.79	0.29	0.51	-0.17	0.44	0.04	-0.15	0.23	0.80	0.26	

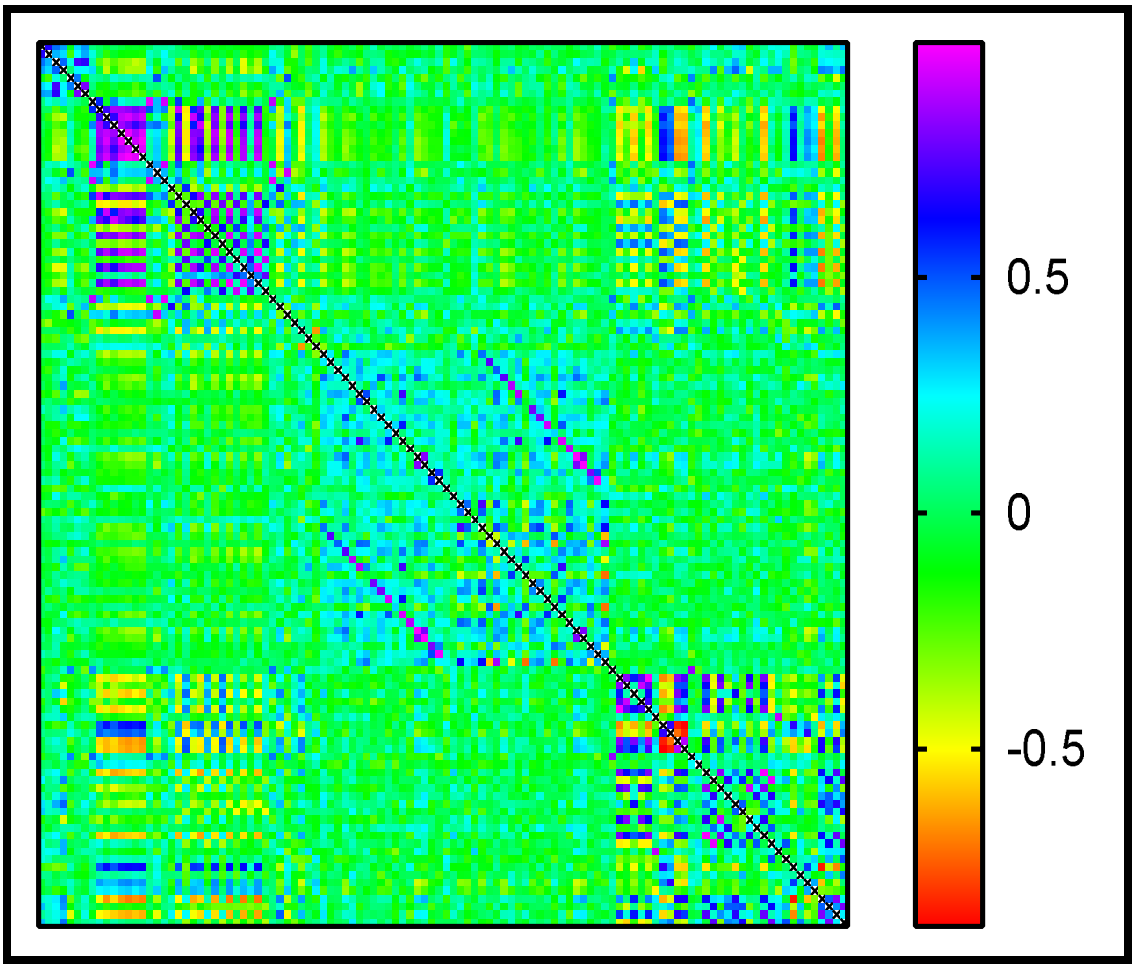


Figure A12.1: Heat map representing the Rho (v) values with different shades of colours representing the r value from -1 to +1, of all 113 variables analysed with each other in the correlation matrix

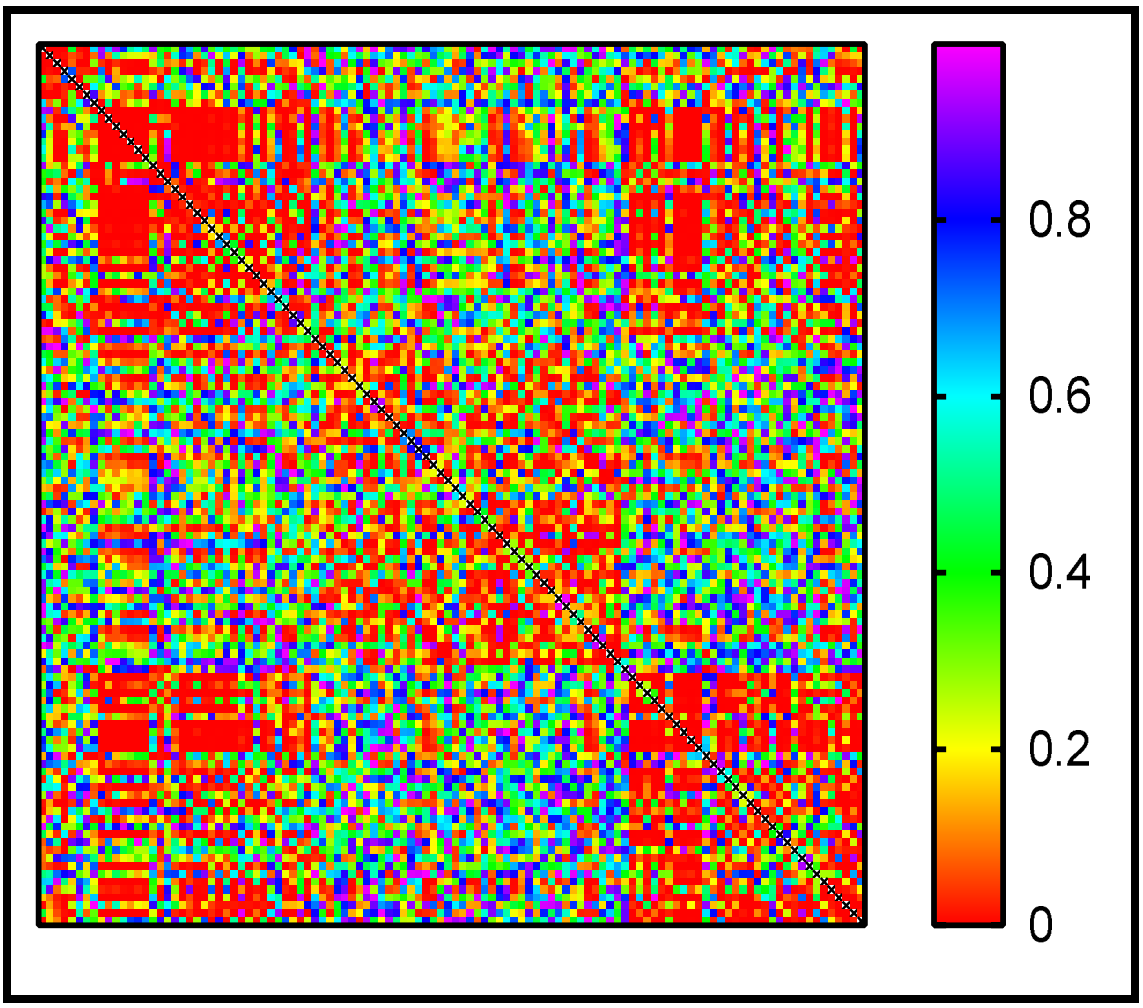


Figure A12.2: Heat map representing the p values with different shades of colours representing the p value from 0.00 to 0.99, of all 113 variables analysed with each other in the correlation matrix