Pharmacogenetics and chiral aspects of anti-asthma therapy

by

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Declaration

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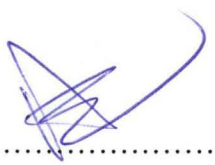

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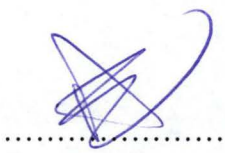

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Abstract

Introduction: Asthma is a chronic inflammatory airway disease characterised by recurrent symptoms. The available treatment options for the disease to date are concentrated on symptom control. Treatment responses to most anti-asthma medications show wide variation among populations and individuals. There is evidence that genetic variations can affect the pharmacokinetic and pharmacodynamic effects of medications, hence influencing treatment outcomes. The objective of this research is to investigate the effect of some common genetic variations in enzymes and proteins associated with the pharmacokinetic and pharmacodynamic properties of anti-asthma medications.

Method: Observational studies were first carried out to examine plasma levels of salbutamol enantiomers during disease exacerbation. Animal models were then employed to study the distribution of salbutamol enantiomers into various tissues and the role of the organic cation transport (OCT) system. The genetic determinants (single nucleotide polymorphism (SNP) in the SULT1A3 enzyme) on the pharmacokinetics of salbutamol enantiomers were investigated with a controlled clinical study. A retrospective study was employed to investigate the genetic determinants (microsatellite in ALOX5 and SNPs in ALOX5, LTC4S, LTA4H and cysLTR1) of treatment outcome (pharmacodynamics) with montelukast therapy. A comparison study was designed to explore the relationship between genetic variations (SNPs in activator protein-1 (AP-1) related genes) and refractory asthma, with the intention to gain more understanding of the role of genetic variations in the pathophysiology of airway inflammation.

Result: The studies revealed wide variability among individuals in both the pharmacokinetic and pharmacodynamic parameters of anti-asthma medications. The studies observed some relatively high levels (>20 ng/ml) and uneven presentation of salbutamol enantiomers (R:S ratio of 0.43) among patients who presented at the emergency department with disease exacerbation. Animal models indicated an enantioselective uptake and disposition of salbutamol (R:S ratio ≥2.9) in cardiac and skeletal muscle, but the model was unable to demonstrate the role of OCT systems. The study did not find significant genetic influences in the pharmacokinetic parameters of (R)- and (S)-salbutamol, mean difference (95%CI) in AUC (0-4h) were -1.1 (-38 – 36) and -5.8 (-97 – 88) respectively. There was also no significant relationship found between genetic determinants and the treatment response to montelukast ($p=0.12$ for ALOX5 microsatellite, $p>0.35$ for all SNP determinants). The study examined patients with refractory asthma and found no significant differences in genetic determinants at AP-1 related genes ($p>0.40$) compared to 'regular' asthma.

Discussion: The results suggest that salbutamol enantiomer levels in the circulation are widely variable between individuals. This work also suggests that there are no overwhelming pharmacogenetic effects in the anti-asthma medications examined. The sample size of the studies and the heterogeneous nature of genetic effects should be taken into consideration in future asthma pharmacogenetic studies.

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"*Asthma is a chronic disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. During an asthma attack, the lining of the bronchial tubes swells, causing the airways to narrow and reducing the flow of air into and out of the lungs.*

The causes of asthma are not completely understood. However, risk factors for developing asthma include inhaling asthma "triggers", such as allergens, tobacco smoke and chemical irritants. Asthma cannot be cured, but appropriate management can control the disorder and enable people to enjoy a good quality of life."

(World Health Organisation (WHO) definition of asthma in 2007 [1])

Abbreviations

AAI	Acute Asthma Index
ADRB1	β_1-adrenoceptor gene (gene ID)
ADRB2	β_2-adrenoceptor gene (gene ID)
AGRF	Australian Genome Research Facility
anti-LTs	anti-leukotrienes
ALOX5	5-lipoxygenase gene (gene ID)
AP	antarctic phosphatase
AP-1	activator protein-1
ASM	airway smooth muscle
AUC	area under the curve
B cell	bone marrow-derived lymphocytes
β-agonist	β-adrenergic-receptor-agonists
β_2-agonist	selective β_2-adrenergic-receptor-agonists
BSL	blood sugar level
C_{max}	maximum concentration
CI	confident interval
COPD	chronic obstructive pulmonary disease
CRHR1	corticotropin releasing hormone receptor 1 gene (gene ID)
CSL	Central Scrience Laboratory (UTAS)
CYP	cytochrome P450
cysLTR1	cystenyl-leukotriene receptor-1 gene (gene ID)
DDD	defined daily dose
DEM	Department of Emergency Medicine (RHH)

DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECG	electrocardiogram
ECM	Extra-cellular matrix
EDTA	ethylenediaminetetraacetic acid
Exo	exonuclease I
FEV_1	force expiratory volume in 1 second
FOS	c-Fos gene (gene ID)
GR	glucocorticoid receptor
HPLC	high performance liquid chromatography
HR	heart rate
IFN	interferon
ICS	inhaled corticosteroids
IL	interleukins
JNK	c-Jun-N-terminal-kinase (protein)
JNK1	c-Jun N-terminal kinase 1 gene (gene ID, also known as MAPK8)
JUN	c-Jun gene (universal gene ID)
LABA	long acting β_2-agonist
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography – hybrid mass spectrometry
LFT	lung function test
LLoQ	lower limit of quantification
LTA4H	leukotriene A4 hydrolase gene (gene ID)
LTC4S	cys-leukotriene-C_4-synthase gene (gene ID)

MDI	metered dose inhaler
MS	mass spectrometry
MTD	maximum tolerable dose
NAC	National Asthma Council (Australia)
OCT	organic cation transporter
PCR	polymerase chain reaction
PDE	phosphodiesterase isoenzymes
PEF	peak expiratory flow
QTc	corrected QT (interval)
rac-	raecemic mixture
RHH	Royal Hobart Hospital
RSD	relative standard deviation
SIA	steroid insensitive asthma (categorised subject group)
SD	standard deviation
SDS	Sodium Dodecyl Sulfate
SMA	spinal muscular atrophy
SMN	survival motor neuron
SNP	single nucleotide polymorphism
SPE	solid phase extraction
SSA	steroid sensitive asthma (categorised subject group)
SULT	human cytosolic sulfotransferase
SULT 1A3	human catecholamine sulfotransferase
SULT1A3	human catecholamine sulfotransferase (gene ID)
T cell	thymus-derived lymphocytes
$T_{1/2}$	half life

TAE buffer	Tris-acetate EDTA buffer
Th-cell	T-helper cell (also identified as T_H cell)
TNF	tumour necrotic factor
Treg cell	regulatory T cell
UTAS	University of Tasmania
UV	ultra violet (light)
WHO	World Health Organisation
W/T	wild-type (genotype)

Chapter 1

Background

1.1 Epidemiology of asthma

Asthma is a chronic respiratory disease that affects people from around the word.[1-3] The WHO has estimated 300 million people (~5% of world population) suffer from asthma world wide.[1,3] It has also been estimated 255,000 people die from asthma per year, where 80% of the deaths occur in low-income and lower-middle-income countries.[1] In Australia, the prevalence of asthma is among the highest in the world,[2,4,5] with an estimated 3.5 million people diagnosed with asthma and 2 million people having active disease.[4,6,7]

Asthma affects people from all age groups, but is much more common in children.[1,2,4,8] Asthma affects 14-20% of children and 10-12% of adults in Australia, and is one of the leading causes of childhood admission to hospital.[4,6,9] Asthma related deaths among Australians are moderately high by international standards, and represent about 0.3% of all deaths, with 2/3 of the cases occurring in patients aged over 65 years.[4] Asthma is also found to be one of the leading causes of morbidity among Australians, leading to poor life satisfaction, physical inactivity, social inactivity and emotional problems.[4,5,9,10] Asthma was identified in the National Health Priority for Australia as 'a disease of significance' (with the fourth highest prevalence),[4] with significant social and economic impact on the Australian community (estimated AUD 500-750 million per year).[6]

There are more females than males who suffer from active asthma, but the incidence of asthma during a lifetime is higher in males.[4,11] The prevalence of asthma is also found to vary by 2-32% between countries and populations.[2,8,11-14] Asthma is more

common in English-speaking and Latin-American countries, but less common in Eastern-European and Asian countries.[11-15] In Australia, the incidence of asthma is higher in boys than girls during childhood, but is the opposite during adulthood.[4,9,11] The incidence of asthma is found to be higher in some population groups such as Indigenous Australians, and lowest in populations from non-English speaking backgrounds.[4,16,17] Geographic location and urbanisation are not found to be associated with the incidence of asthma in Australia, but asthma related death is more common in regional Australia and in socioeconomically disadvantaged areas.[4]

Over the last 50 years, the prevalence of asthma has increased significantly, especially in industrialised and urbanised areas.[1,14,18-21] Compared to the peak in the 1990s, the prevalence of asthma is found to be stable or even decreased, with improvement in the mortality and morbidity rates.[4-6,9,21-25] However, it is argued that there is little evidence of improvement in asthma management that corresponds to the observation.[22-24] In addition, due to inconsistent interpretation of asthma between studies and changing definitions of the disease over the years, the data should be interpreted with caution.[4,5]

1.2 Aetiology of asthma

Asthma is a disease influenced by multiple factors, including genetics, the immune system, environmental allergens, airway pathology and infections.[2,8,20,21,26-29] Combinations of various disease risk factors are believed to contribute to different asthma phenotypes.[21,26,30] A number of studies have indicated that genetic factors and a dysregulated immune response have a causal relationship with asthma.[20,21,26,28,29,31,32] In addition, environmental factors are found to be an important determinant in asthma development,[8,26,27] where exposures to certain stimuli are associated with increases in the prevalence of asthma.[20,21,26-28] These include smoking, chemicals, allergens and respiratory infections.[20,21,26-28] However, there is evidence that exposure to some allergens and respiratory infections during childhood may provide a protective effect against asthma.[26-28]

1.2.1 Immune system

The immune system is an important part of the body's defence mechanism, and environmental interactions play an important role in the system's development.[20,26,31] The cellular component of adaptive immunity has two primary groups of lymphocytes, the thymus-derived lymphocytes (T cell) and bone marrow-derived lymphocytes (B cells).[26,33] B cells are mainly found to regulate the humoral immunity (e.g. antibody production), where the T cells (which include T-helper cells (Th-cells), cytotoxic cells and killer cells) are mostly responsible for cellular immunity.[20,26,33,34]

The mature Th-cells are commonly characterised into two main groups (Th-1 and Th-2) depending on their response to, and the ability to produce, various cytokines.[20,26,29,31-33,35,36] Some cytokines, in particularly interleukins (IL) -2, IL-12, tumour necrosis factor (TNF) -α and interferon (IFN) -γ are mainly produced by the Th-1 cells (sometimes referred as Th-1 cytokines), where as cytokines such as IL-4, IL-5, IL-10 and IL-13 are mostly produced by the Th-2 cells (sometimes referred as Th-2 cytokines).[20,26,29,31-33,35] However, some cytokines are produced by both types of T-cells.[20,31-33] In general, the Th-1 cytokines are commonly associated with cellular immunity, whereas the Th-2 cytokines favour the promotion of humoral immunity such as inflammation and antibody production.[26,28,31,32,35,36]

The development of an individual's immune system starts from the time of conception.[26,36] It is believed that the immune development strongly leans toward the Th-2 immune response during foetus development, but matures during childhood with the development of the Th-1 immune response.[26,36] However, disturbance of the immune development results in immune response imbalance, where the dominance of Th-2 immune response is believed to cause atopic diseases such as asthma (the Th1/Th2 paradigm; Figure 1.1).[20,21,26,33,34,36,37]

In a typical airway model, the immune response is triggered by allergen(s) which intrude into the airway (Figure 1.2).[26,33] During the allergen intrusion, local cells in the airway produce cytokines and present the allergen to regional lymph nodes to activate the adaptive immune system.[26,32,38] Upon receiving the signals, the adaptive immune system produces antibodies and cytokines, in particularly Th-2 cytokines, to combat the intruding allergen.[26,31-33,38] During the immune response, mast cells and

T cells will also release chemo-attractant molecules (chemokinases) to initiate the migration of other inflammatory cells (e.g. eosinophils, macrophages and neutrophils).[26,31-33,38] In healthy individuals, the Th-1 cells will also be activated during the immune response and produce Th-1 cytokines, which are believed to serve as a feedback system to maintain the balanced immune response.[20,26,29,31-34] However, in asthmatic individuals, the immune responses are found to be dominated by the Th-2 responses.[20,28,29,32-34,36]

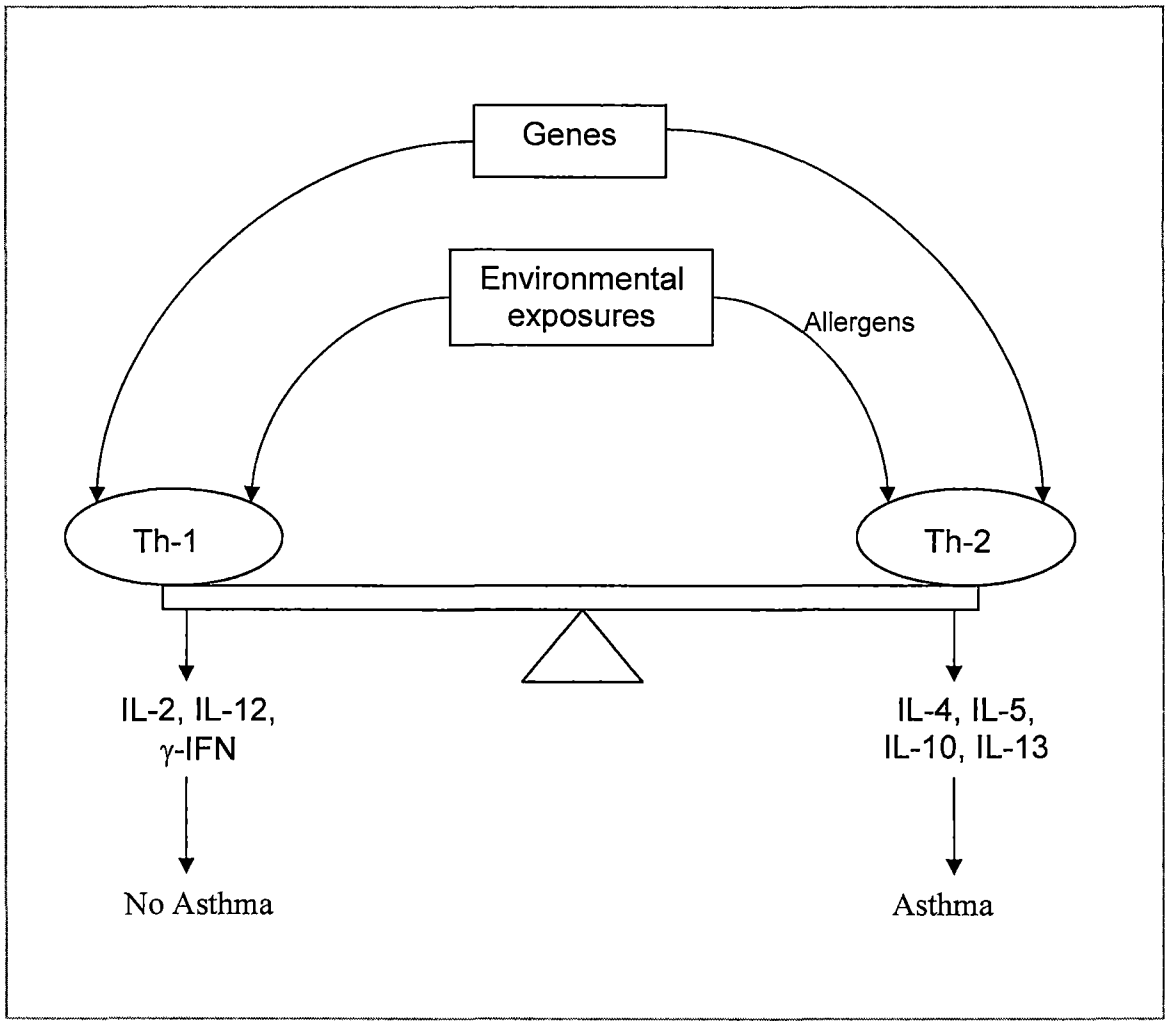


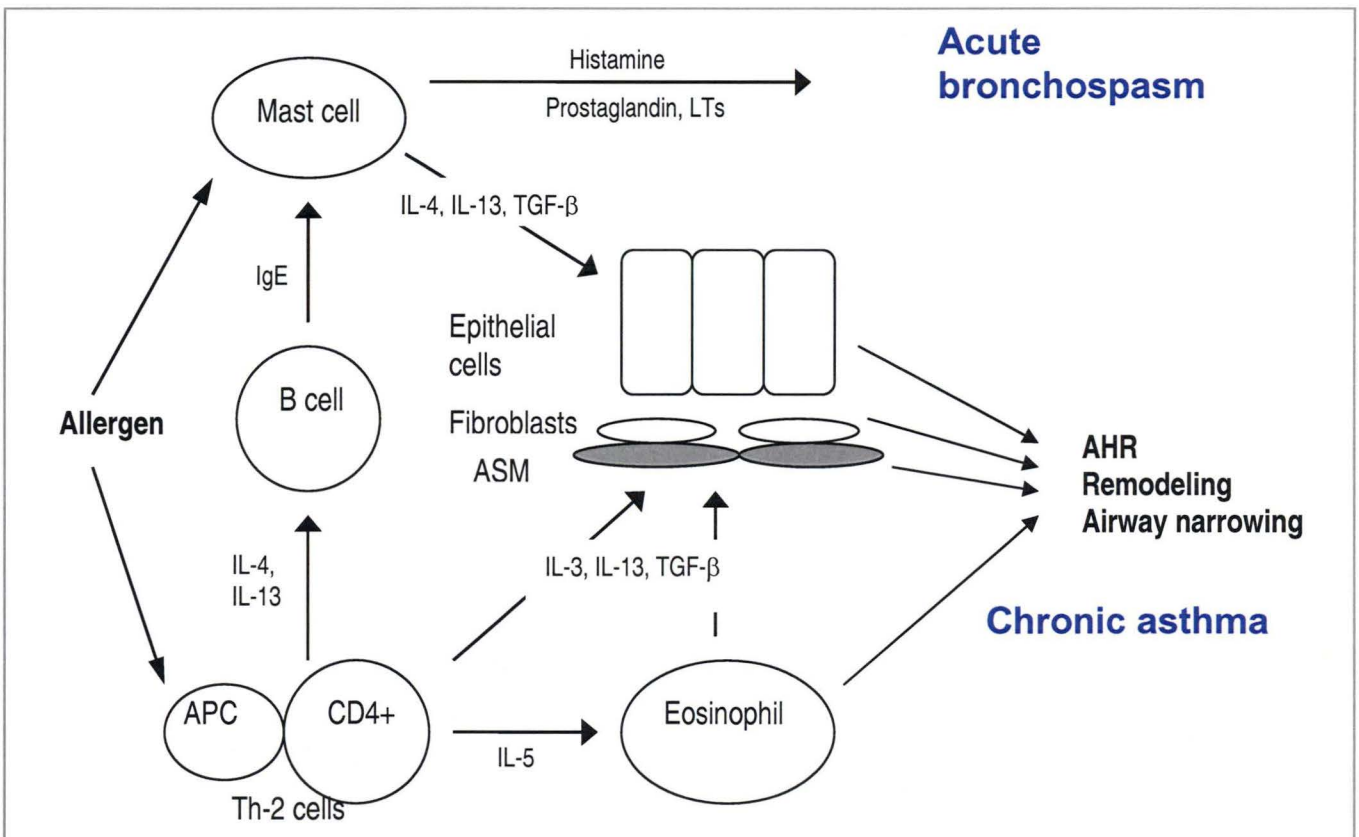
Figure 1.1 Role of Th1/Th2 paradigm in asthma. (Adapted from Maddox and Schwartz 2002 [26])

Table 1.1 T cell-related cytokines in asthma (Adapted form Larche, Robinson and Kay 2003[32])

Cytokine	T_H 1/T_H 2 /Treg associated	Actions	Producer cells in human patients	Animal models of airway inflammation	Human asthma
IL-2	T_H 1	T-cell growth factor	T_H 1 cells, Eos	Decreased	Increased
IL-3	T_H 1/T_H 2	Differentiation and activation of Eos, Neu, Baso, MC	T_H 1/T_H 2 cells, Eos, MC, Baso, Mac/Mono, Fib	Increased: blocking decreases eosinophilia	Increased
IL-4	T_H 2	B-cell switch to IgE synthesis, MC development, Eos and Baso activation, mucus, secretion, favors T_H 2 production, increased Endo VCAM expression	T_H 2 cells, Eos, MC, Baso	Increased; blocking decreases AHR (but some residual)	Increased; soluble IL-4 receptor had some steroid-sparing effect
IL-5	T_H 2	Eos, Baso differentiation, maturation, and activation	T_H 2, Eos, MC, Baso	Increased; knockout or blocking decreases Eos and AHR	Increased; antibody decreased eosinophils but not AHR
IL-6	T_H 1/T_H 2	T- and B-cell growth factor, cofactor for IgE synthesis	T_H 1/T_H 2 cells, Mac, Endo	Transgene showed increased AHR and inflammation, knockout less	Increased (in severe asthma)
IL-8		Neu activation-chemotaxis; weak Eos chemotaxis	Endo, Epi, Mac, Fib, T cells	IL-8 receptor knockout decreased AHR and neutrophils	Increased
IL-9	T_H 2	MC and Eos development, AHR, mucus secretion	T_H 2 cells, Eos, MC, Baso	Transgene had Eos, AHR, and mucus; knockout no effect	Increased
IL-10	Treg	Suppresses T_H 1/T_H 2 function, Eos/MC/Baso activation, favors Treg production, B-cell switch to IgG4	T cells, Mac	Adenoviral transfer decreased inflammation	Decreased/decreased
IL-11		AHR, eosinophilia, mucus hypersecretion, airway remodeling	Fib, Mac, Endo, Epi	Transgene had AHR and changes of remodeling	Increased
IL-12	T_H 1	Favors T_H 1 production, inhibits IgE synthesis	Mac, B cells	Exogenous IL-12 blocked eosinophils and AHR	Decreased; exoge-nous IL-12 decreased Eos but not AHR
IL-13	T_H 2	MC development, B-cell switch to IgE production, eosinophilia, AHR, mucus hypersecretion	T_H 2 cells, Eos, MC, Baso	Increased; soluble IL-13 receptor blocked AHR but not Eos or IgE	Increased
IL-15	T_H 1	T-cell growth factor; expands Treg	Many non–T cells	?	Decreased
IL-16		Chemoattractant for CD4 cells, Mono, Eos	$CD8^+$, MC, Eos	Increased	Increased
IL-17		Induces inflammatory cytokine production by Fib, Mac, Epi, Endo	CD4 memory cells	Provokes airway neutrophilia	Increased
IL-18	T_H 1	Induces IFN-γ production by T cells, NK cells; favors T_H 1 expansion	Mac	Knockout increased Eos and AHR, exogenous IL-18 (with IL-12) decreased AHR and Eos	Reduced
IL-23	T_H 1	Cofactor for T_H 1 development, activates DCs	Various hemopoietic cells	Not studied	Not studied
IL-25	T_H 2	Favors T_H 2 development and IL-4, IL-5, and IL-13 production	T_H 2 cells	Adenoviral transfer induces T_H 2 cytokines, Eos, AHR, and mucus	Not studied
IL-27	T_H 1	Favors T_H 1 expansion	APC	Not studied	Not studied
GM-CSF	T_H 1/T_H 2	Differentiation and activation of Eos, Neu, Easo, MC	T_H 1/T_H 2, Mac, Eos, MC, Baso, Fib, Epi, Endo	Increased; transgene has airway inflammation	Increased
TNF-α		Activation of Endo and Epi	Mac, NK cells, T cells	Increased	Increased
TGF-β	Treg	Suppresses T_H 1/T_H 2 function, favors Treg induction, cofactor for IgA secretion, fibrosis	Eos, MC, Baso, T cells, Mono, Mac	Increased	Increased
IFN-γ	T_H 1	Inhibits IgE synthesis, inhibits T_H 2 induction, activates Eos and Mac	T_H 1 cells, NK cells	Decreased	Increased in viral infection and exacerbations

Treg, T-cell regulatory cell; Eos, eosinophil, Neu, neutrophil, Baso, basophil; MC, mast cell; Mac, macrophage; Mono, monocyte; Fib, fibroblast; VCAM, vascular cell adhesion molecule; Endo, endothelial cell; Epi, epithelial cell; APC, antigen-presenting cell; TGF, transforming growth factor.

In recent years, epidemiological data has shown that, compared with non-atopic individuals, patients with atopic diseases also have a higher prevalence of the Th-1 dominated autoimmune diseases, including type I diabetes, inflammatory bowel disease and rheumatoid disease.[20,28,34] It is also found that the Th-1 and Th-2 cells are not always counteracting each other's function, and an unadulterated Th-1 response, rather than counteracting the effect of Th-2 response, may actually exacerbate the symptoms of atopic diseases such as asthma.[20,28,34,35,37] In addition, T-cell specific therapies have uniformly failed to improve asthma control in many clinical trials.[21] It has become apparent that the Th1/Th2 paradigm has oversimplified the immune system, and additional immunological principles must regulate the system and the



AHR – airway hyper-responsiveness; APC – antigen-presenting cell; IL – interleukins; LTs – leukotrienes; TGF – transforming growth factor; Th – T-helper cell.

Figure 1.2 A simplified overview of the allergen triggered immune response model of an asthmatic airway. (Adapted from Larche, Robinson and Kay 2003 [32])

related diseases.[20,21,29,34,35,37] In recent years, the regulatory T cells (Treg cells) are believed to play an important role in mediating the immune responses.[20,29,34,37] The Treg cells actively influence the activities of other immunological cells (Figure 1.3).[20,34,37] Although the mechanism is not fully understood, it appears to control the development of the individual's immune system and plays a role in the development of immunological related diseases such as asthma.[20,29,34,37]

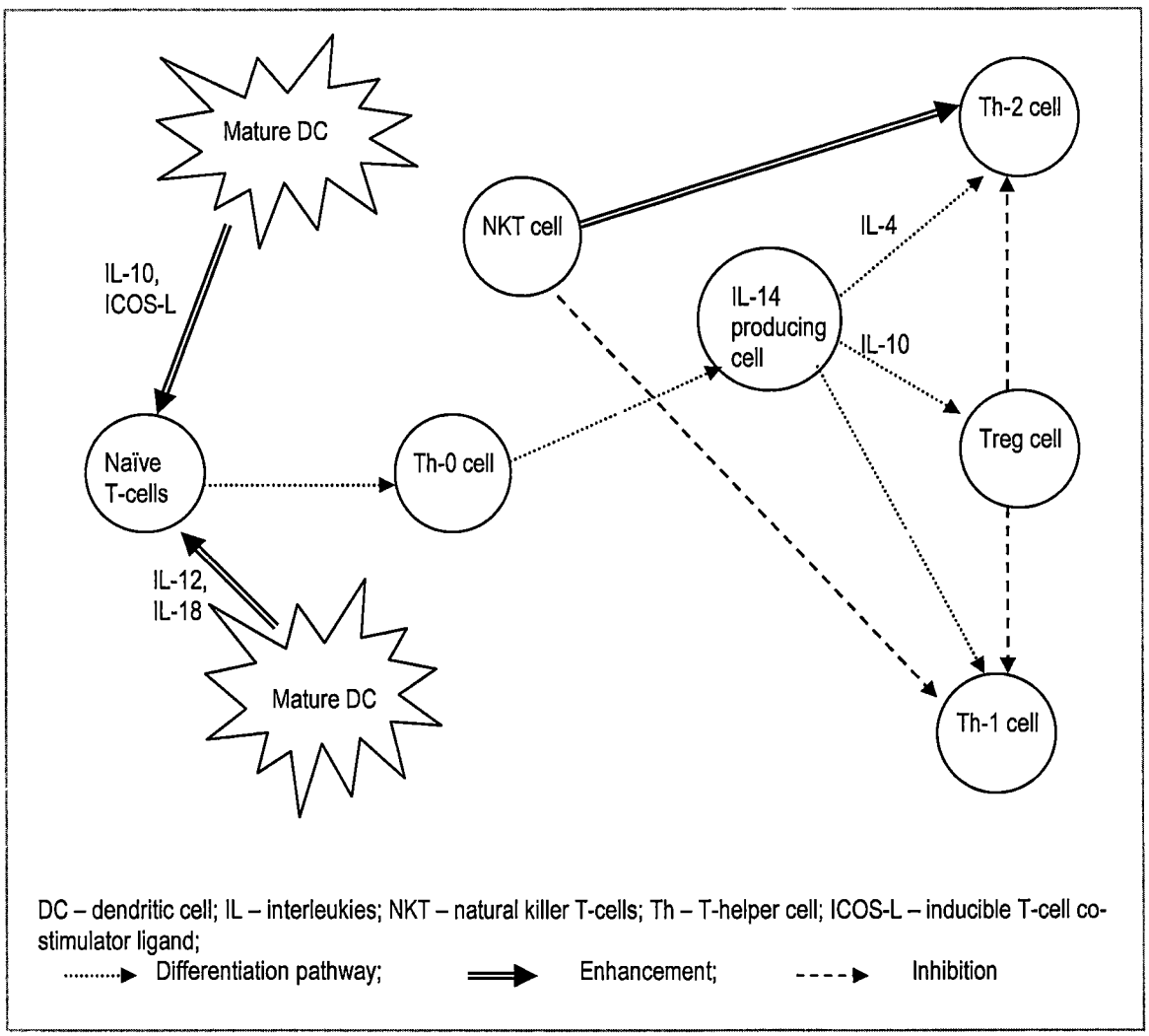


Figure 1.3 Role of Treg cells in asthma (Adapted from Umetsu et al 2003 [39])

It was previously believed that non-allergic individuals are unresponsive to allergens due to insufficient previous exposure, therefore lack allergen-specific immune response.[20,26,28,36] However, linkage studies have shown that these individuals have

been exposed to higher quantity of allergens than have asthmatic individuals.[20,26-29,36] Over the past few decades, the prevalence of asthma and other allergic diseases has increased dramatically, and found to be related to changes in environmental exposures, including decreases in childhood infections, reduced animal contact and increased in vaccination and antibiotic usage.[20,26-29,36] These findings have since given rise to the 'hygiene hypothesis', which suggests the lack of exposure to infections and antigens has prevented the maturation of the immune system and therefore contributes to the imbalance in immune responses.[20,26-29,36]

1.2.2 Genetics

Over the past few decades, many studies have sought to identify the genetic association with various diseases, including asthma, with mixed results.[26,40,41] To date, studies have indicated that asthma is unlikely to be the result of a single genetic-locus variation.[26,28,42,43] However, heredity has been found to play an important but complex role in asthma.[3,21,26,30,42,44-49] Many studies which have investigated the genetic association with asthma have particularly focussed on genes that related to IgE antibody production, cytokine production, airway cells and T cells responsiveness, and have had some positive findings.[3,21,30,42,44,45,47-53] In addition, studies have also found that genetic backgrounds from different populations are highly influential in the prevalence of asthma.[21,43,48,54]

To date, there are over 100 genes found to be related to asthma.[21,41,44,50,51] Many of the genes are found to be risk factors or protective factors for developing asthma (e.g. ADAM33), but the mechanisms remain unclear.[26,41,44-47,51,55] Table 1.2 lists some of

the candidate genes that have been identified with asthma. Most of the genes found to be related to asthma are concentrated at certain sections of the genome, in particular chromosomes 2q, 5q, 6p21, 11q13, 12q, 16q and 17q.[26,44,47,48,50,51] In addition, some studies have identified asthma associated with genetic variations, such as polymorphisms, on common functional genes (e.g. IL genes and β_2-adrenoreceptor genes; ADRB2).[26,44,45,56] These genetic variations are believed to affect the activity of the gene(s) and/or the encoded proteins, resulting in alteration of the airway function or response to therapy.[41,44,45,56-58] However, many of the findings are inconclusive and there is often inconsistency between studies.[26,44,47,48,50,51] It is believed that environmental factors, such as gene-environment interactions, play an important role in the outcome.[26,44,47,48,50,51]

Despite the advances in technology in genetic research, the clinical outcome of genetic influence(s) is often difficult to determine, because the outcome is also often affected by gene expression and the corresponding protein production.[44,57] Some studies have therefore suggested proteomic studies will elucidate the molecular basis of health and disease.[57] However, unlike the situation with nucleic acids, disease-protein associations are much more complicated because in addition to quality and quantity variations, proteins also exhibit complex dynamic behaviours such as protein-protein interactions, protein-environment interactions, regulation, local expression and relationships with other biochemical substances.[57]

Table 1.2 Candidate genes found related to asthma and asthma endotype from linkage and microarray studies. (Adapted from Anderson 2008 [21])

	Genetic	Biology
Lung function, basal FEV_1, airway hyper-responsiveness, ASM	EDN1, ADAM33, B2ADR, CREB, CCR5, COL29A1, CSTA, CYSLTR1, CYSLTR2, EP2, FCER2, GSTM, HNMT, KCNS1, LELP1, MMP, MUC7, MLCK, NK2R, PDGFRA, PLA2, PLAU, PTGDR, PTGER, PTGIR, TBX21, VDR	CREB, GSNOR, NOS, NR3C1
Immunity	FLG, IL17F, TGβ; IL6, RORα, RORγ, BDNF, chemokines, CD14, CD40,CD86, DPP10, FCER2, FLG, HLA-G, ICOS, IGHG, IL2, IL4, IL6, IL9, IL10, IL12B, IL13, IL16, IL17, IL18, IL27, IL33, IRAKM, ITK, MICB, MMP, MRP1, MUC1, NOD, PHF11, PLA2, PPARG, PTGDR, PTGER, RIP2, RUNX1, SFTPC, SOCS, SPP1, STAT6, TBX21, TIM1, VDR, VEGFR	CREB, HCK, IL23, IL33, LYN, NFATc, NOS, NR3C1, PTEN, RIP, ROR, SHIP, SHP, TSLP
Inflammation and remodelling	EDN1, ADAM33, IL17A, IL17F, NRF2, SOD, CREB, VDR, CAT, chemokines, COL29A1, CSTA, DPP10, ECP, EP2, FYN, GSTM, IGHG, IL2, IL5, IL9, IL13, IL17, IL18, IL33, PLA2, PLAU, SOCS, STAT6, TNFA, UTG, VEGFR	AMCase, ARG, C3AR1, c-kit, C3\5, EGFR, CSF2 (=GM-CSF), HCK, HMGB1, LYN, NOS, NR3C1, NRF2, PTEN, RAGE, RIP, SCF, SHIP, SHP, SOD, TIMP, TSLP
Resolution and repair	VDR, LEP	IL-10, FAS, NR3C1, RAGE, TIMP, Lipoxin A4 (15LOX, 5 LOX), presqualene phosphates
Exacerbations, smoke and environmental irritants	VDR, AOAH, CAT, CYP24A1, GSNOR, HLA-G, IL2B, IL2, IL6, IL12, IL17, IL23, IL33, IRAKM, MMP, MRP1, NOD, SFTPC, UTG, NRF2	CD200, EGFR, IFN, NR3C1,SOD, TIMP
BMI abd nutrition	ADRB2, FEBP, NR3C1, FABP4, NR3C1	
Perception	KCNS1, GAD65	
Genes of unknown function from linkage	DCNP1, GCLM, ORMDL3, SCGB3A2	

Definitions of the genes are available at the NCBI website OMIN database (http://www.ncbi.nlm.nih.gov/sites/entrez).

1.2.3 Airway smooth muscle and autonomic nervous system

Asthmatic airways are often found to have different cellular pathology compared to non-asthmatic airways, particularly the airway smooth muscle (ASM) which relates to most of the asthma symptoms.[21,59-61] The ASM tone is also influenced by multiple intrinsic and extrinsic mechanisms, in particular chemical mediators and innervations.[62,63] There are a range of signalling mediators (e.g. acetylcholine, histamine, bradykinins and leukotrienes) and pathways that mediate the ASM contraction and relaxation, which act on various G-protein-coupling-receptors (GPCRs) including muscarinic-3-receptors, histamine-1-receptors and cystenyl-leukotriene-1-receptors.[64] In addition, the airway wall, the ASM and the secretary glands are also innervated and mediated by the autonomic nervous system.[62,63,65-68] The autonomic nervous system at the airway is largely dominated by the parasympathetic nervous system, where the sympathetic nervous system only exerts its influence through circulating neurotransmitters.[62,65,66]

Abnormal structure and function of these systems is often found in asthmatic airways, and some of these findings have been used to derive hypotheses of the origin of asthma, in particularly the abnormal ASM cell hypothesis and the mast cell hypothesis.[38,59,69] However, most of these hypotheses are unable to appropriately accommodate all the pathogenesis findings from investigations over the years.[59,63,67-70] It is understood that most of these hypotheses have been derived from detailed *in vitro* study of individual cell types, but the dynamics of airway hyper-reactivity and inflammation are believed to be much more complicated.[68,69]

1.3 Pathogenesis of asthma

Asthma is a disease characterised by airflow limitation and airway inflammation, which involves complex mechanisms that are not yet fully understood.[27,36,71,72] Throughout their lifetime, some asthmatic patients will achieve disease remission but others may experience relapses or continuous symptoms.[8,27,36]

Although not fully applied to all asthma phenotypes, an inhaled allergen challenge and the two-phase reaction model is the best way to illustrate the mechanism of asthma exacerbation.[73] Under the model, asthma reaction is triggered by an intruding allergen that activates local airway cells, in particular the mast cells and the macrophages, to release IgE antibodies and pro-inflammatory mediators.[8,32,33,38,72,73] The release of these substances results in contraction of ASM, increased mucous secretion and airway vasodilatation, which are the characteristic features of the early phase reaction.[38,73] The pro-inflammatory mediators and cytokines released during the early phase reaction also promote the recruitment and activation of inflammatory cells such as eosinophils.[8,26,31-33,38,73] These inflammatory cells then maintain and intensify the airway inflammation, resulting in further airway obstructions, which form the characteristics of the second phase reaction.[26,31-33,38,73]

Airway inflammation is a key component of asthma pathology that appears to involve the whole bronchial tree, and with complex inter-cellular interactions.[69,71-74] Although the asthma symptoms are often characterised as reversible airflow obstructions, recent studies indicate that the lung function of chronic asthmatic patients continues to decline.[26,73,75,76] This observation indicates that the airway obstructions may not be

fully reversible and this is likely due to the changes in airway structure.[26,73,75,76] Although the exact mechanism remains unclear, continuous inflammation is believed to play an important role in airway remodelling by promoting cellular de-differentiation and connective tissue deposition.[8,26,72,73] The structural changes of asthmatic airways are found to involve both the airway epithelial and sub-epithelial layers, and are also believed to be responsible for the resistance to therapy.[21,69,73,74,77] To date, the majority of studies that have investigated airway remodelling are mainly focussed on the ASM.[74,76]

The long-term effects of asthma have the characteristics of chronic inflammation, persistent airway hyper-reactivity and airway remodelling.[8,26,73] The airway wall of asthmatic patients also exhibits structural and cellular abnormalities, including inflammatory cell infiltration, ASM hypertrophy and hyperplasia, mucus gland hyperplasia, thickening of the lamina reticularis, thickening of the sub-epithelial extracellular matrix, increased vascularisation, and increased in myofibroblasts and epithelial cell denudation.[8,59,61,69,71,73]

1.3.1 Inflammatory cell infiltration

Inflammatory mediators such as chemokines play an important role in promoting the recruitment, activation and survival of the inflammatory cells that are important in sustaining the airway inflammation.[38,73,76] Inflammatory cells that are commonly found recruited to the asthmatic airway are eosinophils, lymphocytes, basophils, macrophages and neutrophils.[71-73] Inflammatory cell infiltration, in particular eosinophilia is one of the common pathological characteristics of asthma, and plays

an important role in orchestrating the second phase reaction as well as maintaining the airway inflammation.[71-73] Although the degrees of eosinophilia differ among individuals, it is commonly found that the activated eosinophils are significantly elevated in the airway epithelial and submucosal layers among asthma patients.[69,71,73]

1.3.2 Airway smooth muscle mass

The ASM cells are located at the outer region of the airway subepithelial layer that surrounds the airway from trachea to alveolar ducts, and is the primary component of controlling the airway resistance.[59,61,74] Many cytokines, chemokines and growth factors released during airway inflammation are known to modulate ASM activities.[64,73,74,76]

ASM hypertrophy and hyperplasia is one of the common pathological characteristics of the asthmatic airway, and is believed to be the result of airway remodelling.[59,61,69,71-74,76] Although myocyte hyperplasia is a common finding in asthmatic airways, the findings of myocyte hypertrophy are inconsistent.[69,76] Nonetheless, the abnormal ASM cells are believed to cause most of the symptoms of asthma and airway hyper-responsiveness. [59,61,71,74,76] It is also suggested that the ASM may mediate the inflammation process.[59,61,74,76]

1.3.3 Mucous glands and airway epithelium

Changes in the pathology of mucosal glands in severe asthma are associated with abnormal mucus secretion, both quantitatively and qualitatively.[69,71,73] However, other than goblet cell hyperplasia and hypertrophy, changes of airway epithelial

layers among asthmatic subjects are inconclusive and remain the subject of many debates.[59,69,73]

1.3.4 Reticular basement membrane

The basement membrane of the airway is made up of a basal lamina membrane and laminar reticularis (composed of immunoglobulins, collagen, fibronectin and various types of cells), where the thickened laminar recticularis is often found in chronic asthmatic airways.[69,71,73] However, the thickening of the laminar reticularis is not unique to asthma, and is also commonly found in rhinitis patients and chronic obstructive pulmonary disease (COPD) patients.[73]

1.3.5 Extra-cellular matrix (ECM)

The airway subepithelial layer also contains a complex structure of protein known as ECM, which is believed to play a critical role in maintaining the airway's structure and function.[73] In asthmatic subjects, the subepithelial layer is found to be thicker and with abnormal composition of ECM, including higher composition of fibronectin, collagen, hyaluronan and laminin.[59,69,73,76] Alteration of the ECM is also believed to affect the growth and the function of ASM cells.[76]

1.3.6 Blood vessels

Increases in number and size of blood vessels, as well as increases in the endothelial gap in bronchial mucosal microvasculature, are the other common features of chronic asthmatic airways.[69,72,73,76] It is believed that the microvascular remodelling

contributes to airway oedema and increased mucus/sputum secretion during airway inflammation, as well as enhancing airway inflammation, airway hyper-responsiveness and myocyte hyperplasia.[69,73,76]

1.4 Diagnosis and Management

To date, there is no clear definition for the disease of asthma, and asthma is generally recognised and diagnosed by the symptoms including recurrent episodes of wheezing, breathlessness, chest tightness, cough and airway inflammation.[3,5,7,8,30,78-80] Since asthma was recognised as a major public health issue in the mid-1980s, a number of national and international guidelines were developed to help practitioners and patients make appropriate health care decisions.[81,82] Many of the current guidelines also recommend the severity (during diagnosis) and control (during follow-up) approach for systematic asthma assessment.[3,7,30,81,82]

In most patients, the identification of asthma is relatively straightforward, being mainly based on the presenting symptoms, change in lung function, biochemical measurements, medical history review and improvement with treatment.[3,7,8,30,78-81,83] However, confirmation of diagnosis can be difficult in some patients, in particular patients with respiratory co-morbidity and among young children.[3,7,8,30,78-80,83] In addition, most of the diagnostic features for asthma are not unique, and it is not necessary that patients with asthma exhibit all of the symptoms.[8,78-80] Over the years, some of the key characteristic symptoms and diagnostic features that are used for identifying asthma have also changed, due to redundant advances in knowledge.[8,80,81,84]

To date, there is no cure for asthma, and the primary treatment options are to provide optimal symptom management, including environmental control, utilisation of pharmacotherapy, education and self management.[3,7,8,30,78,79,81,85,86] Most of the current

national and international guidelines have emphasised the importance of environmental control in the management of asthma, including both the primary prophylaxis strategy (eg. avoiding triggering factors and immunotherapy) and the secondary prophylaxis strategy (e.g. smoking cessation and avoiding environmental pollutants).[3,7,30,81] Educational strategies (for practitioners, patients and the public) and self management strategies (treatment compliance and individual asthma action plans) are also extensively discussed and recommended in the guidelines.[3,7,30,81,82] However, the most effective and most extensively covered topic of asthma management is pharmacotherapy.[3,7,30,81]

1.4.1 Pharmacotherapy

The use of medication is a major part of managing the disease of asthma.[8,79,83,85,87] Anti-asthma medications have various mechanisms of action, but with the common objective of achieving and maintaining control of the asthma symptoms.[8,73,78,79,83] According to various guidelines, most of the current mainstream anti-asthma medications are categorised into two main groups, the reliever medications and the controller medications.[3,7,30,81] In addition, most studies and guidelines suggest combinations of various therapies and a stepwise pharmacological approach (Figure 1.4) to best manage the asthma symptoms.[3,7,8,30,78,88]

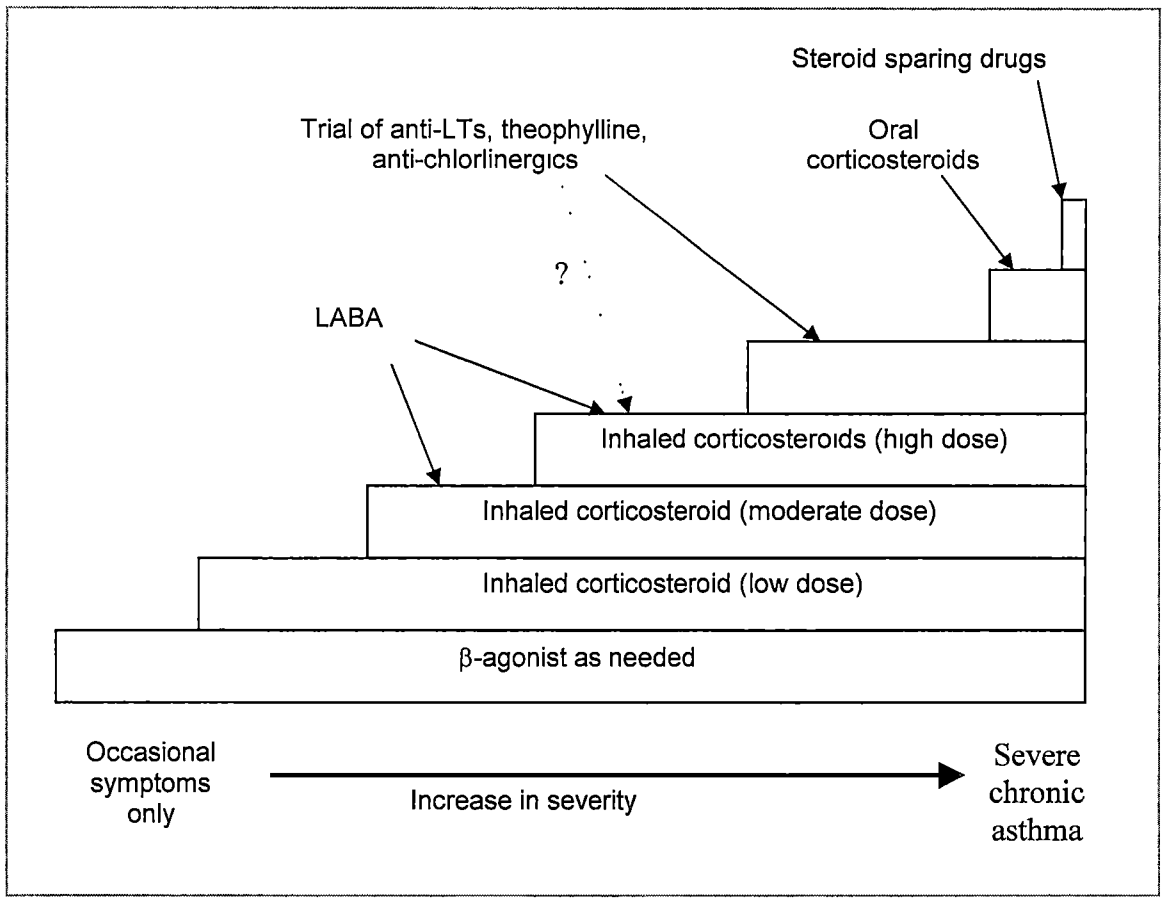


Figure 1.4 Stepwise pharmacological approach of asthma management (Adapted from Tattersfield et al 2002 [8])

1.4.1.1 Reliever medications

The primary characteristic of reliever medications is to provide immediate relief for shortness of breath and acute symptoms occurring during asthma exacerbation.[3,7,8,30,83] The most commonly recommended reliever medications are rapid/short acting β_2-agonists, followed by anti-cholinergics.[3,7,30,81,83] Corticosteroids, in particular systemic corticosteroids, and theophylline are also used in acute severe asthma, where the use of anti-leukotriene agents is also being investigated.[3,7,30,83]

The objective of administering reliever medications is to provide rapid symptomatic relief during asthma exacerbation (e.g. inducing bronchodilatation), but it does not

necessarily eliminate the underlying problem such as ongoing inflammation.[8] According to current guidelines, patients who require regular use of reliever medications (e.g. more than twice a week) should be assessed by a practitioner, and the initiation or intensified treatment with controller medications is recommended.[3,7,30]

1.4.1.2 Controller medications

Controller medications are the foundation of current asthma management, which is intended to reduce the incidence and severity of asthma exacerbation.[3,7,30,81] Corticosteroids, in particular inhaled corticosteroids (ICS), are the most effective and most recommended controller medications in current practice.[3,7,8,30,73,77,81] Anti-leukotrienes and long acting β_2-agonists (LABA) are also found to be effective alternatives, but are often recommended in conjunction with ICS, in particular for patients with more severe symptoms.[3,7,30] Other controller medications include theophylline and mast cell modifiers (e.g. cromones), but they are usually considered as the second or third-line alternatives.[3,7,8,30,73] In addition, anti-IgE monoclonal antibody is also included in some of the more recent guidelines.[3,7,30,81]

1.4.1.3 β_2-agonists

β-adrenergic-receptor-agonists (β-agonists) are one of the oldest classes of drugs used in medical practice, and mimic the effects of endogenous catecholamines.[83,87,89,90] The β-agonists bind to β_2-adrenergic receptors (also known as β_2-adrenoceptor) that are found in abundance in the ASM, and result in smooth muscle relaxation.[87,89,90]

New generations of β-agonists (β_2-agonists) are designed to selectively interact with the β_2-receptors and with minimal interaction with the β_1-receptors, hence minimising adverse effects.[87,89,90] In current practice, β_2-agonists are commonly given as inhalers, which deliver the drug to the site of action (airway wall) and minimise the systemic absorption.[83,89] To date, there are two main groups of β_2-agonists, categorised by the onset and the duration of action.[89,91]

1.4.1.3.1 Rapid/short acting β_2-agonists

Rapid/ short acting β_2-agonists have the characteristic of fast onset (within a minute) and often with a short duration of action (4-6 h), with the peak of efficacy generally being achieved within 5 minutes.[89,92] Studies have shown that the rapid/short acting β_2-agonists are the most effective drugs available for inducing bronchodilatation, regardless of the cause of asthma exacerbation.[8,83,89,91] Some studies have also found that rapid/short acting β_2-agonists have anti-inflammatory effects and can reduce mucus secretion.[87] The commonly used rapid/short acting β_2-agonists include bitolterol, isoetharine, levalbuterol (pure (R)-salbutamol), metaproterenol, pirbuterol, reproterol, salbutamol (also known as albuterol) and terbutaline.[3,87,89,91]

Since their development in the 1960s, rapid/short acting β_2-agonists have been used as the first line treatment for acute asthma exacerbation, and play a vital role in asthma management and in emergency medicine.[8,83,89]

1.4.1.3.2 Long acting β_2-agonist (LABA)

LABAs have the characteristic of a long duration of action (usually >12 hr).[89-91] LABAs achieve the longer duration of action with a hydrophobic side chain, which anchors the drug molecule on the cell membrane next to the β_2-receptor and allows the molecule to interact with the β_2-receptor continuously.[89,90] LABA produce bronchodilatation effects similar to the rapid/ short acting β_2-agonist, but usually have a slower onset of action.[89] The LABAs that are commonly available for the treatment of asthma include salmeterol, eformoterol and formoterol, where they are all designed to be administered by inhalation.[89]

The early version of LABAs (e.g. fenoterol) was associated with increases in asthma mortality in the 1970s and was withdrawn from therapeutic use, but the new generation of LABAs have not been found to share the same mortality profile.[88,89,91] However, as a result of some recent investigations, many studies and guidelines have recommended that LABAs are to be used in conjunction with corticosteroids, and as prophylaxis agents.[88,89,91]

1.4.1.4 Corticosteroids

Corticosteroids are potent anti-inflammatory agents, which exert their anti-inflammatory effect by binding to and activating the glucocorticoid receptors (GR) in the airway cells and the inflammatory cells (Figure 1.5).[87,93-98] The activated GR then bind to inflammatory transcription factors (activated) and deactivate the transcription factors by forming a protein-protein complex.[93-98] The activated GR can also bind to the corresponding DNA and prevent it from binding to the transcription factors and

therefore reduce the production of inflammatory proteins and subsequently inhibit the inflammatory process.[93-99] Some studies have also found that corticosteroids exert their therapeutic effect by increasing the production of anti-inflammatory mediators.[94,95,97,99] Although the mechanisms of interactions are yet to be fully understood, studies have discovered that in any cell, there are between 10 and 100 genes that can be directly regulated by the GR, and many more genes are indirectly regulated.[93-98] The corticosteroids that are commonly used for the management of asthma include ICS (beclomethasone, budesonide, ciclesonide, flunisolide, fluticasone and triamcinolone), oral corticosteroids (e.g. prednisolone) and parenteral corticosteroids (e.g. hydrocortisone).[3,7,95,96,100,101]

Since the recognition of the role of inflammation in the pathogenesis of asthma, corticosteroids, in particularly ICS, have become the corner stone of the treatment of asthma.[8,77,88,90,95,96,102] Although some patients may be responsive to one corticosteroid but the not another, evidence to date suggests all corticosteroids are equally effective, at equivalent doses.[95,101] However, the desirable therapeutic dose and the dose-response relationship of corticosteroids are still the area of much debate.[95,100] Oral corticosteroids, although not being used regularly in current practice, retain an important role in asthma therapy, in particular in treatment of resistant asthma and in emergency medicine.[83,95,96,103]

Although effective, regular use of oral corticosteroids often produces undesired systemic effects that limit the widespread use of the medication.[95,96,101] The development of locally acting corticosteroids, administered as inhalers, has shown

outstanding efficacy in asthma control and significantly improves the side effect profile.[95,96,101]

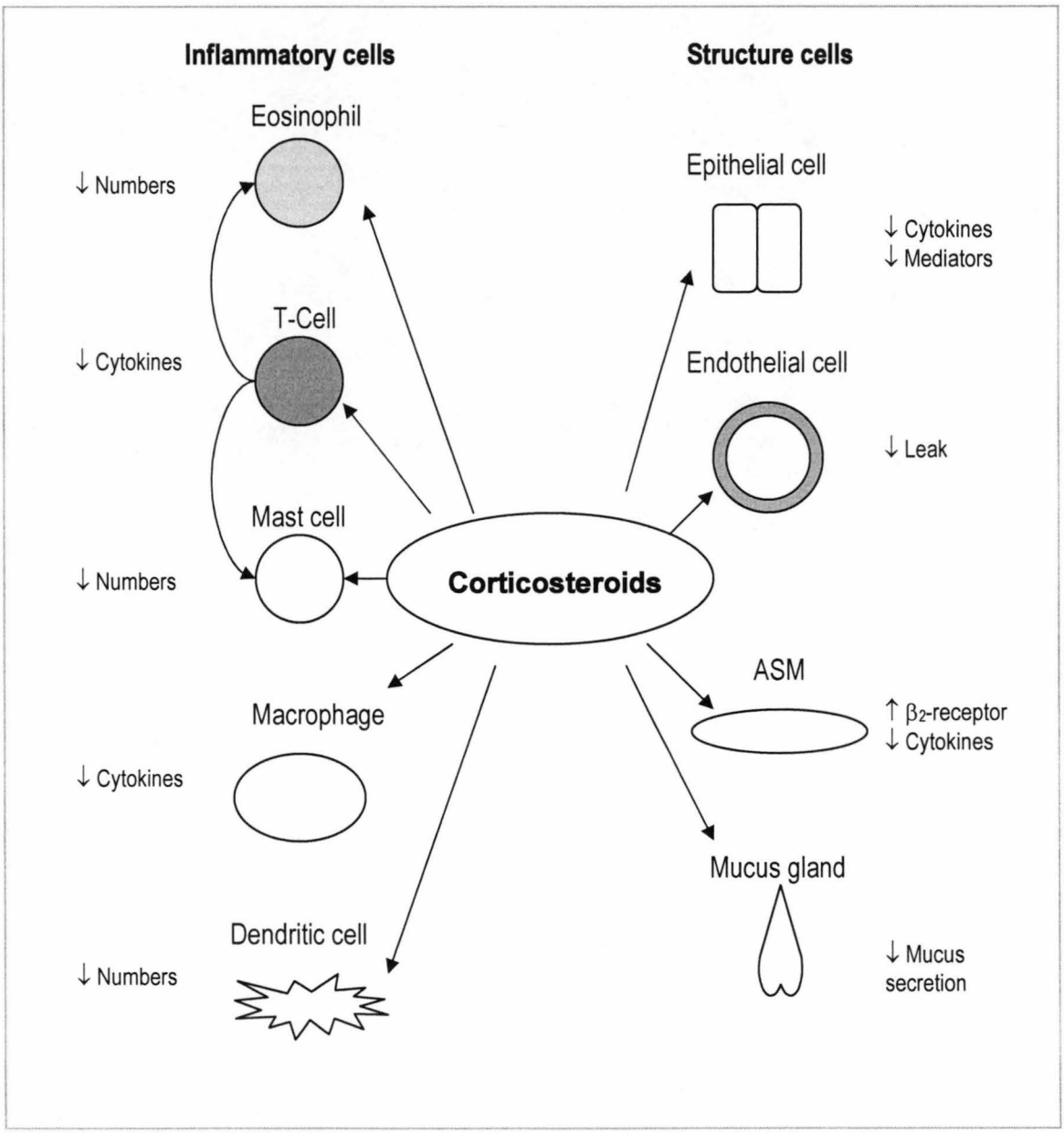


Figure 1.5 Cellular effect of corticosteroids (Adapted from Barnes 2003 [93])

1.4.1.5 Anti-leukotrienes

Anti-leukotrienes (anti-LTs) are a new group of medications designed to reduce the influence of LT, a group of potent inflammatory mediators that play an important role

in asthma pathogenesis.[104,105] Anti-LTs exert their therapeutic effects by either blocking the LT receptor(s) or inhibiting LT production.[105] Anti-LTs include three drugs that inhibit the cysteinyl-leukotriene type-1-receptor (montelukast, zafirlukast and pranlukast) and a leukotriene synthesis inhibitor (zileuton).[104,106]

The anti-LTs are designed to be administered orally. They have a fast onset of action (within 15 min), and will exhibit significant efficacy within a few days, with regular dosing.[106,107] However, the full efficacy of anti-LTs was often observed after 1-3 months of treatment.[106-108] The anti-LTs have both anti-inflammation and bronchodilation effects, and are found to provide additional anti-inflammation and bronchodilation effects when co-administered with corticosteroids and β_2-agonists, respectively. [104,106,107,109-111] The anti-LTs are found to be effective in preventing and reducing the severity of asthma symptoms, in particular allergen-induced asthma, exercise-induced asthma and aspirin-induced asthma [106-108,112] as well as being effective in treating rhinitis (which often co-exists in asthma patients).[8,107,108] However, evidence has suggested that anti-LTs are slightly inferior to ICS in reducing airway inflammation and asthma symptoms.[104,106,113]

It is suggested anti-LTs are effective in inhibiting some inflammatory pathways that are not properly suppressed by corticosteroids.[111] In addition, studies have shown that anti-LTs have matching efficacies with LABA,[104,107,110] and can be used as steroid sparing anti-asthma medications.[109,113]

1.4.1.6 Anti-cholinergics

Anti-cholinergics comprise a group of anti-asthma medications used since the 1800's, which exert their therapeutic effects by competitively antagonising the actions of acetylcholine.[83,87,114,115] Acetylcholine is a common neurotransmitter of the parasympathetic nervous system, which influences ASM activities.[114,115] The commonly used anti-cholinergics are atropine, ipratropium, thiazinamium, oxitropium and glycopyrrolate.[114]

Anti-cholinergics are commonly delivered by an inhaler, and have been shown to produce an improvement in airway calibre of mild asthmatics that is comparable to that of the β_2-agonists.[83,87,90,115,116] However, due to their slower onset of action, lack of anti-inflammatory effect and ineffectiveness in reversing the non-cholinergic triggering of bronchoconstriction (e.g. in allergen induced asthma), the therapeutic role of anti-cholinergics in asthma management is inferior to that of the β_2-agonists.[87,115,116] When administered in conjunction with β_2-agonists, anti-cholinergics have been found to produce additional bronchodilatation and lower hospital admission rates,[117-119] in particularly among children and patients with severe asthma.[83,114-116,120,121]

The earlier generation of anti-cholinergics (e.g. atropine) had serious unwanted systemic effects that limited their therapeutic role in asthma management.[114-116] However, the new generation anti-cholinergics have overcome most of these issues by selectively targetting the M_1 and M_3 receptors and/or having reduced lipid solubility (e.g. ipratropium, which contains a quaternary ammonium group) to minimise

systemic absorption from the lung.[90,114-116,118] The long acting selective-M_3 anti-cholinergic (tiotropium) has been found to be effective in managing COPD, but its role in asthma management is yet to be confirmed.[114]

1.4.1.7 Xanthines/ Theophylline

Xanthine derivatives (eg. theophylline, aminophylline, oxtriphylline, dyphylline and caffeine) have been used in the treatment of asthma for nearly a century.[83,87,122] However, most studies which have investigated the xanthines were only conducted with theophylline. The exact mechanism of action of theophylline is still unknown, but it is believed to produce bronchodilation by non-selective inhibition of the phosphodiesterase isoenzymes (PDE).[87,90,122-125] Some studies have also suggested that the therapeutic effect of theophylline is delivered by antagonism of adenosine receptors,[87,122,124,125] inhibition of intracellular calcium release,[122] an effect on catecholamine secretion,[122] a decrease in microvascular leakage and/or modulation of the immune response.[122,124,125] In addition, recent studies have found that theophylline exerts anti-inflammatory effects at low doses.[123,124,126] It is believed that it reduces the production of inflammatory proteins through the activation of histone deacetylase, a group of enzymes that regulate gene expression.[123]

Theophylline is usually administered orally but parenteral drug (in the form of aminophylline) can also be given, in particular during severe asthma exacerbation.[83,90,124] Theophylline was a commonly used anti-asthma medication until the 1990s, both as reliever and controller medication,[122,124] and it is particularly effective in managing nocturnal asthma.[124] However, the use of theophylline has

declined, especially in Australia and the UK,[127] and it is only recommended as a secondary treatment option in today's practice, due to the narrow therapeutic margin, the variable pharmacokinetic properties, the potentially serious side effects and drug interactions, as well as the development of new medications with superior efficacy and side effect profiles.[83,87,90,122,124-126]

The majority of side effects and drug interactions of theophylline result from PDE inhibition, but the effects are minimal when low doses are used.[123] Studies have also found that theophylline produces additional anti-inflammatory effect when co-administered with corticosteroids (believed to be exerted through a novel mechanism), and it has been suggested that theophylline can be used as a steroid sparing medication or in the treatment of resistant patients.[123,124,126]

1.4.1.8 Cromones and Nedocromil

Cromones and nedocromil are group of drugs that are commonly referred to as 'mast cell stabilisers', which provide asthma symptoms control by reducing the irritant induced airway inflammatory responses. [87,90] However, their mode of action is not yet understood and they are also found to work on other cell types.[87,90] They are believed to inhibit the release of inflammatory mediators, reduce airway hyper-reactivity and reduce the immune response to allergens.[87,90] Cromones and nedocromil are similar in 'modes of action' and effectiveness, but differ in some chemical properties.[3,7,87] Cromoglycate (cromolyn) is the member of cromones that is commonly used in current practice.[3,7]

Both cromoglycate and nedocromil are administered by inhalers, and are used as prophylactic medications.[3,7,87,90] Although their efficacy does not rival that of the ICS, cromoglycate has less reported side effects and is often recommended for the treatment in children, as a steroid sparing medication.[87,90] However, cromoglycate and nedocromil are only recommended as second-line medications in asthma management.[3,7,87]

1.4.1.9 Others

Omalizumab is a monoclonal antibody that targets the circulating IgE antibodies, where IgE is the basis of most allergic responses such as asthma.[77,128] Omalizumab binds to the circulating IgE and prevents it from triggering the subsequent cascade of immune responses.[77] Omalizumab, delivered subcutaneously as an add-on therapy, has been found to be effective in reducing the severity and incidence of asthma exacerbation.[77,128] Omalizumab is recommended to be used as an add-on therapy in asthma management in moderate to severe asthmatics.[77,128]

Some immunosuppressants, such as methotrexate and cyclosporine, have also been investigated for the treatment of asthma, and have been found to be effective alternatives for the treatment of steroid resistant asthma.[102] In addition, anti-TNF-α,[88] immunotherapy, first generation-antihistamines, inhaled frusemide, magnesium, helium-oxygen gas mixture and macrolides have also been investigated, with mixed results.[53,79,83,102] Based on current knowledge, there is minimal value from these medications for the management of asthma.[77,83,102]

Many investigations are being carried out to develop a new generation of anti-asthma medications, in particularly medications that will provide compatible or superior efficacy to current ICS and with a superior side effects profile.[8,102,129] Some of these studies are currently at different stages of animal and/or early clinical investigation and have produced some promising results.[102,130,131] The new drugs being investigated include novel steroids,[77,130,132,133] anti-IgE monoclonal antibodies,[8,53,90] anti-IL antibodies (e.g. sIL-4R),[77,129] anti-TNF-α antibodies,[77] a new generation of xanthines (e.g. selective PDE-4 inhibitors),[77,90,134,135] chemokine receptor antagonists and transcription factor inhibitors.[77,90,102,129]

1.4.2 Non-pharmacotherapy treatment

One of the most important aspects of managing asthma is to identify the triggering factors, such as allergens, and to avoid them if possible.[8,53,78,79,85] Moderate exercise, maintaining healthy weight and smoking cessation have also been strongly associated with the improvement in asthma control.[8,21,77-79,85] Some studies have also found that dietary factors, such as vitamin D and anti-oxidants, can reduce the risk and severity of asthma.[21,79,136] According to current guidelines, education on the use of medication, avoiding triggering factors and life style modification are among the most critical factors in successfully managing asthma.[8,53,78,137]

Over the years, a number of chemical and non-chemical agents have been investigated for the treatment of asthma, including pro-biotic treatments, breathing exercises, yoga and acupuncture, but these have shown minimal effectiveness.[8,79,137-142]

1.5 Personalised therapy

The treatment objective of current mainstream anti-asthma medications is to provide symptom management, but like many other medications, the efficacies of these medications are not equal and can be widely variable among individuals.[41,49] It has been estimated that genetic differences are responsible for 15 to 95% of variability in therapeutic responses of all major classes of drugs, including anti-asthma medications.[49,143-146]

1.5.1 Mechanisms of pharmacogenetic variability

Although there are a number of studies investigating the pharmacogenetic aspects of anti-asthma therapy, much of the current knowledge in pharmacogenetics has been derived from wider areas outside asthma specific studies, These findings have often been used to demonstrate the potential effects of pharmacogenetics and pharmacogenomics, as well as provide a broad perspective in all areas of pharmacogenetic research, including anti-asthma therapy.[145146147]

In general, there are three common ways that genetic variation has been found to influence the therapeutic outcome of a drug: (1) by affecting the pharmacokinetic properties (e.g. metabolism); (2) by affecting the pharmacodynamic properties (e.g. drug-receptor interactions); (3) by interference with unintended target(s) (e.g. adverse effects).[41,58,143-146]

A major portion of current knowledge of pharmacogenetic effects is concentrated on drug metabolism, particularly of cytochrome P450 (CYP).[146-149] For example, genetic variations in CYP2C9, CYP2D6, CYP2C19 and CYP3A4 +/- CYP3A5 were found to be associated with treatment responses to phenytoin,[150,151] anti-depressants,[152,153] proton-pump-inhibitors [154,155] and isonizid respectively.[145-149] Genetic variations were also found to influence other aspect of pharmacokinetic properties, for example genetic variations at the P-glycoprotein gene with the reduced bioavailability and renal clearance of a number of drugs (e.g. digoxin and HIV protease inhibitors),[156,157] Although many studies have focussed on the genetic effects in pharmacokinetics, a number of genetic variations were also found to affect the pharmacodynamics of drugs, in particular the transporter proteins and receptors. For example, genetic variations at apolipoprotein E (APOE) were found to be related to treatment response to anti-cholinerase therapy for Alzheimer's disease.[158,159145,146] In addition, genetic variations at cellular receptors such as the bradykinin B2 receptor were found to be associated with an adverse effect (persistent cough) of ACE-inhibitors [160,161] and variations at the β_2-adrenoceptor were found to be associated with treatment outcome of β_2-agonists.[145,148,162]

However, most of the clinical outcomes from genetic determinants have also been found to involve combinations of various mechanisms, including many which are unknown, and difficult to predict.[145,147,163-165] For example, variations at CYP2C9 were found to affect warfarin metabolism and increase the risk of bleeding,[166,167] but the clinical outcome was also found to be largely influenced by the polymorphisms at a gene that encodes for vitamin K epoxide reductase complex 1 (VKORC1).[146,147,165,168-170]

1.5.2 Effects of genetic variations

The majority of current pharmacogenetic studies focus on a small selection of candidate genes, based on the understanding of pharmacokinetic and pharmacodynamic targets,[58,145,147,163,165,166,171-174] but provide limited information regarding the influence from the genome wide variations (pharmacogenomics).[58,145,147,163,165,174] However, the clinical outcomes of these genetic variations often difficult to predict given the large diversity of genetic factors and genetic interactions,[163,165,169,175-178]

In recent years, pharmacogenetic studies that have investigated candidate genes have observed that both the nature of genetic variations and the location of genetic variations were associated with the variable treatment outcome of a drug. The common genetic variations that were associated with pharmacogenetics include single nucleotide polymorphism (SNP),[178,179] tandem repeat/microsatellites [173,178] and gene expression/presentations.[145,146,163,180,181] The common locations of genetic variations found to be associated with molecular variations include non-synonymous SNPs at the coding region (resulting in alteration of amino acid sequences),[182] genetic variations at non-coding regions (resulting in gene splitting, and isoform formation),[169] and genetic variations at the gene promoter regions (resulting in changes of gene/protein expression).[163,173,183]

Some of the classical findings include alteration of amino acid composition at β_2-adrenoceptors that are related to treatment responses to β_2-agonists,[162,179] CYP-

enzymes isoforms that are related to fast- and slow- metabolism of drugs,[184-186] and overexpression of HER-2/neu gene affecting treatment outcome with trastuzumab.[145,163,187181,188]

However, experience from previous studies has suggested that the location and/or the nature of genetic variations can not predict the *in vivo* pharmacogenetic outcome, especially in a clinical setting.[163,169,186] Given that the pharmacogenetic effects were often a combination of genetic factors,[145,163,164,169,177] it has been suggested that identification of genetic phenotype (e.g. haplotype), preferably based on genome-wide pharmacogenomic investigation, will improve research outcomes and the application of this information into clinical practice.[51,146,149,163-165,176,189]

1.5.3 Pharmacogenetics and pharmacogenomics of anti-asthma medications

Despite the advances in understanding the disease and sophistication in therapeutic design, most anti-asthma medications have a wide variability in therapeutic response among individuals.[49,144] These variables are believed to be the result of multiple factors, but it is reasonable to believe that genetic differences would be one of the most influential factors (estimated 60 to 80% of treatment outcomes).[49,58,144,164] To date, many studies have found that genetic differences are responsible for a number of variations in treatment outcome among some of the most commonly used anti-asthma medications, in particular β_2-agonists, anti-LTs and corticosteroids.[41,49,53,58,143,190]

Recent studies had identified a number of genetic variations at the β_2-adrenoceptor gene (ADRB2),[179,183,191182] including three non-synonymous SNPs located at codon 16, 27 and 164 with amino acid substitution of Arginine(Arg) → Glycine(Gly), Glutamine(Gln) → Glutamic acid(Glu) and Threonine(Thr) → Isoleucine(Ile) respectively.[182,183,192,193] The amino acid substitutions at codon 16 and 27 are located in the amino terminus of the receptor that sits outside the cell membrane (the substrate binding terminal),[182,183,192,193] where the codon 164 amino acid is located within the 4th transmembrane spanning domain of the receptor.[182,183,192,193] *In vitro* studies have found that Arg16 genotype (wild-type SNP on cordon 16 of ADRB2) and Glu27 genotype (mutated SNP on codon 27 of ADRB2) were associated with β_2-agonist induced down regulation of the β_2-receptor,[194172,195171183]

The following pharmacogenetic studies have found that asthmatic patients carrying the Arg16 genotype (wild-type SNP on cordon 16 of ADRB2) experience deterioration in asthma control while using short β_2-agonists (salbutamol) on a regular dosing regimen, but patients with Gly16 genotype experience improvement in asthma control.[143,144,162,179,196,197] It is believed that the findings were related to the receptor desensitisation among Gly16 genotype by endogenous adrenergic substratea and therefore could not express much of the additional desensitisation when challenged with regular salbutamol, as opposed to the Arg16 genotype.[198] In recent years, cross-tolerance between short acting β_2-agonist and LABA has also been observed.[89] Patients who carry the Arg16 genotype were found to be associated with disease deterioration from LABA use, but the findings remained inconsistent among studies.[191,199-203]

On the other hand, SNPs at codon 164 were found to alter the coupling function between receptors and substrates, and is associated with reduction in duration of action with LABA.[193183] However, the clinical significance of many of the SNPs remain largely undetermined.[179,194] In addition, a study has also found that the outcome of these pharmacogenetic influences were affected by the subject's ethnic background,[54] which suggest that the previous observations may be affect by a combination of other genetic variations.[54189,204]

Studies which have investigated the pharmacogenetics of anti-LTs and corticosteroids are less conclusive. Some studies have found that naturally occurring mutations at the 5-lipoxygenase gene's (ALOX5) promoter region and SNP on nucleotide -444 of LTC_4 synthase gene (LTC_4S; genotype A_{-444}) are related to diminished clinical responses to anti-LTs.[143,144,173,205 173,178]A number of genetic variations (eg. SNP) were also identified on the ALOX5 gene,[206] LTC_4S gene,[176205] and CysLT1 receptor gene (CYSLTR1)[176], with suggestion that the variations may contribute to the differences in treatment outcome.[176175207] Studies which investigated the pharmacogenetics of corticosteroids have identified several variations in the GR gene, including α- and β-GR subtype, which are found to be related to a lack of therapeutic response to corticosteroids.[94,95,97,208209210180] Genetic variations at the corticotropin releasing hormone receptor genes (CRHR) were also found to be associated with variable treatment outcome with ICS.[211,212] (More detail in Chapter 7 and Chapter 8)

In addition, other pharmacogenetic effects on anti-asthma medictions have also been reported in recent years, but the findings were much less consistent.[19058213-215] Studies have so far identified over 2000 SNP and over 220 candidate genes for the response

of ICS and β_2-agonists, and genome wide association study have been proposed.[164,174,177]

1.5.4 Clinical implications: expectations and complications

Until recently, most of the pharmacogenetic and pharmacogenomic discoveries were driven by clinical observation.[149,174,216] However, the advance in knowledge in molecular biology has driven the inclusion of genetic analysis in new drug designs and in clinical trials, for example the development and requirement of genetic analysis prior to treatment with trastuzumab (Herceptin®) and cetuximab (Erbitux®).[147,174,216-218187] Based on developments in pharmacogenetics and pharmacodynamics, it is anticipated that future advances will be able to verify and predict the drug reactions of individual patients, and allow customised drug therapies based on the individual's genetic makeup.[41,42,86,187,190,219,220] The anticipated benefits include improvement in effectiveness of drugs, improvement in efficacy of treatment options, determining appropriate dosages, improvement in disease screening and the reduction in health care costs.[41,48,58,86,145,190,219,220] There are currently over 20 drugs which include pharmacogenetic references in their product information, and many more drugs are expected to follow, particularly the newly designed drugs.[147,163,174,218] To date, the clinical use of pharmacogenetic information is most advanced in oncology, and the development of pharmacogenetics in other disciplines has also shown some promising results, in particular in asthma, schizophrenia, depression and cardiovascular medicine.[164,174,190,217]

However, personalised therapy is still in its infancy and faces some significant barriers, including the complexity of pharmacogenetic and pharmacogenomic investigations, limited availability of alternative therapies, cost effectiveness of genetic analysis, economic incentives of pharmaceutical companies, and education as well as some ethical, legal and social issues.[41,58,216,220] Besides the difficulties in determining the genetic variation(s) that influence the pharmacogenetic properties of a drug, it is also improbable that the treatment outcome of a drug can be predicted solely based on genotyping.[145,174] Therefore, when the eligibility of a treatment option is determined based on pharmacogenetic information, some individuals may be denied the particular treatment (which might still be effective), due to the person carrying the 'wrong' gene.[174] This scenario is particularly likely when the treatment is relatively expensive , and it will become an even more complicated ethical issue if there are minimal alternative therapies.[174] In addition, despite the advance in development of genotyping technology, genetic screening is still considered not to be cost effective in many applications, in particular clinical practice as well as some clinical trials.[163,174]

Although pharmacogenetic and pharmacogenomic studies have shown some promising findings, the use of this information in clinical practice is still not very common.[174] Some studies have suggested extensive education (to practitioners, patients and the public) is important to overcome some of the resistance to employing genotyping as one of the tools to aid therapeutic options.[163,174,216]

1.6 Study overview

The aim of this study was to investigate the relationship between genetic variations and the pharmacokinetic and pharmacodynamics of some of the commonly used anti-asthma medication.

The study first examined the application of an anti-asthma medication (salbutamol), including the dose, efficacy and safety profile, at a typical emergency department setting. Analytical techniques were developed for the extraction of salbutamol and genetic material from various sources, as well as accurate measurement of salbutamol enantiomers and determination of genetic profiles. The information gathered from these studies was then used as reference for the following pharmacokinetic and pharmacogenetic studies, and relate to current asthma management. The study also examined the pharmacogenetic of other anti-asthma medication, including anti-LTs and ICS.

This investigation was intended to explore the application of pharmacogenetics knowledge into current practice of asthma management in Australia, such as the improvement in treatment strategy and the prevention of potential adverse effects.

Chapter 2

Circulating salbutamol enantiomers levels in acute severe asthma and acute COPD presentations in the emergency department setting

2.1 Abstract

Background: This observational study was designed to investigate plasma levels of salbutamol enantiomers among patients with acute severe asthma or COPD presenting to the emergency department.

Method: Blood samples were collected and plasma/serum levels of (R)- and (S)-salbutamol enantiomers were determined by LC-MS and LC-MS/MS assay. Extra-pulmonary effects measured at presentation included ECG measurements, serum potassium level and blood sugar level which were collected from the hospital medical records.

Results: High plasma levels of both enantiomers were observed in some individuals, with median (range) concentrations of 8.2 (0.6-24.8) and 20.6 (0.5-57.3) ng/mL for (R)- and (S)- salbutamol respectively among acute asthma subjects, and 2.1 (0.0-16.7) to 4.1 (0.0-36.1) ng/mL for (R)- and (S)- salbutamol respectively among COPD subjects. Levels were not associated with an improvement in lung function or the potential adverse cardiac effect (prolonged QTc interval).

Discussion: High plasma concentrations of salbutamol were observed, in both asthma and COPD patients presenting to the emergency department. Extra-pulmonary cardiac adverse effects were not associated with plasma level of salbutamol when administered by inhaler in the emergency department setting. Long term effect(s) of continuous high circulating salbutamol enantiomer concentrations remain unknown and further investigations are required.

2.2 Introduction

Salbutamol, a β_2-agonist, plays an important role in emergency medicine and is the first line medication for relief of shortness of breath during acute asthma exacerbations. Salbutamol is also used on a regular basis for the management of chronic obstructive pulmonary disease (COPD), both during stable periods and acute exacerbations.[221-225] Many recent studies and guidelines have indicated that the use of short acting β_2-agonists on a regular basis will not improve asthma control, and may even cause deterioration.[162,226,227] However, regular use of short acting β_2-agonists such as salbutamol is still very common for the management of COPD.[221,224,225]

Salbutamol is a chiral compound consisting of (R)- and (S)- enantiomers, and is most commonly administered as a 1:1 racemic mixture (*rac*-). The therapeutic effect of salbutamol is supposedly delivered by the (R)-enantiomer.[228] Although the drug has been used since 1969, some fundamental issues including the dosage, pharmacological effects of the enantiomers, and therapeutic and toxic levels remain controversial and unresolved.[83,229]

The pharmacokinetics of (R)- and (S)-salbutamol are quite different, irrespective of the route of administration and dosage form.[230-233] It has been found that (S)-salbutamol always exibits greater bioavailability (AUC ratio of S/R-salbutamol were 20.0, 7.7 and 3.1 for oral, inhaled and intravenous dosage forms respectively) and a longer half life (S/R-salbutamol C_{max} ratio of 12.9, 1.9 and 1.5; $t_{1/2}$ ratio of 2.1, 2.2 and 1.9 for oral, inhaled and intravenous dosage forms respectively) than (R)-

salbutamol.[230,232,233] The nature of enantioselective metabolism and enantioselective renal excertion were believed to be the major factors for these finding.[231]

These differences in pharmacokinetics of salbutamol enantiomers ($t_{1/2}$ of inhaled salbutamol is approximately 5 h and 2.5 h for (S)- and (R)-salbutamol respectively) can contribute to the accumulation of the (S)-salbutamol after repeated dosing.[230-232] Some studies have claimed that the (S)-salbutamol is not inert, but rather has detrimental physiological effects, including pro-inflammatory and pro-constriction effects,[234,235] and that it increases airway responsiveness,[236,237] and acts as a functional antagonist.[238] Potential adverse effects of (S)-salbutamol have also been suspected when studies found that pure (R)-salbutamol is superior in treatment outcome compared to the equivalent dose of *(rac)*-salbutamol.[239-242] However, these findings are usually difficult to interpret and are often not translated into clinical studies that compare the therapeutic outcome.[229,243-245] There are number of studies indicating that both the immediate therapeutic effects and immediate adverse effects of *rac*-salbutamol are delivered solely by the (R)-salbutamol.[233,246-248] The weight of evidence to date suggests that (S)-salbutamol is inert, but the effects of high levels of (S)-salbutamol remain unclear.[229,245]

Most of the pharmacokinetic and pharmacodynamic studies of salbutamol are performed on healthy, mild asthmatic patients, within the generally recommended dose.[230,233,238,246] However, patients presenting to the emergency department with exacerbations of asthma and/or COPD are usually heavily reliant on short acting β_2-agonists for symptom relief prior to the presentation, and would be expected to use much higher doses of salbutamol. A study has shown that patients who have died

from asthma have up to 2.5-fold higher plasma salbutamol levels than asthma patients using salbutamol at the emergency department.[249] In addition, studies have shown that significant extrapulmonary effects of inhaled salbutamol include increased heart rate,[233,247,250,251] increased QT interval [250] and decreased plasma potassium level [233,247,250,252] can all occur within the maximum recommended dose.

In addition, some recent studies have discovered that individuals with particular genotype (single nucleotide polymorphism at β_2-adrenoceptor gene; SNP at ADRB2) can develop tolerance to salbutamol after repeated dosing, resulting in worsening asthma control and may require a higher dose to achieve clinical efficacy.[162,179,183,253] Recent studies have also discovered some genetic variations at the human catecholamine sulfotransferase (SULT 1A3; enzyme responsible for salbutamol metabolism), and suggested that these genetic variations may alter the emzyme's activity (detail refer to Section 6.1),[254,255] resulting in slow metabolism and hence the accumulation of salbutamol enantiomer(s). Althought most β_2-agonists, including salbutamol, were designed to selectively interact with the β_2-adrenocepter, studies have found that they still exibit some β_1-adrenoceptor binding potency and are less selective at higher drug concentration which results in cardiovascular adverse effects as observed in clinical settings.[92] Furthermore, there is some evidence that some genetic variations at the β_1-adrenoceptor gene (ADRB1) are related to a higher risk of heart failure and cardiovascular events.[92,256,257] It has been suggested that the present of β_2-agonists can aggravate the risk of these cardiovascular events, in particular among individuals that have long term exposure to accumulated doses of β_2-agonist.[92,251]

Our preliminary investigations in emergency department presentations have revealed relatively high plasma levels in acute severe asthma patients, with up to 5-fold difference in concentrations of (R)- and (S)-salbutamol.[258] The objective of this study was to observe the relationship between (R)- and (S)-salbutamol levels and lung function measures, as well as potential extra-pulmonary adverse effects, in presentations of acute disease exacerbation seen in a typical emergency department setting.

2.3 Methods

2.3.1 Study design

The study was observational in design, and conducted in two separate phases. The first phase was designed to observe the relationship between salbutamol enantiomer levels and lung function measures among patients presenting with an acute asthma exacerbation. The second phase was designed to observe the relationship between salbutamol enantiomer levels and potential extra-pulmonary adverse effects among patients presenting with exacerbation of COPD.

The study was conducted at the Department of Emergency Medicine (DEM), Royal Hobart Hospital (RHH), Tasmania, Australia. The study was approved by the State Human Research Ethics Committee and written informed consent was obtained from the subjects prior to the investigation.

Unless otherwise specified, all chemicals used in this Chapter were laboratory grade chemicals.

2.3.1.1 Acute asthma

2.3.1.1.1 Study subjects

Potential subjects of the study were patients who presented to the DEM with an acute exacerbation of asthma. The inclusion criteria were adult patients, aged between 18 and 65 years, and self-reported *rac*-salbutamol utilisation within 24 h prior to the

presentation. Recruitment was convenience sampling in nature over the time frame of the study. Patients who had presented to the emergency department for over 12 h prior to blood sampling were excluded. Moderate to severe asthma exacerbation was diagnosed by independent emergency physicians, in consulation with the National Asthma Council Australia (NAC) guideline [7].

2.3.1.1.2 Sample and data collection

Blood samples (10 mL) were collected from each subject in potassium EDTA tubes by medical or nursing staff at the DEM. The blood sample was then centrifuged, and the plasma harvested and stored at -20°C until analysis.

History of *rac*-salbutamol use by subjects within the previous 24 h was obtained from subjects by interview and from medical records. The salbutamol utilisation was also converted to defined daily dose (DDD) [259] which was designed to standardise the dose between different types of formulation. One DDD of *rac*-salbutamol was considered equivalent to 800 μg of *rac*-salbutamol delivered by pressurised metered dose inhaler (MDI) or 10 mg delivered by nebuliser. The DDD was only used as an estimation for the number of doses of salbutamol required during the asthma exacerbation (between different dosage forms), and does not represent the amount of salbutamol being delivered or to reflect the recommended dose.

Basic demographic information and details of medical treatment during hospital presentation and on the way to hospital were obtained from the hospital medical records. Concomitant use of other asthma medication was recorded. Clinical measures of severity and response to therapy included improvement in percent predicted PEF

after 60 min and a four-point severity score. This severity score is similar to the Acute Asthma Index (AAI) designed and validated by Rodrigo and Rodrigo,[260] which was shown to able to predict the asthma therapeutic response at emergency department, and early identification of patients who required more extensive care However, a 60 min PEF was used instead of the 30 min PEF as used in the AAI, as it was more achievable by emergency department staff in our setting. Respiratory function tests were performed with a Vitalograph® Compact spirometer (Buckingham, UK).

2.3.1.2 Acute COPD

2.3.1.2.1 Study Subjects

Potential subjects of this study were patients presenting to the DEM with exacerbation of COPD. Subjects were excluded if they did not have a routine serum sample collected within 4 h of presentation, or were not admitted to the general ward after the DEM presentation. Confirmation of the diagnosis (according to the GOLD guideline [261]) and subject recruitment (convenience sampling) was carried out at the general ward by an independent medical officer from the Department of Respiratory Medicine, RHH.

2.3.1.2.2 Sample and data collection

Serum aliquots were obtained from remaining samples after routine blood examination was performed according to DEM procedures, Routine tests undertaken include full blood examination, electrolyte examination and ECG measurement. The Department of Clinical Chemistry (Pathology), RHH, was informed of each subject's participation, through a secure collaborative network, after written informed consent

was obtained. The remaining serum samples (collected in VACUETTE® Z Serum Sep C/A tubes), were then transferred to the investigators after being kept at the Pathology Department (at 4-8°C) for 7 days as required in accordance of the RHH Pathology serum protocol. After the transfer, serum samples were stored at -20°C until analysis.

Information regarding the potential extra-pulmonary adverse effects of salbutamol within the 4 h of DEM presentation, including heart rate (HR), corrected QT (QTc) interval, serum potassium level, and blood sugar level (BSL), were collected from hospital medical records. Demographic information and relevant medical history were extracted from medical records. Medication history prior to the ECG measurement and the blood sampling, in particularly medications known to affect the measurements clinically, was also recorded. ECG measurements were examined by an independent clinician to determine if the recorded QTc intervals were affected by underlying cardiac condition(s) (eg heart block). Subjects with a medical or medication history that may interfere with the measurement(s) were excluded from the association analysis.

2.3.1.3 Analysis of salbutamol

Salbutamol enantiomer analysis was performed at the School of Pharmacy laboratory and the CSL laboratory, University of Tasmania. A previously published method [262] was modified and used for salbutamol analysis (details referred to in Chapter 3). In brief, the samples were brought to room temperature and salbutamol was extracted from the plasma and serum sample (from acute asthma subjects and acute COPD subjects respectively) using solid-phase extraction and analysed by LC-MS or LC-

MS/MS. The lower limit of quantification (LLoQ) was 0.156 ng/mL (from 500 μL) and reproducibility (RSD) was <15%. This method was modified as described in Chapter 3 (section 3.3),

2.3.2 Statistical analysis

Previous study by Lipworth et al[233] observed a significant increase of mean (95% CI) heart rate and serum potassium level of 10.5 (4.3; 16.8) beats/min and 0.41 (0.22; 0.61) mmol/L respectively, with only 12 subjects and corresponding plasma salbutamol concentration of less than 2 ng/mL. Based on a two-tailed $\alpha = 0.05$ and $\beta = 0.20$, with the mean salbutamol level observed during the first phase of the study, a sample size of 25 would allow a minimum change in heart rate and potassium level of 8.6 beats/min and 0.27 mmol/L respectively, to be detected.

One-way factorial ANOVA was used to assess the relationship between severity score and plasma salbutamol and Fisher's protected least significant difference (PLSD) post hoc test was used to assess any statistical significance. Linear regression was used for the relationship between continuous variables. Spearman rank correlation and Mann-Whitney tests were used to assess the relationship between serum salbutamol level and extra-pulmonary effects (heart rate, QTc interval, serum potassium level and BSL), which do not exhibit Gaussian distributions. Statistical analyses were undertaken with Statview 5.0.1 (SAS Institute Australia Pty Ltd, NSW, Australia) and SPSS 15.0 for Windows (SPSS Australasia Pty. Ltd. Chatswood, NSW, Australia).

2.4 Results

2.4.1 Acute asthma

Fifteen patients were recruited for the study. Basic demographic and salbutamol utilisation in the previous 24 h are summarised in Table 2.1. The initial baseline respiratory test (PEF) was not performed in three subjects, partly due to the severity of their symptoms, but was estimated by clinicians to be less than 25% predicted.

Table 2.1 Subject's demographic and *rac*-salbutamol utilisation among patients presenting to DEM with acute asthma

	Median (range) n=15
Age	38 (22-65)
Gender	6 M; 9 F
Smoking history (medical record)	
Current smoker	5
Ex-smoker	2
Respiratory test, % predicted PEF (n=12)	
Baseline	51 (21-69)
60 min post-initial test	60 (31-78)
Total *rac*-salbutamol utilisation in preceding 24 h (DDDs)	3.0 (0.8-11.0)
Total dose delivered via MDI	1.5 (0.0-5.3)
Total dose delivered via nebuliser	2.0 (0.0-5.5)
Total dose delivered by healthcare officer	1.5 (0.0-0.25)

Plasma salbutamol enantiomer levels were measured in all subjects (Table 2.2 and Figure 2.1). There were no relationships between plasma salbutamol enantiomer levels and severity or response to treatment, measured both by the four-point severity score (Table 2.3) and percent improvement in predicted PEF at 60 min. Patients with higher levels of 24 h *rac*-salbutamol utilisation (DDDs), consistent with greater

morbidity, had a lower percent predicted PEF at baseline (r^2=0.33, p=0.03), but not a poorer response to therapy measured using the severity score [F(2,12)=1.83, p=0.20].

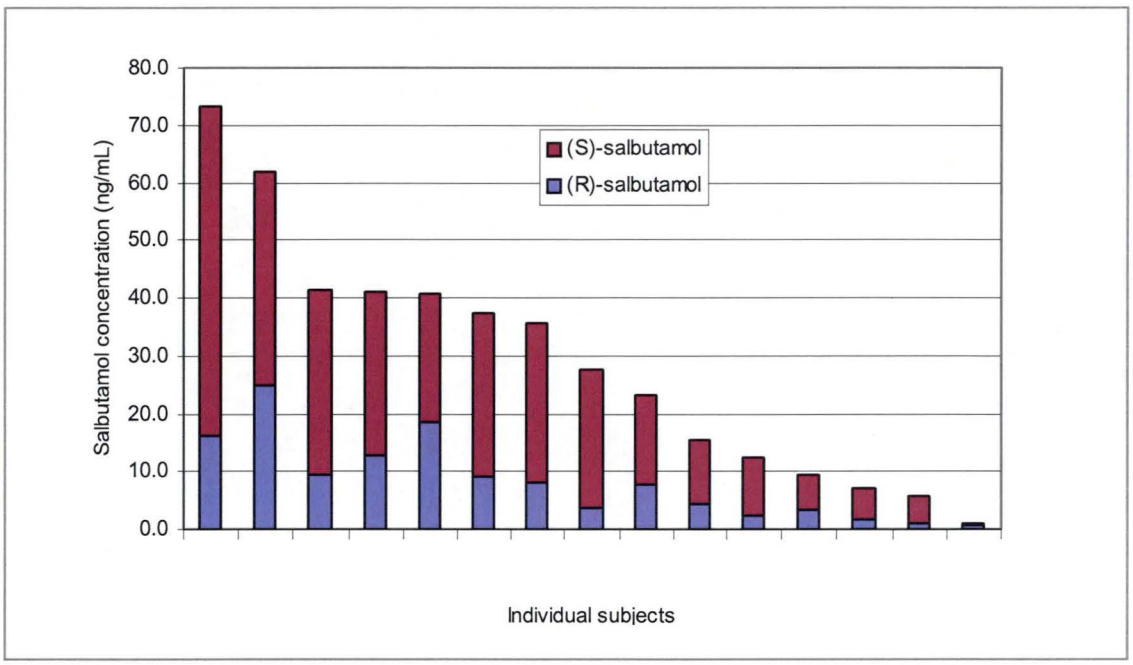


Figure 2.1 Plasma salbutamol enantiomer levels observed among subjects presenting with acute asthma exacerbation (n=15)

Table 2.2 Correlation between *rac*-salbutamol dose utilisation [median (range)] and serum salbutamol enantiomer levels [median (range)], among acute asthma subjects.

		Salbutamol utilisation	
	Serum level	Total dose utilisation [a]	Recorded dose utilisation [b]
		20.0 (0.6-55.0) mg	15.0 (0.0-50.0) mg
(R)-salbutamol	8.2 (0.6-24.8) ng/mL	r^2=0.22	r^2=0.54*
(S)-salbutamol	20.6 (0.5-57.3) ng/mL	r^2=0.50	r^2=0.33
Total salbutamol	28.9 (1.1-73.3) ng/mL	r^2=0.43	r^2=0.42

2-tailed Pearson Correlation test

[a] Dose administered in the preceding 24 h, including dose administered prior to the hospital presentation

[b] Dose administered by healthcare officer, as recorded in hospital medical history

* p<0.05

Neither smoking history nor the use of inhaled corticosteroids was associated with the dose of salbutamol used (DDD), the percent improvement in predicted PEF at 60 min or the severity score. Subjects who had been using long acting β_2-agonists were found

to be more likely to use less *rac*-salbutamol in the previous 24 h before presentation (p=0.02).

Table 2.3 Severity Score* and salbutamol plasma levels

	Median (range) plasma levels ng/mL			
Severity Score	Total salbutamol	(R)-salbutamol	(S)-salbutamol	S:R ratio
2 (n=8)	21.5 (1.1-61.9)	4.1 (0.6-24.8)	17.4 (0.5-37.1)	3.0 (0.8-6.6)
3 (n=4)	32.3 (9.5-73.3)	10.3 (3.2-16.0)	22.0 (6.3-57.3)	2.1 (2.0-3.6)
4 (n=3)	35.5 (5.6-40.8)	8.1 (0.9-18.6)	22.1 (4.7-27.4)	3.4 (1.2-5.2)

* modified from the Acute Asthma Index; AAI [260]

2.4.2 Acute COPD

Thirty patients were recruited for the COPD phase of the study (Table 2.4), where 25 of the subjects had a recorded medical history of cardio-vascular co-morbidity (Table 2.4).

Serum salbutamol enantiomers levels were measured in all subjects (Table 2.5 and Figure 2.2), with a weak correlation observed between salbutamol dose and both (R)- and (S)-salbutamol enantiomer levels (r^2= 0.55 and 0.60 respectively; p<0.01).

Table 2.4 Subject demographics and *rac*-salbutamol utilisation among acute COPD patients presenting to DEM

	Median (range) (n=30)
Age	70 (51-85)
Gender	11 M; 19 F
Smoking history (medical record)	
Current smoker	12
Ex-smoker	13
Co-morbidity with asthma	4
Cardio-vascular co-morbidity	
Ischaemic heart disease	8
Heart failure	3
AF	1
Past AMI	3
Total *rac*-salbutamol delivered (by healthcare professional [a])	0.5 (0.0-4.0)

[a] Healthcare professionals include paramedic, doctor and nursing staff

ECG measurements were available in the medical records for 23 subjects, but two subjects ECG measurements were excluded from analysis due to a concurrent digoxin toxicity and a probable atrial flutter respectively. Five subjects (2 male and 3 female) were identified with prolonged QTc intervals (>440 msec and >450 msec for males and females respectively), however, these were not associated with higher serum levels of salbutamol enantiomer. Results of serum salbutamol levels, heart rate and QTc interval are summarised in Table 2.5

Table 2.5 Corelation between *rac*-salbutamol dose utilisation [median (range)] and serum salbutamol enantiomer levels [median (range)], among acute COPD subjects.

	Serum level	Salbutamol utilisation [a] 5.0 (0.0-40.0) mg
(R)-salbutamol	2.1 (0.0-16.7) ng/mL	r^2=0.55 *
(S)-salbutamol	4.1 (0.0-36.1) ng/mL	r^2=0.60 *
Total salbutamol	6.1 (0.0-53.0) ng/mL	r^2=0.59 *

2-tailed Pearson Correlation test

[a] Dose administered by healthcare officer, as recorded in hospital medical history

* $p<0.01$

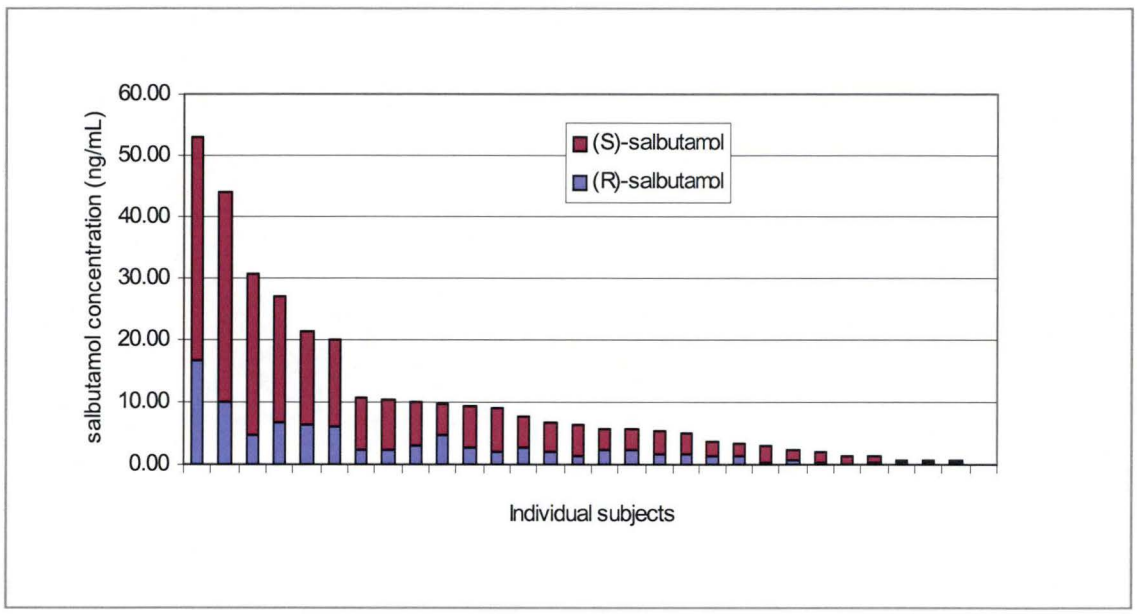


Figure 2.2 Serum salbutamol enantiomer levels observed among subjects presenting with acute exacerbation of COPD (n=30)

The serum potassium levels were recorded in 27 subjects, and the BSL were recorded in 26 subjects. However, 20 of the serum potassium results were considered inconclusive and excluded from the analsysis due to the subject's medication history (potassium supplement, diuretics and iv fluid infusion) and/or faulty specimen (suspected haemolysed sample). Similarly, 13 of the BSL results were also excluded from analysis due to the subject's medical history (diabetes) and medication history (oral/iv corticosteroid and iv fluid infusion). Other than one subject who was indentified with slightly lower serum potassium level and four subjects identified with elevated BSL, the serum potassium level and BSL for the remaining subjects were recorded within the 'normal' physiological range (3.7-5.2 mmol/L and 4.0-7.5 mmol/L respectively). The subjects identified with 'abnormal' serum potassium level and BSL were not associated with higher salbutamol enantiomer levels (Table 2.6).

Table 2.6 Median (range) ECG measurements (HR and QTc interval), serum potassium level and BSL for each tertile (n=10) of salbutamol enantiomer serum level.

Salbutamol concentration (range)	(R)-salbutamol Lower (0.0-1.2 ng/mL) Middle (1.3-2.5 ng/mL) Upper (2.8-16.7 ng/mL)	(S)-salbutamol Lower (0.0-2.1 ng/mL) Middle (2.7-6.4 ng/mL) Upper (6.8-36.3 ng/mL)	Total salbutamol Lower (0.0-3.2 ng/mL) Middle (3.6-9.2 ng/mL) Upper (9.7-53.0 ng/mL)
HR (/min)	89 (70-120)	87 (70-116)*	89 (70-116)
(n=21)	102 (82-127)	102 (59-120)	102 (59-120)
	109 (96-137)*[a]	109 (96-137)*[a]	109 (96-137)*[a]
QTc interval (msec)	428 (386-486)	425 (374-460)	425 (374-479)
(n=21)	427 (374-461)	448 (377-486)	448 (377-486)
	382 (376-404)	386 (363-427)	386 (363-406)
Serum potassium level (mmol/L)	4.7 (4.4-5.3)	4.7 (4.0-5.3)	4.7 (4.0-5.3)
(n=7)	3.5 (-)	3.8 (3.5-4.1)	3.8 (3.5-4.1)
	4.7 (4.1-5.1)	5.0 (4.6-5.1)	5.0 (4.6-5.0)
BSL (mmol/L)	6.7 (5.9-13.3)	6.7 (5.9-13.3)	6.7 (5.9-13.3)
(n=13)	6.0 (5.4-7.8)	6.7 (5.8-7.8)	6.1 (5.4-7.8)
	7.4 (6.2-10.4)	6.8 (5.4-10.4)	7.4 (6.2-10.4)

Mann-Whitney test
* $p<0.05$
[a] comparing upper tertile with lower tertile

2.5 Discussion

This study reflects the variations in the presentation of acute exarcerbations of asthma and COPD in a typical emergency department setting, both in disease severity and the treatment required. However, the relationship between dose and plasma/serum level of salbutamol appear to be minor ($r^2 \leq 0.6$).

In comparison with some previously reported data (1 ng/mL and 3 ng/mL for mean (R)- and (S)-salbutamol respectively),[230,232,233] the levels of salbutamol enantiomer observed in this study (Table 2.2 and Table 2.5) appeared to be considerably higher, in particular among acute asthmatic patients. In addition, the accumulation of (S)-salbutamol and variation in the R:S ratio (Table 2.2 and Table 2.5), highlight the need for enantioselective assays when measuring salbutamol in a clinical setting.

The use of severity score, modified from the AAI, in order to accomodate the existing DEM protocol may be considered less than ideal. However, pervious studies had demonstrated the PEF measured at 30, 60, 120 and 180 min were all valuable in early prediction of the asthma therapeutic response at emergency department.[260,263,264] Although the sensitivity of the scoring system may be less than the AAI, it should be considered as an effective measuring system that can appropriately serve the purpose for this study.

Recent reviews have raised concerns about the safety of using large doses of β_2-agonists, especially in patients with underlying cardio-vascular co-morbidity.[92,251,252] It has been demonstrated that significant extrapulmonary effects can be observed in

subjects given nebulised *rac*-salbutamol at a dose of as little as 6.5 mg.[233,247,250] In this study, we observed relatively high salbutamol levels in the circulation (some more than 10 times the level observed in the study by Lotvall et al [247]), but we observed no corresponding variation in extra-pulmonary parameters among these patients. The QTc intervals showed minimal change from the commonly regarded normal physiological range, and had no significant relationship with either (R)-, (S)- or total salbutamol levels (Figure 2.3). However, evaluation of other metabolic effects of salbutamol were more difficult, due to complex medication regimens, disease co-morbidites, as well as potential psychological (e.g. emotional stress) and physiological (e.g. compensation to respiratory stress) effects. The result of this investigation are in line with a previous study that found minimal change in QTc intervals after repeated dosing of high dose of β_2-agonist.[265] The findings suggests that the potential extra-pulmonary effects of salbutamol do not appear to be problematic among patients who use inhaled *rac*-salbutamol for the acute relief of shortness of breath, even among patients with underlying cardiovascular co-morbidity. However, lower salbutamol enantiomer levels than those observed in phase one of the study, in combination with the high exclusion rate significantly reduced the power of this study to examine the metabolic effects of circulating salbutamol enantiomers. A more comprehensive study is required to provide more conclusive evidence on the metabolic effects of salbutamol enantiomers in severe disease exacerbation. In addition, the long-term effects of accumulation of high concentrations of salbutamol enantiomer remain unknown and are the subject of ongoing work.

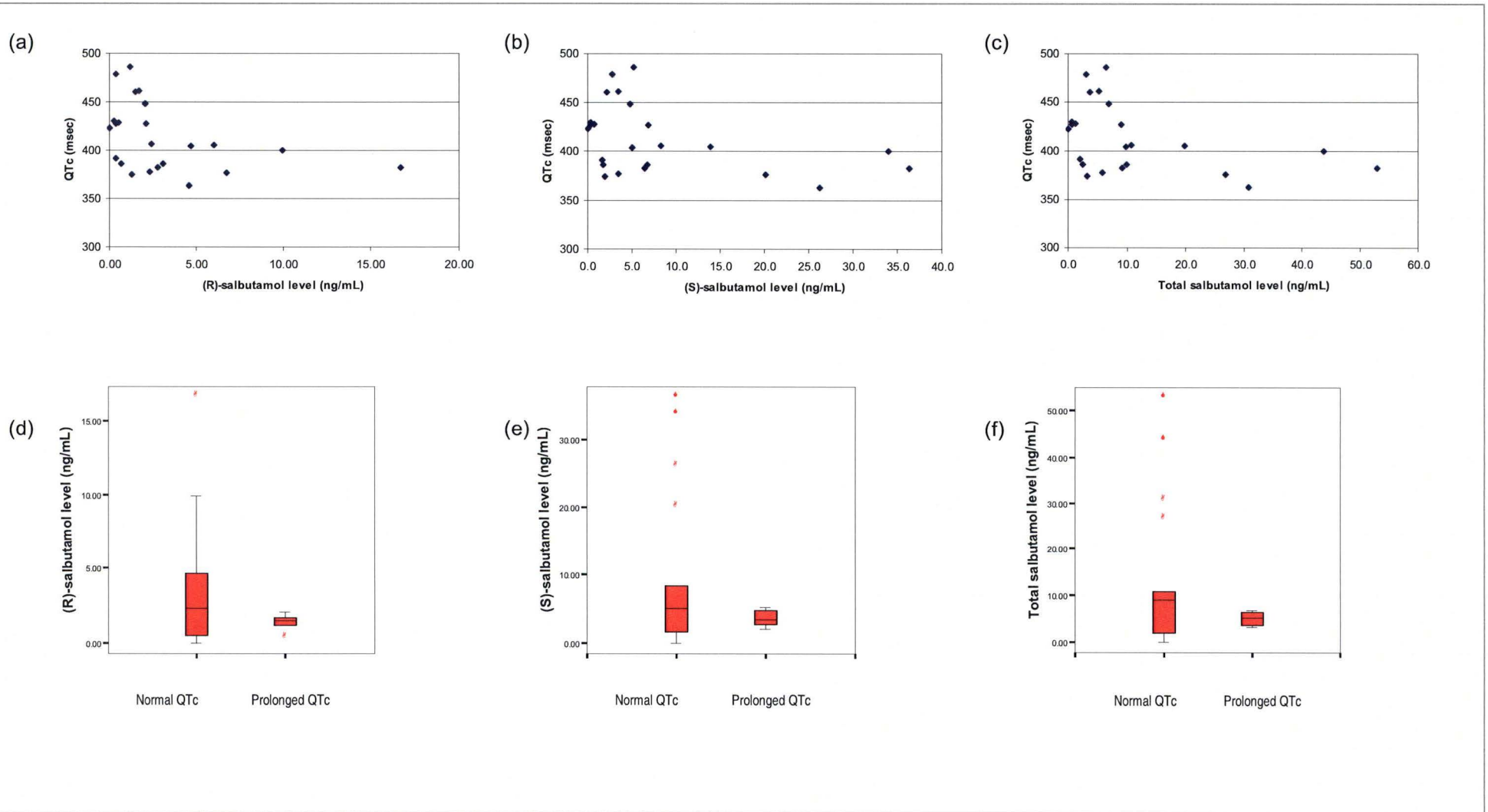


Figure 2.3 Relationship between (R)-, (S)- and total salbutamol levels and recorded QTc interval, (a), (b) and (c). (R)-, (S)- and total salbutamol levels between subject with normal QTc interval and subjects with prolonged OTc interval,(d), (e) and (f).

The wide variation in the relationship between dose and levels have also indicated the difficulties in spot sampling methodology without a population pkarmacokinetic model,[266] as well as the potential impact from the subject's inhalation technique, particularly when an MDI device was used.[267-269] In addition, the potential effect of genetic variations in both the pharmacokinetic (including metabolism by enzyme SULT 1A3 [254,255] and transport by the OCT system [270-272]) and pharmacodynamic (β_1- and β_2-adrenoceptor) [92,162,179,183,253,256,257] parameters should also be taken into consideration. It is reasonable to suggest that the clinical effects of these genetic variations can be interrelated, as patients with reduced bronchodilation effect because of ADRB2 SNP may use larger doses of salbutamol to achieve symptom control, accentuating the accumulation of salbutamol enantiomers (e.g. in heart muscle). The event may be further exacerbated by genetic variation at the SULT 1A3 gene (SULT1A3) and OCT genes, resulting in adverse effects attributable to salbutamol enantiomers, in particular among patients with higher risk of cardiovascular event (e.g. patients with particular ADRB1 genotype).

Chapter 3

Determination of salbutamol enantiomers in tissue matrices: application in pharmacogenetic and disposition studies

3.1 Abstract

Background: Salbutamol is a chiral compound consisting of (R)-salbutamol and (S)-salbutamol with different pharmacokinetic parameters, but is most commonly administered as the (1:1) racemic (*rac*) mixture. Surprisingly, there have been relatively few reports of salbutamol tissue distribution, and distribution of salbutamol individual enantiomers into tissue has not been reported. This is a particularly important capacity when exploring the genetic variability in metabolism, disposition and uptake of salbutamol in tissue.

Method: The method presented here explores the use of an HPLC tandem mass spectrometry (LC-MS/MS) system (LTQ Orbitrap^{TM} hybrid mass spectrometer) with deuterated standard and solid-phase extraction to determine low levels of salbutamol enantiomers.

Result: The assay was linear over the calibration range of 0-5.0 ng/g in muscle tissue (r^2>0.98). The lower limit of quantification (LLoQ) was 0.156 ng/g, with a precision RSD <15% for both enantiomers. The assay was successfully applied to muscle and liver tissue samples from animals fed with grains supplemented with salbutamol, as well as subsequent investigation into enantioselective tissue disposition of salbutamol and the involvement of an active drug transport system, in both a mouse and rat model.

Discussion: By utilising a deuterated internal standard and LC-MS/MS detection, this assay can be used to measure salbutamol enantiomers in muscle and other tissue in addition to blood. This assay has also identified that salbutamol uptake appears to be stereoselective and enantioselective assays are warranted for metabolism and disposition studies involving administration of *rac*-salbutamol

3.2 Introduction

Salbutamol is a selective short-acting β_2-agonist used to relieve bronchoconstriction in asthma (Figure 3.1). Like other β_2-agonists, it also exerts some extrapulmonary effects on various organs and tissues, including heart, muscle and fat. Salbutamol is a chiral compound consisting of (R)-salbutamol and (S)-salbutamol with different pharmacokinetic parameters, but is most commonly administered as the (1:1) racemic (*rac*) mixture.

The extrapulmonary effects of salbutamol, including the potential adverse effects examined in Chapter 2 and skeletal muscle tremor are often observed with higher doses of salbutamol in clinical settings.[92,246] In addition, muscle (including cardiac muscle) and fat repartitioning have also been observed with salbutamol use, along with increases in muscle strength and bulk, in which the magnitude appears to vary with route of administration.[273,274] This has led to salbutamol being used for performance enhancement doping in athletes as well as applications in animal primary production. The therapeutic potential of the muscular effects of salbutamol is also been explored in muscle wasting diseases.[275-278]

There have been many studies reporting the tissue distribution of clenbuterol, a related β_2-agonist, mainly due to its potential abuse in sport and meat production. Surprisingly, there have been relatively few reports of salbutamol tissue distribution, and distribution of salbutamol individual enantiomers into tissue has not been reported. Studies have demonstrated the tissue distribution of various β_2-agonists can be largely different from each other, as well as other pharmacokinetic

parameters.[279,280] In comparison with the plasma level, animal models have shown that salbutamol tends to concentrate in some tissues, in particular the feather and liver tissues (>10 times the plasma concentration), followed by kidney and eyes tissues in chickens.[281] On the other hand, muscle tissue and fat tissue were found to have lower tissue/plasma salbutamol concentration ratios (<1.5). Although similar tissues were identified with a high concentration of clenbuterol, the clenbuterol tissue/plasma concentration ratio in those tissues were found to be much higher than salbutamol.[281] In addition, a French study has found that salbutamol distribution is significantly higher in heart tissue but lower in brain tissue compare to clenbuterol.[279]

Over the years, many assays have been developed to detect and analyse salbutamol and salbutamol enantiomers. The methods most commonly used for analysing salbutamol enantiomers in blood plasma and serum samples include HPLC with fluorescence detection, LC-MS and GC-MS, usually following a solid phase extraction (SPE) technique. A previous SPE LC-MS method developed in this laboratory demonstrated the ability to detect salbutamol enantiomers at clinical levels in plasma and serum samples.[258,262]

This previous assay has been performed well within the validated range. However, the performance of the assay was reduced when applied to lower levels of salbutamol enantiomers, small amounts of samples, and/or poor recovery rates from different sample matrices. This current assay was developed based on the previous method,[262,282] with the specific objective of overcoming these limitations, including the detection of salbutamol enantiomers at low levels and in various tissue matrices,

icluding skeletal muscle, cardiac muscle, liver, and brain, using state-of-the-art analytical instrumentation.

The main differences of this current assay over the earlier assays include the use of tandem mass spectrometry with deuterated internal standard (D_3-salbutamol, Figre 3.1). Analyses were performed using an LTQ Orbitrap^{TM} hybrid mass spectrometer combined with a linear ion trap MS with an Orbitrap^{TM} mass analyser. The hybrid mass spectrometer selectively fill an ion storage device with ions of interest and analyses the ions with improved sensitivity over our previous instrumentation, as well as limiting the signaling noise encountered at normal MS low signal intensities. This assay was developed at the School of Pharmacy laboratory and CSL laboratory, University of Tasmania, and applied to investigations into tissue distribution of salbutamol in subsequent chapters

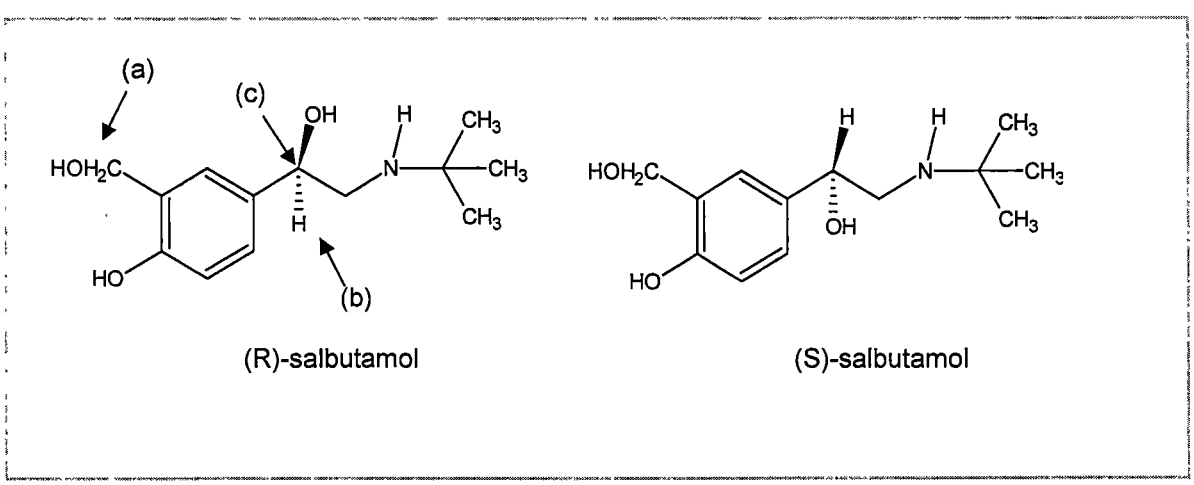


Figure 3.1 Structures of salbutamol enantiomers. Arrows indicate the location of three deuterium molecules in deuterated salbutamol – two at location (a) and one at location (b). Location (c) indicates the chiral centre.

3.3 Methods

3.3.1 Chemicals

Rac-salbutamol and ammonium formate were obtained from Sigma-Aldrich (Sydney, NSW, Australia). Deuterated *rac*-salbutamol (D3-*rac*-salbutamol; 3-hydroxymethyl-D_2, α-D_1) was obtained from Medical Isotopes, Inc. (Pelham, NH, USA). Other chemicals include analytical reagent grade ammonia solution 28%, HPLC grade methanol and distilled water. Unless otherwise specified, all other chemicals used in this Chapter were laboratory grade chemicals.

Ammonia buffer (2.5 mM, pH 10) was prepared by dissolving 0.1577 g of ammonium formate in about 90 mL of distilled water, adjusting the pH by adding ammonia solution, before adjusting the final volume to 100 mL. The buffer was stored at 4°C until use.

3.3.2 Salbutamol standard

Salbutamol primary solution was prepared by accurately dissolving approximately 1 mg of *rac*-salbutamol in 10 mL of methanol. Salbutamol working standard was prepared by diluting the primary solution with distilled water, to produce a 100 ng/mL *rac*-salbutamol solution.

Deuterated salbutamol primary solution was prepared by accurately dissolving approximately 100 µg of D3-rac-salbutamol in 10 mL of methanol. A working

solution was prepared by diluting the stock solution with distilled water, to produce a 100 ng/mL D3-rac-salbutamol solution. The amount of internal standard (D3-*rac*-salbutamol) added into each sample was determined based on the salbutamol concentrations anticipated in the sample, ideally between 10-100% of the anticipated mean concentration.

3.3.3 Sample preparation

3.3.3.1 Solid tissue

Excess wet tissue sample was first made into paste using a mortar and pestle and 500 mg of the tissue paste was accurately weighed (± 5%) into an Eppendorf™ tube. An equal volume (500 µL) of distilled water was added and vortex mixed to create a homogenised sample. Ten microlitres of D3-*rac*-salbutamol working solution (1 ng of D3-*rac*-salbutamol) was added to each sample. The samples were then placed in an ultrasonic bath for 30 min, before centrifuging at 12,000 *g* for 15 min. Supernatants were extracted by pipette into new tubes and equal volume (500 µL) of ammonia buffer solution (0.25 mM, pH10) were added, before continuing with the SPE. Solid tissues including skeletal muscle, heart muscle and liver were all prepared using this method. Five nanograms of D3-*rac*-salbutamol was added to each 500 mg of liver sample, due to the likely higher salbutamol concentration in tissue. In order to assist the process of making the sample paste, liquid nitrogen was used to deep freeze the tissue sample, and added into the mortar during the grinding process.

An additional step of protein precipitation with acetonitrile was used to pre-treat samples with high levels of protein contaminants, such as liver tissue. Three times volume (1500 μL) of acetonitrile was added to the homogenised sample (containing internal standard) and vortex mixed before mixing in an ultrasonic bath. The mixture was then centrifuged at 12,000 *g* for 15 min. The supernatant was extracted into a separate tube by pasteur pipette and dried in a heating block (45°C) with a constant flow of nitrogen gas. The sample was reconstituted with distilled water, and ammonia buffer was added before proceeding to SPE.

3.3.3.2 Semi-solid tissue

Samples of semi-solid tissue (brain tissue weighing between 50 to 500 mg) were prepared by first accurately weighing tissue (±1 mg) into an Eppendorf™ tube. The sample was then made into an homogenised paste using disposable Eppendorf™ micro-pestles. An equal volume (1 μL for each mg of sample) of distilled water was added and vortex mixed to create a homogenised sample. Twenty microlitres of D3-*rac*-salbutamol working solution (containing 2 ng of D3-*rac*-salbutamol) was added to each sample. The samples were then placed in an ultrasonic bath for 30 min, before centrifuging at 12,000 *g* for 15 min. Given that the brain tissue contained a significant amount of fat, the aqueous extract was positioned between the upper fat layer and the pellet after centrifugation. The aqueous layer was extracted by Pasteur pipette into new tubes and an equal volume (1 μL for each mg of initial sample) of ammonia buffer solution (0.25 mM, pH10) was added, before continuing with the SPE.

3.3.3.3 Liquid sample (plasma, serum and blood)

Liquid sample was prepared by accurately transferring 500 μL of the sample into an Eppendof™ tube, and 10 μL of D3-*rac*-salbutamol working solution (1 ng of D3-*rac*-salbutamol) was added to each sample. An equal volume (500 μL) of ammonia buffer solution (0.25 mM, pH10) was added, before continuing with the SPE. Liquid samples including blood, plasma and serum were all prepared using this method.

An additional step of protein precipitation, as described in Section 3.3.3.1, was also employed for the preparation of blood samples, before ammonia buffer solution was added.

3.3.4 Solid phase extraction

SPE was carried out in accordance with the previously performed and validated method, using Oasis™ HLB 60 mg (3 mL) extraction cartridges (Waters Corporation, Milford, Massachusetts, USA) and Vac-Elut™ (Analytical International, Alltech Australia Pty Ltd., Victoria, Australia).[262] In brief, each cartridge was first conditioned with 500 μL of methanol followed by 500 μL of distilled water. The sample was then loaded onto the cartridge, followed by a 500 μL distilled water wash. Extracted salbutamol in the SPE cartridge was eluted into a tapered glass test tube with four consecutive 250 μL volumes of methanol using positive syringe pressure, and evaporated at 45°C in a heating block with a constant gentle flow of nitrogen gas (~30 psi). The dry extract was than reconstituted in 100 μL of methanol, transferred into a new Eppendorf™ tube and centrifuged at 12,000 *g* for 5 min. Supernatant was transferred into an autosampler vial with a 150 μL insert and stored out of light at 4°C

until analysis. Samples beyond the calibration range were diluted appropriately and re-analysed.

3.3.5 LC-MS/MS system

The analytical HPLC linear ion trap tandem MS (LC-MS/MS) consisted of a Surveyor MS HPLC system and a LTQ Orbitrap™ hybrid mass spectrometer (Thermo Scientific, CA, USA). A teicoplanin-based Astec CHIROBIOTIC™ T Chiral HPLC column (250 x 4.6 mm ID), purchased from Alltech Associates Australia Pty. Ltd., (Victoria, Australia) was used for chiral separation, together with a C18 guard column (SecurityGuard™) from Phenomenex Inc. (NSW, Australia).

The HPLC was operated with a mobile phase consisting of methanol, acetic acid and 28% (w/v) ammonia (1000:5:1, v/v/v) at a flow rate of 1.3 mL/min. The retention times of the salbutamol enantiomers were 6.6 and 7.4 min for the (R)- and (S)-enantiomers respectively, with a total run time of 10 min. Positive ion atmospheric pressure chemical ionisation (APCI) ionisation was used with an APCI vaporizer temperature of 400°C, source current of 4 μA, capillary temperature of 200°C, sheath gas flow (50 unit), and auxillary gas flow of 5 unit.

The samples were analysed using a selective reaction monitoring (SRM) scan. The parent masses of the deuterated and non-deuterated enantiomers were isolated together in the 2D ion trap (isolation range - 241.6 +/- 5 Da). The parent masses were fragmented in the ion trap (normalised collision energy 60%), and detected in the Orbitrap, giving high resolution information on the fragment ions (range 218.6-

228.6 Da). Under these conditions, (R)- and (S)-salbutamol gave a fragment daughter of 222.1490 Da, with the deuterated form giving a daughter of 225.1680 Da. A filter for both these masses with a mass tolerance of 5 ppm was applied post analysis to the SRM scan, separating the deuterated and non-deuterated forms.

3.3.6 Calibration and measurement

Calibration samples were prepared from the working standard solution of *rac*-salbutamol (100 ng/mL in water). Working solutions were prepared by 1:1 serial dilution with water, to produce standard concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 0.781 and 0.195 ng/mL *rac*-salbutamol. Fifty microlitres of each dilution (and water for blank) was added to 500 mg (or 500 µL for liquid sample) of homogenised blank muscle tissue sample and thoroughly mixed using a vortex mixer to give spiked concentrations equivalent to 10, 5, 2.5, 1.25, 0.625, 0.313, 0.0781, 0.0195, and 0 ng/g of *rac*-salbutamol in tissue. Samples were prepared as outlined in Section 3.3.3, extracted as outlined in Section 3.3.4, and analysed as outlined in Section 3.3.5.

The calibration for brain sample, liver sample and serum sample (representing liquid sample) were prepared in the same manner, with the spiked concentration range from 50 to 1 ng/g, 500 to 1.0 ng/g and 20 to 0.3125 ng/mL of *rac*-salbutamol respectively. Two hundred microlitres (instead of 500 µL) of serum sample was used for the calibration.

The signal ratio between salbutamol enantiomer and D_3-salbutamol enantiomer was first measured against the spiked concentration, to determine the calibration curve.

The calibration curve was then used to determine the concentration of salbutamol enantiomers in each sample.

3.3.7 Assay performance

Intra-batch reproducibility was assessed by spiked samples (n=4) at a chosen level of 0.313 ng/g (0.156 ng/g for each enantiomer) from muscle samples and 1.0 ng/mL (0.5 ng/mL for each enantiomer) from serum samples. The samples were spiked in the same manner as the calibration samples as outlined in Section 2.3.6.

Recovery was assessed by spiked samples at the chosen levels of 0.313 ng/g (0.156 ng/g for each enantiomer), 10.0 ng/g (5.0 ng/g for each enantiomer), 50 ng/g (100 ng/g for each enantiomer) and 1.0 ng/mL (0.5 ng/g for each enantiomer) for muscle, brain, liver and serum sample respectively, however, D3-*rac*-salbutamol was added only after the elution step, representing theoretical 100% recovery of D3-salbutamol. The samples were fortified in the same manner as the calibration samples as outlined in Section 2.3.6, except for the addition of D3-*rac*-salbutamol.

The lower limit of quantification (LLoQ) was estimated from the lowest concentration with a coefficient of variation of less than 15% by analysing replicate samples (n=4). The LLoQ selected for the evaluation were muscle sample at the level of 0.078 ng/g and 0.313 ng/g (0.0195 ng/g and 0.156 ng/g for each enantiomer), and serum samples at the level of 0.312 ng/mL and 1.0 ng/mL (0.156 ng/mL and 0.5 ng/mL for each enantiomer).

3.4 Results

The calibration curves for each salbutamol enantiomer in all tissue matrices were linear over the calibration range with $r^2 \geq 0.97$. The calibration curve for (R)- and (S)-salbutamol in spiked samples were similar in all matrices. An example of the calibration curve (skeletal muscle tissue sample) is shown in Figure 3.2.

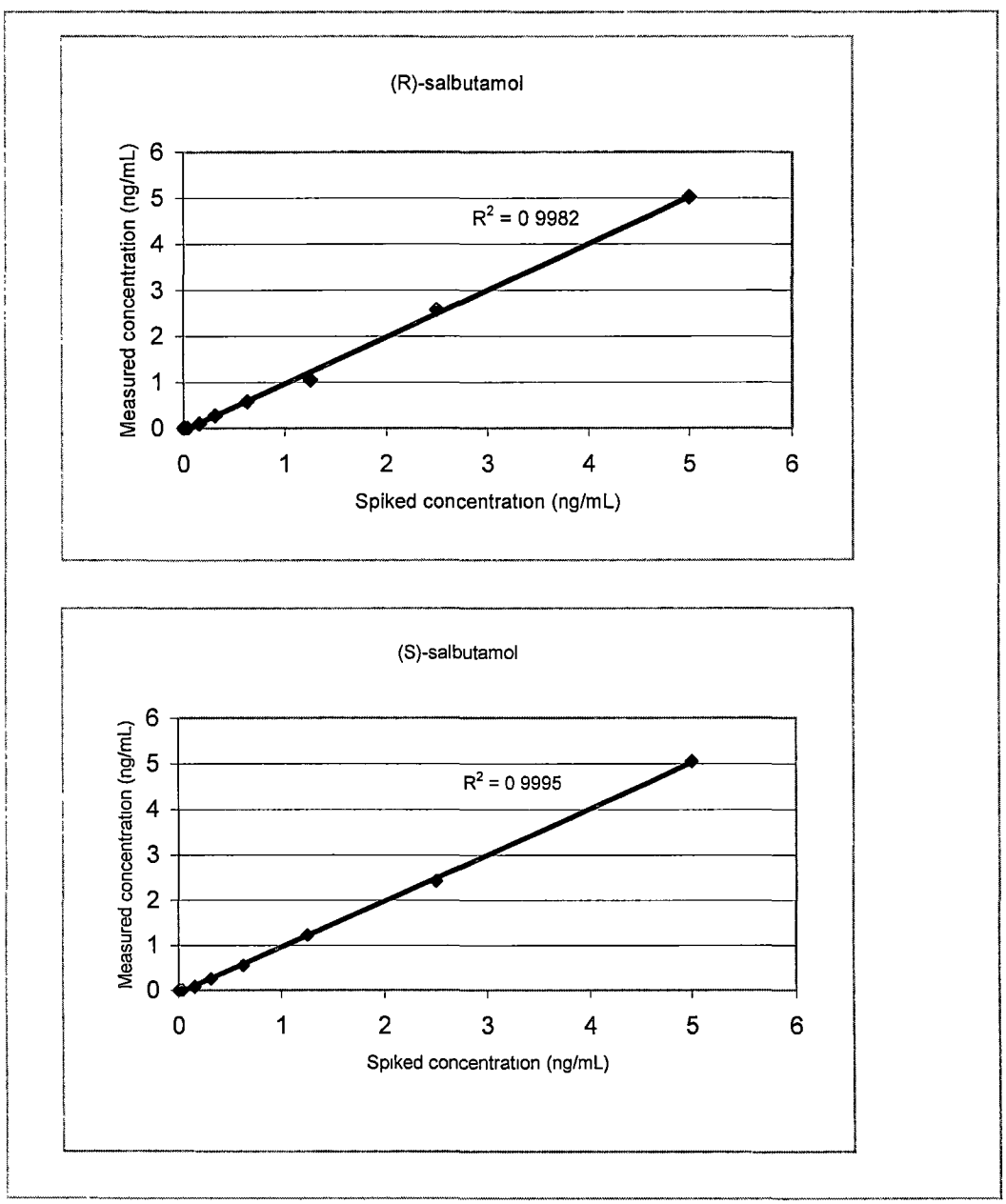


Figure 3.2 Calibration curve of spiked skeletal muscle sample

The assay performance data was summarised in Table 3.1. LLoQ was determined in both the muscle and serum sample equivalent to approximately 30 pg of salbutamol enantioner on column,. An example of the LC-MS/MS chromatogram is shown in Figure 3.3.

Table 3.1 Assay performance data. Intra-batch reproducibility and recovery for muscle, brain, liver and serum[†] were measured at spiked concentrations of 0.156 ng/g, 5.00 ng/g, 25.0 ng/g and 0.500 ng/mL salbutamol enantiomer respectively

	(R)-Salbutamol	(S)-Salbutamol
Calibration curve r^2 (range)		
Skeletal muscle (0.0781-5 ng/g)	0.99	0.99
Brain (0.5-50.0 ng/g)	0.99	0.99
Liver (0.5-250 ng/g)	0.99	0.99
Serum (0.156-10 ng/mL)	0.99	0.97
Intra-batch reproducibility (%RSD)		
Skeletal muscle (n=4)	12.9	11.2
Brian	-	-
Liver	-	-
Serum (n=4)	12.4	12.5
Recovery (Mean±%RSD)		
Skeletal muscle	26.2±4.9	49.8±7.8
Brain	84.8±2.8	84.8±2.8
Liver	87.3±0.7	87.3±0.7
Serum	79.5±6.6	79.5±6.7
LLoQ		
Skeletal muscle	0.156 ng/g	0.156 ng/g
Brain	-	-
Liver	-	-
Serum	0.50 ng/mL	0.50 ng/mL

[†] 200 μL (instead of 500 μL) of serum sample was used for the analysis

Samples that were treated with acetonitrile protein precipitation were found to largely improve the SPE process, with no significant influence on the assay performance. Several methods of preparing homogenised sample from solid tissue (including tissue

grinders and electronic homogenisers) were also investigated without significant variation in the recovery outcomes.

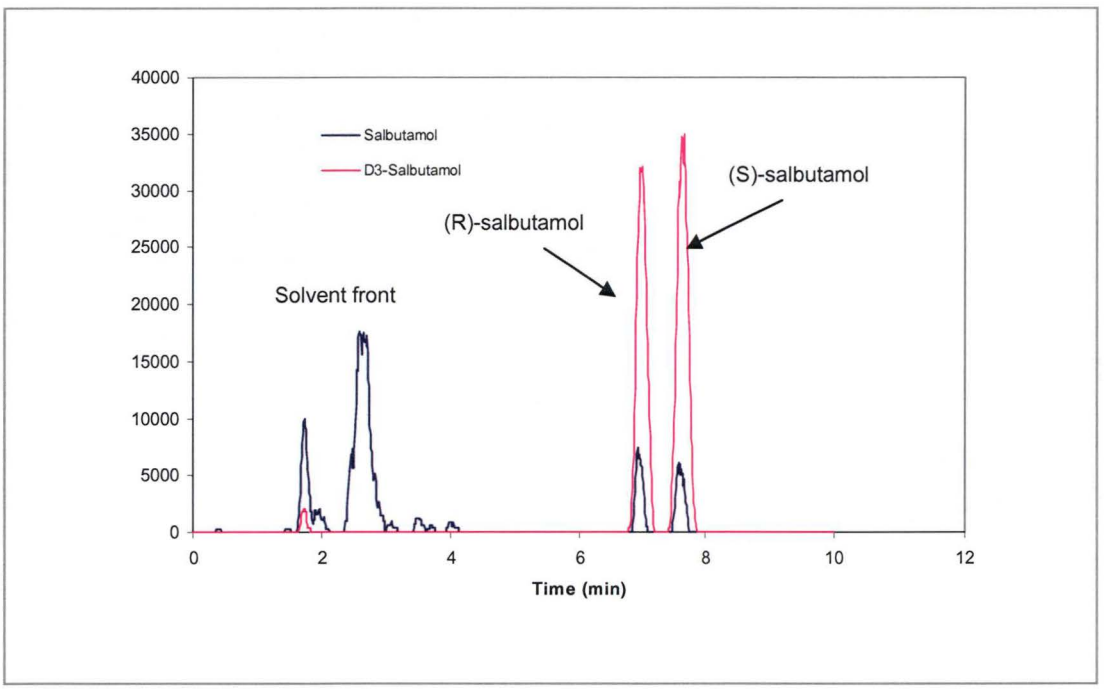


Figure 3.3 LC-MS/MS chromatogram of (R)- and (S)-salbutamol in muscle each at 0.16 ng/g, together with D3-salbutamol internal standard.

The assay was largely developed to investigate the enantioselective disposition of (R)- and (S)-salbutamol in animal tissues and the involvement of the organic cation transport (OCT) system (Chapter 4). In addition, the assay was successfully applied to studies presented in this thesis, including the study of circulating salbutamol enantiomers levels in acute severe asthma and acute COPD presentations in the emergency department setting (Chapter 2), and pharmacogenetic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients, SULT 1A3 SNP (rs1201735) (Chapter 6).The assay has also successfully been applied to investigation(s) not included in this thesis, including the analysis of tissue salbutamol disposition from animals fed grain with 6 parts per million of (R)-salbutamol.

3.5 Discussion

The assay has demonstrated that LC-MS/MS with SPE cleanup methods used for plasma can be applied to other biological matrices, including muscle, liver and brain tissue, with satisfactory assay performance, detecting enantiomers at very low levels (as per Section 4.3). The recovery of salbutamol from muscle tissue was much lower than in plasma or serum (25-50% compared to 80-90% for plasma/serum,[262] brain and liver) but the use of deuterated *rac*-salbutamol as internal standard in current assay has circumvented these problems, particularly in muscle tissue.

Samples prepared from solid tissue appear to contain high quantities of cellular debris despite the SPE clean up, and SPE cartridges were vulnerable to blockage. Centrifuging the sample at 12,000 *g* before SPE has significantly minimised the problem. Full blood samples and samples from organs with high blood content (eg. liver tissue) were found to have high levels of contaminants that could not be easily precipitated by centrifuging force alone, affecting the performance of both the SPE cartridge and HPLC separation. In order to minimize this problem, the additional step of protein precipitation with acetonitrile was successfully undertaken.

The increase in sensitivity of this assay allows the detection and determination of trace amounts of salbutamol enantiomers in various tissues (blood, serum, plasma, skeletal muscle, heart muscle, liver and brain tissue) with a minimum amount of sample required. Given that the LLoQ was determined at around 30 pg on column, procedures that improve the recovery and modifications to the volume of LC injection will allow the determination of salbutamol at lower than currently validated

concentration. This assay has a wide range of applications including respiratory, therapeutic myoanabolic, pharmacokinetic and biochemical uptake studies involving salbutamol.

In conclusion, by utilising a deuterated internal standard and high resolution LC-MS/MS detection, this assay can be used to measure trace amount of salbutamol enantiomers in various tissues.

Chapter 4

Enantioselective disposition of (R)- and (S)-salbutamol in animal tissues and the involvement of the organic cation transport (OCT) system

4.1 Abstract

Background: There is limited data with regard to the relationship between the salbutamol enantiomer levels in the circulation and the levels present in tissue(s). By using animal models, the objective of this study was to examine the distribution of salbutamol enantiomers in tissues and the relationship with blood level, as well as investigating the role of the organic cation transport (OCT) system in salbutamol transport.

Method: Three animal models were employed for this investigation. An oral dosing model was undertaken whereby seven neonatal mice were orally administered with a maximum tolerable dose of salbutamol (10 mg/kg) twice a day for five days, before samples were collected from plasma, muscle and brain. An *in vitro* rat hindlimb muscle perfusion model was employed for the investigation of the role of the OCT system, where isolated hindlimbs were perfused with salbutamol +/- various OCT blockers (corticosterone, beta-estradiol, progesterone) via the circulatory system. Lastly, an *in vivo* infusion rat model was employed for the investigation of salbutamol uptake into cardiac muscle. Plasma and muscle samples were analysed for salbutamol enantiomers with a chiral LC-MS/MS assay developed in the previous chapter.

Result: From the oral dosing model, mean (±SD) levels of racemic salbutamol were 915 (±293) ng/mL in plasma, 2574 (±196) ng/g in muscle, and 53 (±6.6) ng/g in brain. Enantioselective uptake of (R)-salbutamol was observed in skeletal muscle ($p<0.0001$) with an R:S ratio of 3.7. The *in vivo* infusion model demonstrated enantioselective distribution of (R)-salbutamol in heart muscle four hours after the infusion, with an R:S ratio of 2.9, whereas the ratio in plasma was 0.3 (consistent with known enantioselective pharmacokinetic parameters) and 0.9 in skeletal muscle.

Salbutamol distribution into skeletal muscle within four hours was not affected by the presence of OCT blockers and there was no difference between (R)- and (S)-salbutamol.

Discussion: Enantioselective uptake of (R)-salbutamol was most evident in muscle tissue, particularly heart muscle, and is consistent with an enantioselective active transport mechanism. Enantioselective uptake in heart muscle has relevance for cardiac adverse effects of (R)-salbutamol use in airways disease, as a repartitioning agent, or potential therapeutic pathways in muscle wasting diseases. The OCT system does not appear to play a significant role in the skeletal muscle uptake of salbutamol within the first four hours of exposure.

4.2 Introduction

It has long been recognised that β_2-agonists have some systemic adverse effects, including significant cardiovascular effects,[233,247,250,251] and skeletal muscle tremor.[283] Recently, some studies have raised the concern regarding these potential adverse effects, in particularly among high risk patients, e.g. patients with cardiovascular co-morbidity.[92,251,252] On the other hand, β_2-agonists may have significant beneficial effects on muscle strength and bulk. Around a 20% increase in muscle strength may be observed along with a decrease in body fat after oral dosing,[284-287] hence the term "repartitioning" agents has been applied to β_2-agonists.

β_2-Agonist mediated performance enhancement in athletes and applications in animal primary production are well known, particularly for clenbuterol.[288] Significant effects on skeletal muscle function have also been observed with salbutamol,[274] but the effect varies with route of administration. Oral doses of salbutamol result in significant muscle effects which are not observed with inhaled therapy,[273] and infusions of salbutamol can result in the same metabolic effects as clenbuterol in rats.[287,289] Clearly, plasma concentration, duration and muscle uptake are important considerations for any therapeutic application in increasing muscle mass and function.

There has been interest in the application of salbutamol to a variety of conditions and diseases associated with muscle atrophy. In animal models, β_2-agonists including salbutamol have been shown to reduce atrophy in rats after denervation induced atrophy,[290] reduce degeneration in dystrophic mice,[291] retard loss of function in motor neuron degenerative mice,[292] and improve locomotor function in a rat spinal cord

contusion model.[291] Increases in cardiac muscle mass have also been observed in tumor associated atrophy in rats.[293]

There have been many studies reporting the disposition of clenbuterol in animal tissue including chiral assays.[294,295] Von Deutsch et al [295] reported enantioselective uptake in gastrocnemius muscle, eyes and kidney and both S and (R)-clenbuterol had equal anabolic activity. There have been relatively few reports of salbutamol tissue disposition. Salbutamol is a hydrophilic weak base (pk_{a1}=9.22 and pk_{a2}=9.83) which is predominantly charged at physiological pH, yet despite this salbutamol has relatively high bioavailability after inhalation compared to oral delivery. Salbutamol has been reported to concentrate particularly in the liver and cardiac muscle.[279,281] Previous studies had indicated the absorption and elimination of salbutamol after oral delivery exhibit stereoselective behaviour, which can lead to accumulation of (S)-salbutamol in the circulation.[231] However, to the authors' knowledge, enantioselective disposition of salbutamol has not been previously reported in the literature.

The transport of drugs and xenobiotics across plasma membrane (e.g. reabsorption and excretion) are found to involve a group of ATP-dependent transporters, that include four identified super-families.[270] The organic cation transport (OCT) proteins are a sub-family of the organic cation/anion/zwitterion transporter super-family (SLC22).[270] Although the mechanism involved is unclear, salbutamol transport in the lung has been thought to involve the OCT family,[296,297] which can transport organic cationic drugs in either direction across membranes. The OCT proteins have three known members (OCT-1, OCT-2 and OCT-3), which are found in various organs and tissues, including both skeletal muscle and heart muscle.[270,298] In addition,

investigation into human OCT proteins has suggested stereoselective binding with drugs closely related to salbutamol, including acebutolol, fenoterol, atenolol, propranolol and isoproterenol.[270,299,300]

The role of these transporters in the pharmacokinetics of drugs has been demonstrated with some recent studies, which found that transgenic mice (genetic knockout of OCT1 and OCT2) given intravenous injection of cationic drugs have both lower drug concentration in organs (e.g. liver and heart) and increases in drug elimination.[270,301,302] In addition, a number of genetic variations were identified in recent studies, at genes encoding the OCT proteins (SLC22A1-3), including a number of SNPs.[270-272] It was also found that some of the SNPs, in particular the SNP at SLC22A1, were related to a reduction in the protein activities.[270,303,304]

This study was designed to investigate the distribution of salbutamol enantiomers into various tissues, and the involvement of the OCT system. Some common validated animal models were employed for this investigation, including an extensively used hind-limb perfusion method developed by Kolka.[305]

4.3 Method

Unless otherwise specified, all chemicals used in this Chapter were laboratory grade chemicals.

4.3.1 *In vivo* oral dosing model

This model was employed to investigate the tissue distribution of salbutamol enantiomers after regular oral dosing. All animals in this experiment were wild-type sibling controls of spinal muscular atrophy (SMA) transgenic mice, (animals with normal genetic background, $SMN2^{+/+}$; $SMN\Delta7^{+/+}$; $Smn^{+/+}$).[306] The mouse phase of this study was carried out by Psychogenics (Tarrytown, NY, USA) and the study was approved by the Institutional Animal Care and Use Committee (USA). The animals were part of a larger study investigating the therapeutic potential of salbutamol in SMA.

4.3.1.1 Drug administration

Seven mice were administered an oral chronic maximum tolerable dose (MTD) of *rac*-salbutamol by oral gavage using a syringe from the postnatal age of three days. Salbutamol was administered twice daily, for five consecutive days.

The MTD for salbutamol dose was defined as a dose that produced observable but mild to moderate behavioural and non-behavioural side effects such as change in body weight, without seizures or other major physiological change. The MTD for

salbutamol in this study was 10 mg/kg and delivered in phosphate buffered saline at a concentration of 2 mg/mL.

4.3.1.2 Sample collection

One hour after the morning dose on the fifth day, the animals were anesthetised using phenobarbitone. Blood samples were collected via cardiac puncture and plasma samples were extracted. Skeletal muscle samples were extracted from hind limbs. Two different brain samples were collected, partial brain (cerebral only) and whole brain (including cerebellum, brainstem and pituitary gland) given previously reported accumulation outside the blood-brain barrier in the pituitary of rats.[307] All samples were frozen on dry ice and stored at -80°C until analysis.

4.3.2 *In vitro* hind limbs muscle perfusion model

This model was employed to investigate the role of OCT proteins in salbutamol uptake into skeletal muscle. Previously verified competitive OCT blockers (corticosterone, β-estradiol or progesterone, which selectively block OCT-3 proteins, OCT-1 plus OCT-3 proteins, and all OCT proteins respectively) [308] were selected for this experiment. All animals used were male Hooded Wistar rats (n=8), weighing 250 g (+/- 5 g), and the study was approved by the University of Tasmania Animal Ethics Committee, in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). The experiment was performed in the Biochemistry Department laboratory (University of Tasmania).

4.3.2.1 Surgical Procedure

A previously validated and performed surgical procedure [305] was used to prepare the animals for perfusion, as outlined by Ruderman et. al., (1971) [309] with additional modifications as detailed by Colquhoun et. al., (1988).[310] In brief, the animal was anesthetised with intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) (Virbac Pty Ltd, Australia). Once the animal was unconscious, circulation to the tail and tarsus were restricted with strong twine ligates, and an incision was made along the midline of the abdomen. The abdominal wall was then removed from the pubic syphysis to the xiphoid process. The superior epigastric vessels and iliolumbar vessels were restricted with ligates, as well as all vessels supplying the testes, bladder, seminal vesicles, descending colon and duodenum. The bladder was drained and most organs in the abdominal cavity were removed (including testes, seminal vesicles, large and small intestine), to allow better access to the vasculature underneath. Blood flow at the mesenteric vessels and renal vessels was also restricted with ligates.

The aorta and vena cava were carefully separated, two pairs of ligatures were loosely placed around each vessel (above the iliolumbar vessels and below the renal vessels). One IU/g body weight of heparin (Mayne Pharma International Pty Ltd, Melbourne, VIC, Australia) was injected into the vena cava and allowed to circulate through the vasculature. The superior ligature around the vena cava was tied off and the vena cava was cannulated with an 18G catheter before being secured in place by the lower ligature. The aorta was then cannulated in a similar way with a 20G catheter. The animal was then transferred to perfusion cabinet and attached to the perfusion apparatus (described in Section 4.3.2.2). After successful connection to the perfusion apparatus, the rat was euthanased with pentobarbital sodium intracardiac injection,

and flow to the upper torso was restricted with ligature around the abdomen at the L3-L4 vertebrae.

4.3.2.2 Perfusion procedure

The perfusion was performed on both hind limbs and the temperature was maintained at 37°C. The perfusion medium was modified Krebs-Henseleit bicarbonate buffer, which consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $NaHCO_3$, 2.54 μM $CaCl_2$, 8.3 mM D-Glucose and 4% Bovine Serum Albumin (Trace BioSciences NZ Ltd., Hamilton, New Zealand), filtered at 0.45 μm. The perfusion medium was continuously stirred and gassed with carbogen gas (95% O_2, 5%CO_2) and temperature raised to approximately 37°C by passing through a glass, water-jacketed heat exchanger prior to entering a Silastic lung (~4 m coiled up Silastic® Laboratory Tubing (6736), Dow Corning Co., Midland, MI, USA), which was also gassed with carbogen gas to maintain constant arterial PO_2.

The perfusion was performed with a previously validated method,[311-313] and the flow of perfusion medium is shown in Figure 4.1 and Figure 4.2. In brief, the perfusion medium was pumped through the system with a peristaltic pump (Masterflex, Cole-Palmer, USA). The perfusion medium was first pumped through the heat exchange chamber, followed by the Silastic lung. Air bubbles within the perfusion medium were released with a simple bubble trap (eg. clinical infusion port) and 'arterial' pressure measured with a pressure meter, before being pumped into the isolated hindlimb through an aortic cannula. The effluent emerging from the venous cannula was passed thought a Clark-type-electrode (which measures oxygen on a catalytic

platinum surface using the Nett reaction) before being directed into a waste container or recirculated into the reservoir of perfusion medium for re-circulation (detail refered to in Table 4.1).

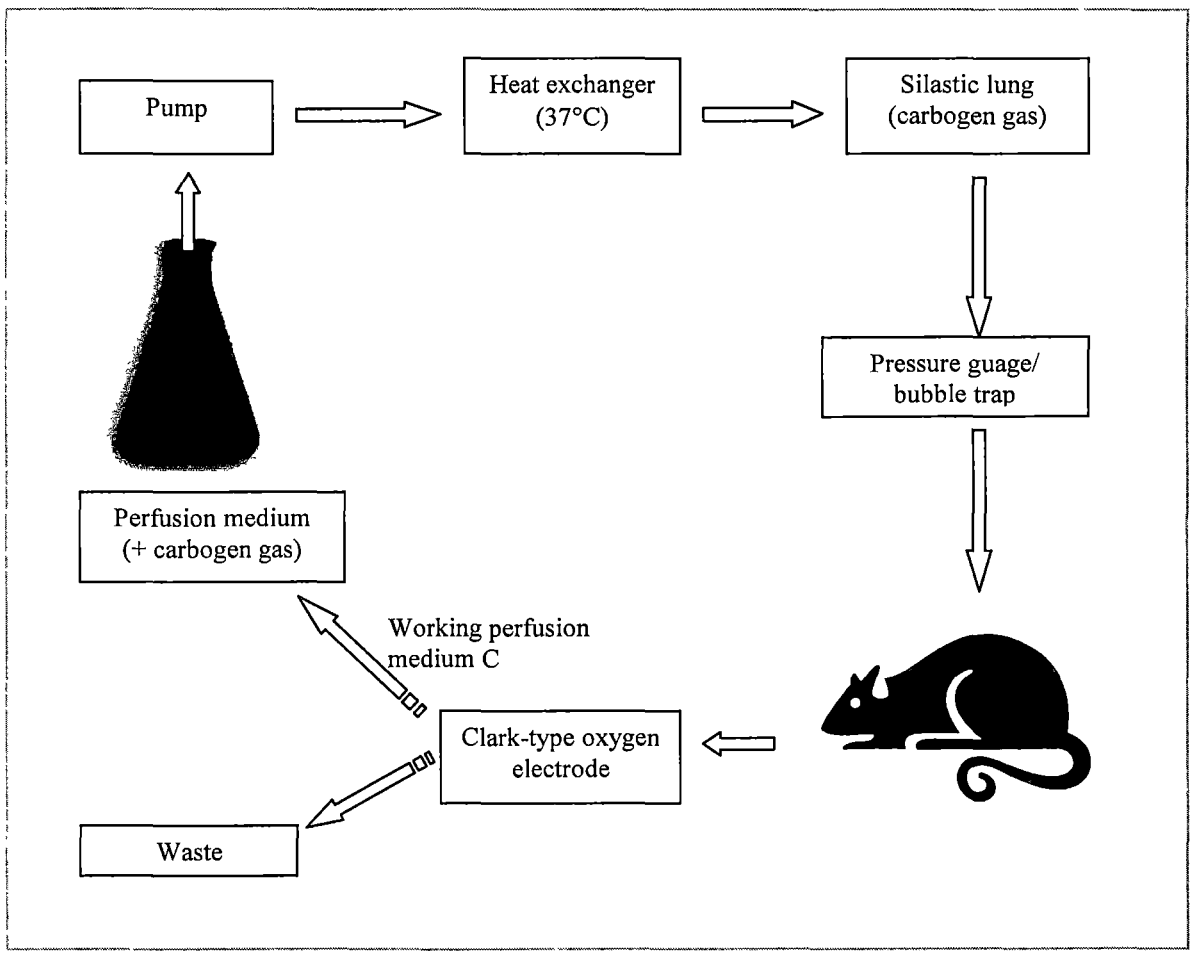


Figure 4.1 The perfusion apparatus setup and direction of flow of perfusion medium in the *in vitro* hindlimb muscle perfusion model. Both hind limbs of the animal were perfused for the duration of 4 h with working perfusion medium C. All perfusion media emerging from the venous line were eventually directed into a waste container, except for working perfusion medium C which was directed back into the reservoir for re-circulation.

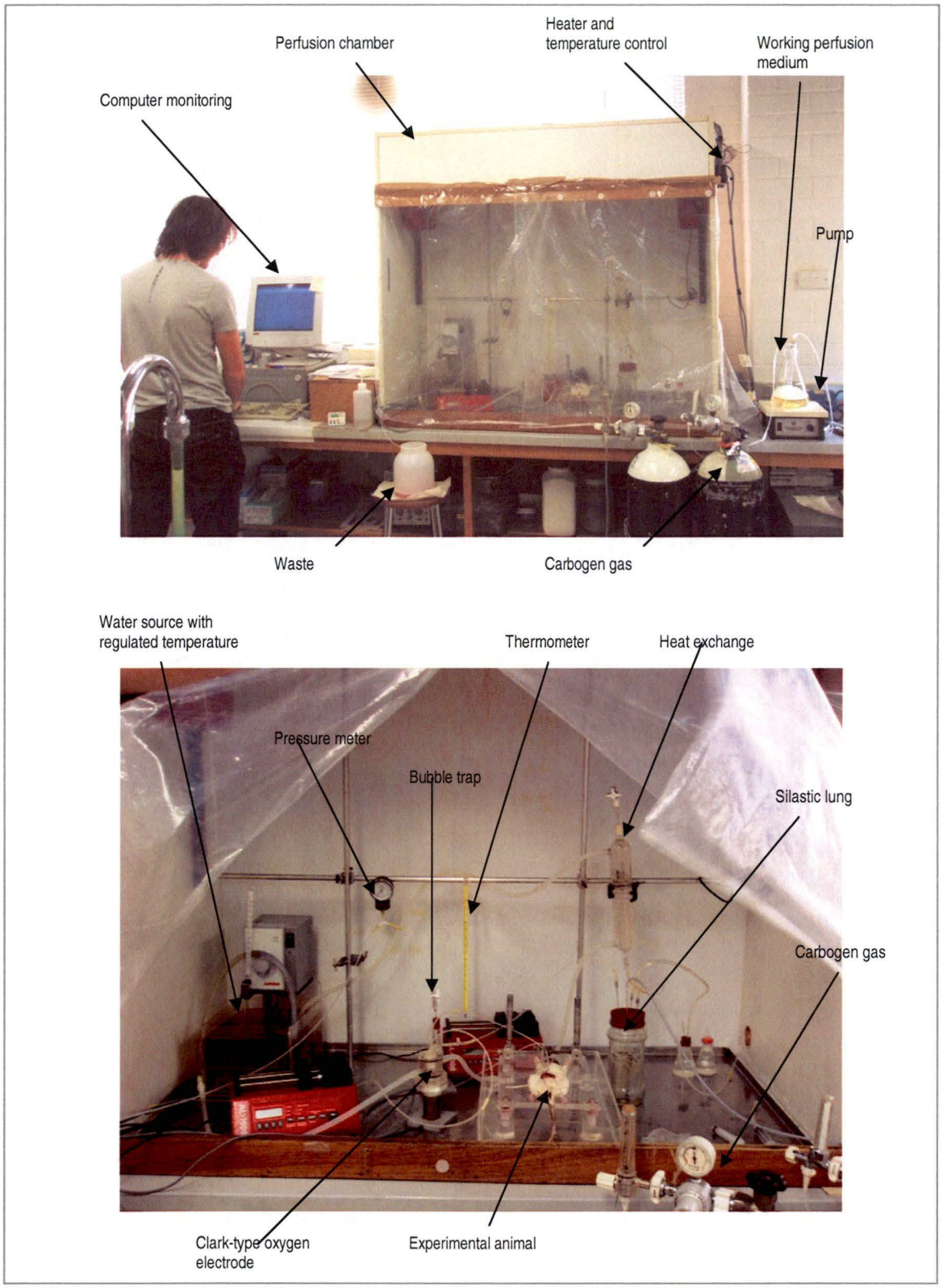


Figure 4.2 The perfusion chamber set up and the perfusion apparatus

The sufficiency of perfusion of the isolated muscles was monitored by examining the vascular resistance, and metabolic activity was monitored by examining the oxygen

uptake of the perfused muscle. The vascular resistance was monitored with continuous arterial pressure, using a pressure transducer proximal to the aortic cannula. Oxygen content of the venous effluent was monitored using a Clark-type oxygen electrode. The perfusion pressure and the venous oxygen content were recorded throughout each perfusion and analysed with WINDAQ data acquisition software (DATAQ Instruments Inc., USA).

Rac-salbutamol dose was calculated according to the estimated perfused muscle weight (10 mg/kg), and the concentrations of OCT blockers were calculated (1x concentration and 10x concentration) based on the IC50 determined by Hayer-Zillgen et al[308]. Working perfusion medium was prepared by adding concentrated salbutamol solution (10 mg/mL in ethanol) and/or a concentrated OCT blockers solution (in ethanol) into blank perfusion medium (Table 4.1). *Rac*-salbutamol, corticosterone, β-estradiol and progesterone were all purchased from Sigma-Aldrich Australia (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW Australia), and all working perfusion medium contained less than 0.1% of ethanol.

At the beginning of the perfusion, blood was first allowed to wash from the hind-limb (with blank perfusion medium), and then the hind-limbs were perfused with working perfusion medium, in the order of perfusion medium A, B, C and then D, as detailed in Table 4.1. The venous effluent of the working perfusion medium A, B and D was directed into the waste container, but the venous effluent of working perfusion medium C was directed back into the reservoirs for re-circulation.

Table 4.1 The working perfusion medium, rate and duration of perfusion for *in vitro* hind limbs muscle perfusion model

	Working perfusion medium A **15 mL/min for 3 min** **Then 8 mL/min for 15 min**	**Working perfusion medium B** **(100 mL)** **8 mL/min for 12 min**	**Working perfusion medium C** **(400 mL)** **8 mL/min (re-circulate) for 4 h**	**Working perfusion medium D** **(100 mL)** **8 mL/min for 10 min**
Rat 1	Blank	Blank	Blank	Blank
Rat 2	Blank	Blank	4 μM salbutamol	Blank
Rat 3	Blank	0.3 μM corticosterone	4 μM salbutamol + 0.3 μM corticosterone	Blank
Rat 4	Blank	3 μM corticosterone	4 μM salbutamol + 3 μM corticosterone	Blank
Rat 5	Blank	6 μM β-estradiol	4 μM salbutamol + 6 μM β-estradiol	Blank
Rat 6	Blank	60 μM β-estradiol	4 μM salbutamol + 60 μM β-estradiol	Blank
Rat 7	Blank	20 μM progesterone	4 μM salbutamol + 20 μM progesterone	Blank
Rat 8	Blank	200 μM progesterone	4 μM salbutamol + 200 μM progesterone	Blank

4.3.2.3 Samples and data collection

The calf muscles of each animal were collected (separated at tendons) after perfusion, quick frozen in liquid nitrogen and stored at -80°C until analysis by LC-MS/MS for (R)- and (S)-salbutamol. Samples of working perfusion medium C were also collected before and every hour during the perfusion. The samples of perfusion medium were stored frozen at -20°C until analysis.

4.3.3 *In vivo* infusion model

This model was employed to investigate salbutamol uptake into cardiac muscle and the rate of salbutamol uptake into skeletal muscle. The animal used in this experiment was a 250 g male Hooded Wistar rat (n = 1), and the study was approved by the University of Tasmania Animal Ethics Committee, in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). The experiment was performed in the Biochemistry Department laboratory (University of Tasmania).

4.3.3.1 Surgical Procedure

Surgery was performed as outlined by Rattigan et al.[311] In brief, the animal was first anesthetised with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight). An incision was made from the throat to bottom of neck, the neck muscle was separated and the vessels on either side of the trachea were separated from connective tissues. An incision was cut between cartilaginous rings and a tracheotomy

tube was inserted before securing in place with thread to maintain the opening of airway.

The left and right jugular veins were first cannulated, followed by a carotid artery, using polyethylene PE50 tubing (BD Intramedic^{TM}, MD, USA) and secured in place with ligates. The carotid line was attached to a pressure transducer, and the jugular lines were attached to pentobarbitone solution (Figure 4.3).

4.3.3.2 Infusion conditions

Anaesthesia was maintained according to the animal's response (guided with change in blood pressure >10 mmHg using WINDAQ data acquisition software), intra arterially at a trickle level of 0.6 mg/min/kg. The animal was first stabilised at this condition for 45 mins before the start of salbutamol infusion. The infusion model was performed at ambient room condition (22 ± 2°C and room air).

A dose of 1.25 mg of salbutamol (10 mg/mL in ethanol) was infused through the right jugular cannula, at a rate of 5 μL/min using an infuser pump. The dose was calculated based on the animal's body weight (5 mg/kg) and half of the original oral MTD based on an assumption of ≤50% oral bioavailability parent drug.[107,231,280] The animal was maintained in a stable condition, as described above, for four hours and euthanised with pentobarbitone sodium through a jugular cannula (100 mg/kg) at the end of the experiment without regaining consciousness. The apparatus set-up for the infusion model is shown in Figure 4.3

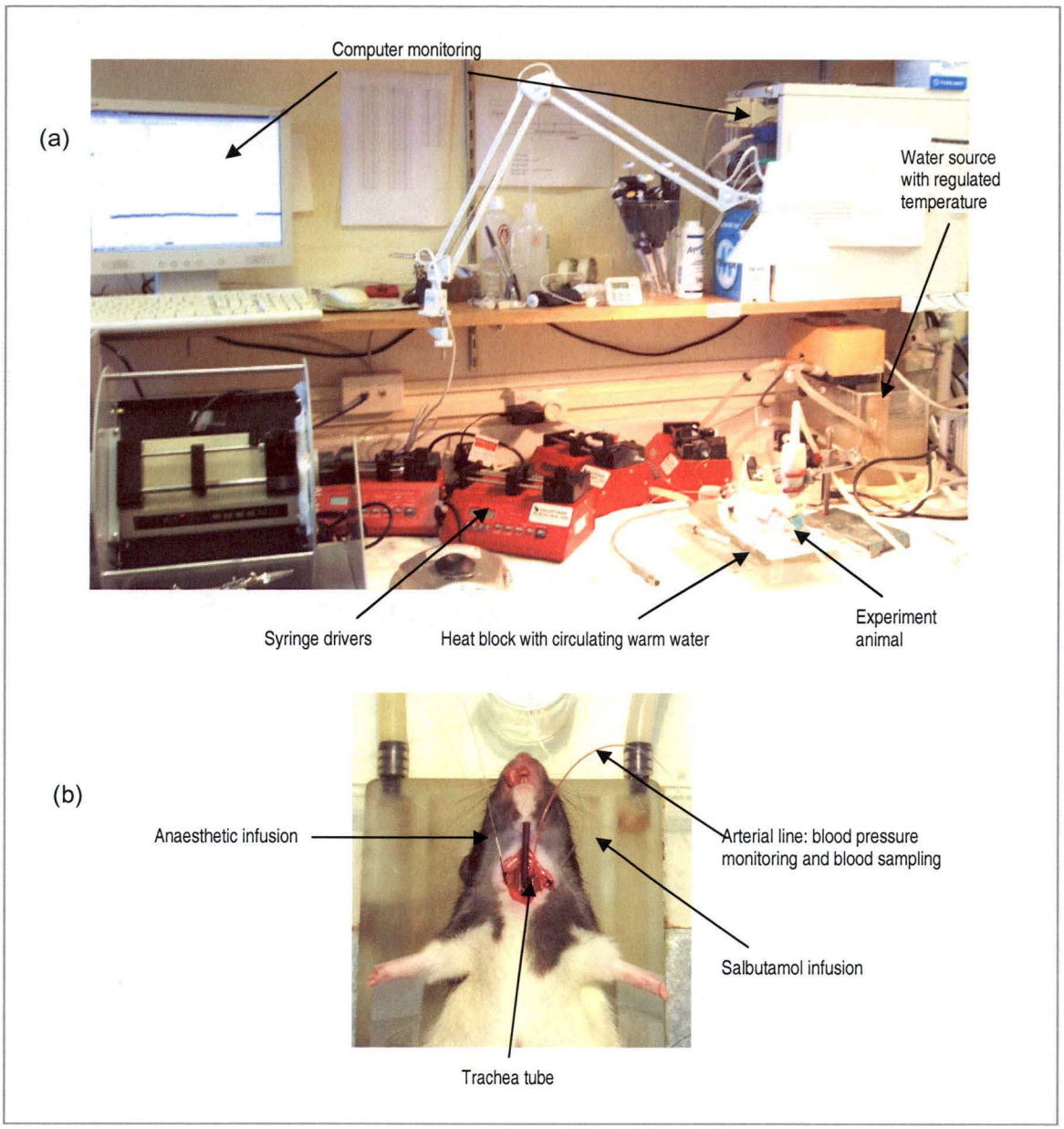


Figure 4.3 The *in vivo* infusion model, (a) set up of infusion apparatus and (b) the organisation of infusion cannula

4.3.3.3 Samples and data collection

Blood samples (~1 mL) were collected, through the arterial cannula every hour for four hours after the initiation of salbutamol infusion. Blood glucose level was measured using a glucose analyser, Model 23A (Yellow Springs Instruments Co., Inc, OH, USA), before the plasma sample (400 μL) was harvested. The remaining blood

cells were resuspended in normal saline and re-introduced to the animal via the arterial cannula. The plasma samples were frozen at -20°C until analysis

Muscle samples (~ 500 mg) were harvested, from the abdominal wall, every hour for four hours, at the same time as blood sampling. The calf muscles and the heart were harvested at the end of the four hours experiment, after the animal was euthanised. The heart chambers were washed and drained with normal saline using positive syringe pressure. All tissue samples were quick frozen in liquid nitrogen after harvest and stored at -80°C until analysis

4.3.4 Sample preparation

Salbutamol enantiomer analysis was performed at the School of Pharmacy laboratory and the CSL laboratory as previously outlined (Chapter 3). Plasma samples were prepared based on a previously reported method (Section 3.3.3.3, Section 3.3.4 and Section 3.3.5).[262] Due to the high dose given (MTD of 10 mg/kg), plasma samples were diluted 100-fold with distilled water prior to solid-phase extraction using Water Oasis™ HLB solid-phase extraction (SPE) cartridges.

Tissue samples were extracted using SPE in a similar manner (Section 3.3.3.1, Section 3.3.3.2, Section 3.3.4 and Section 3.3.5). In brief, the tissues were roughly minced using a scalpel and homogenised using mortar and pastel, with liquid nitrogen. Approximately 50 mg of skeletal muscle tissue, 50 mg of heart muscle tissue and 100-250 mg brain were accurately weighed (±0.5 mg), and placed into Eppendorf™ tubes with an equivalent amount of distilled water to produce

homogenised mixture. The samples were then centrifuged at 15,000 rpm for 15 min, aliquots of homogenate supernatant were taken and diluted appropriately with distilled water, mixed 1:1 with pH10 ammonia buffer and transferred to a Water Oasis ™ HLB SPE cartridge and analysed by LC-MS assay.

4.3.5 Statistical analysis

Differences between (S)-salbutamol and (R)-salbutamol levels in each of plasma, muscle, and brain were assessed using two-tailed paired student's t-test. Analyses were performed using Statview for Macintosh version 5 (SAS Institute, Sydney, AUS) with $p<0.05$ considered statistically significant.

4.4 Results

Assay performance criteria for the plasma, muscle and brain assays were all within acceptable limits as reported in Section 3.4. An example LC-MS chromatogram of muscle tissue extract is shown in Figure 4.4.

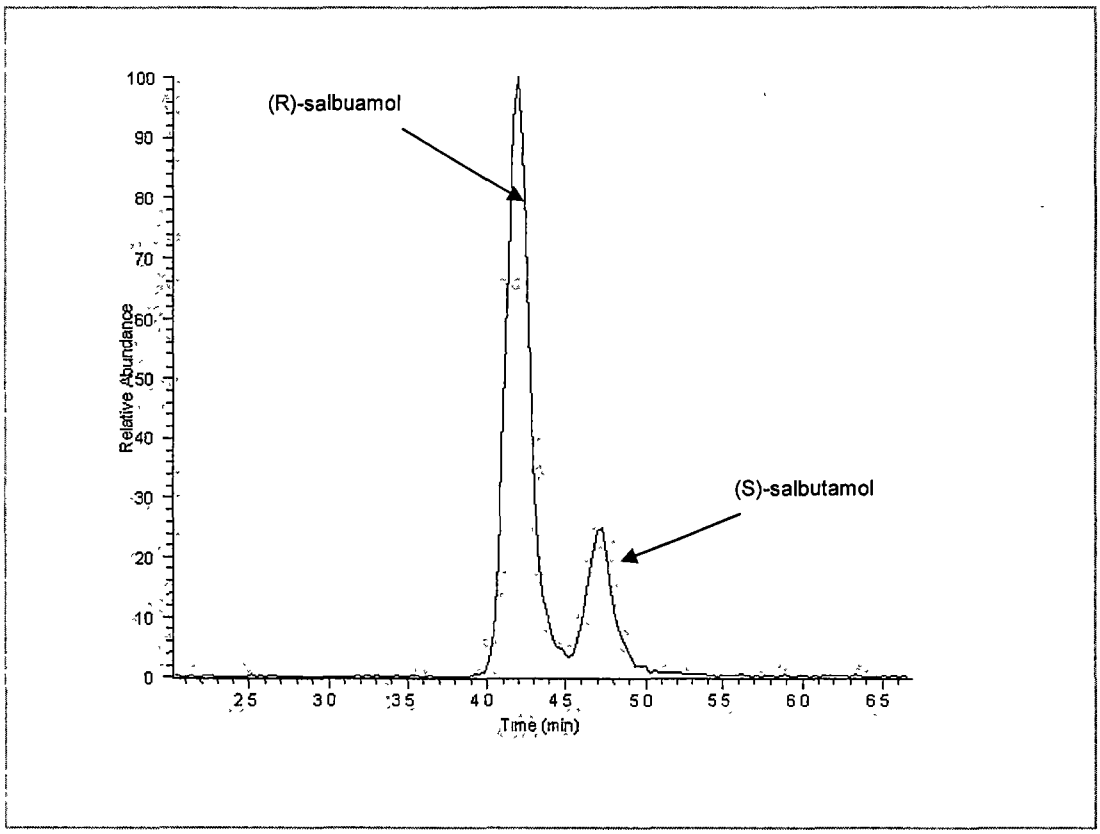


Figure 4.4 An example LC-MS chromatogram of muscle tissue extract is shown equivalent to 1958 ng/g and 522 ng/g for (R)-salbutamol and (S)-salbutamol respectively with a R:S ratio of 3.75 indicative of the selective uptake of (R)-salbutamol into muscle.

4.4.1 *In vivo* oral dosing model

Seven neonatal mice were treated with salbutamol. Plasma samples were collected from all the mice. Four skeletal muscle samples, four partial brain samples and three whole brain samples were collected. Mean levels of *rac*-salbutamol were 915 ng/mL

in plasma, 2574 ng/g in muscle and 53 ng/g in brain suggesting an active uptake mechanism into skeletal muscle and low penetration across the blood-brain barrier. There was no difference between whole brain and partial brain minus pituitary.

Enantioselective uptake of (R)-salbutamol compared to (S)-salbutamol was observed in skeletal muscle ($t=36.3$, $p<0.0001$) whereas differences between enantiomer disposition in plasma and brain were not observed. mean (±SD) (R)-salbutamol to (S)-salbutamol ratio was 3.7 (±0.16) in muscle. The salbutamol enantiomer disposition in plasma, muscle and brain is summarised in Table 4.2.

Table 4.2 (R)-salbutamol and (S)-salbutamol (mean (±SD)) levels in *in vivo* oral dosing model, plasma (n=7), muscle (n=4) and brain (n=7), after maximum tolerable dose of *rac*-salbutamol at 10 mg/kg with partition coefficients K_m (muscle-plasma), K_b (brain-plasma) and R:S ratio (mean (±SD)) of (R)-salbutamol:(S)-salbutamol.

	(R)-salbutamol	(S)-salbutamol	Total	R:S
Plasma (ng/mL)	461 (±134)	454 (±165)	915 (±293)	1.04 (±0.13)
Muscle (ng/g)	2029 (±138)	545 (±58)	2574 (±196)	3.73 (±0.16)
K_m	5.69 (±2.09)*	1.69 (±0.57)	3.80 (±1.35)	
Brain (ng/g)	26.2 (±1.9)	26.5 (±5.1)	52.7 (±6.7)	1.02 (±0.16)
K_b	0.063 (±0.025)	0.066 (±0.029)	0.065 (±0.027)	

4.4.2 *In vitro* hind limbs muscle perfusion model

The experiment found no enantioselective distribution of salbutamol in skeletal muscle tissue, with comparable levels between muscle and perfusion medium. The results also show that the presence of OCT blockers did not influence the distribution of salbutamol in skeletal muscle. The results of this experiment are summarised in

Table 4.3 and Figure 4.3. The salbutamol levels in the perfusion medium did not differ considerably (<15%) over the four hour period.

Table 4.3 Salbutamol levels in skeletal muscle and in perfusion medium after 4 h perfusion (n=1), the ratio between (R)-salbutamol and (S)-salbutamol, and the ratio between salbutamol levels found in skeletal muscle and in perfusion medium.

	Total salbutamol (ng/g in muscle) (ng/mL in medium)	R:S	Ratio of muscle : Infusion medium
Control			
Skeletal muscle	911	1.15	1.08
Perfusion medium	840	0.95	
0.3 μM corticosterone			
Skeletal muscle	832	1.08	0.79
Perfusion medium	1055	0.94	
3 μM corticosterone			
Skeletal muscle	820	0.99	0.92
Perfusion medium	888	1.08	
6 μM β-estradiol			
Skeletal muscle	792	1.04	0.88
Perfusion medium	902	1.04	
60 μM β-estradiol			
Skeletal muscle	756	1.01	0.54
Perfusion medium	1401	0.96	
20 μM progesterone			
Skeletal muscle	811	1.03	0.76
Perfusion medium	1073	0.99	
200 μM progesterone			
Skeletal muscle	975	1.00	0.94
Perfusion medium	1038	0.99	

The arterial pressure and oxygen uptake (measured with WINDAQ data acquisition software) of the perfused muscle remained relatively stable over the four hours perfusion period, with no significant difference among the experiment animals.

4.4.3 *In vivo* infusion model

There was substantial enantioselective distribution of salbutamol in heart muscle, in favour of (R)-salbutamol (R:S ratio was 2.88), but similar findings were not observed in the skeletal muscle sample. The results are shown in Figure 4.5. Plasma samples taken from one hour after the infusion showed substantial differences between (R)- and (S)-salbutamol level, and a gradual decrease in plasma level was observed in (R)-salbutamol, but not (S)-salbutamol over the four hour period (R:S ratio were 0.60, 0.60, 0.45 and 0.34 respectively over the 4 h period). However, similar observation were not found in salbutamol levels from skeletal muscle samples, where both (R)- and (S)-salbutamol level remained relatively stable (mean (±SD)) R:S ratio were 1.07 (±0.09). The salbutamol levels from skeletal muscle were similar to the level observed in the *in vitro* hindlimb perfusion model, with no major differences between samples taken from different muscle groups (Figure 4.6).

A significant drop in blood pressure (from 100 ± 10 mmHg to 60 ± 10 mmHg) was observed at the initiation of the salbutamol infusion and remained stable (60 ± 10 mmHg) for the duration of the experiment. An increase in blood sugar level (> 4 mmol/L) was also observed at one hour post infusion, but slowly returned towards the base line physiological level (6.5 mmol/L) over the experiment period.

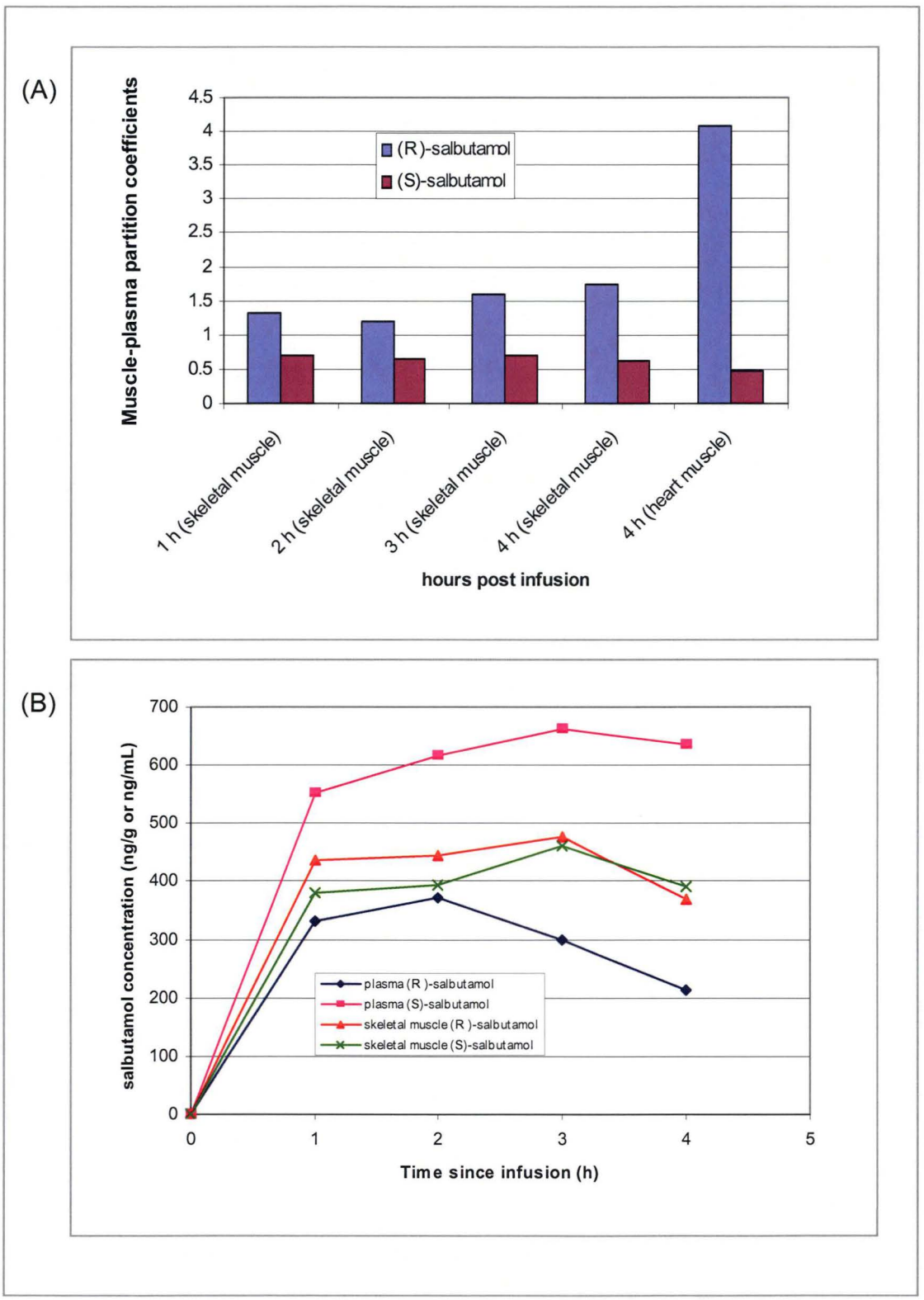


Figure 4.5 The muscle-plasma partition coefficient of skeletal muscle (at one hour interval) and heart muscle (at 4 h post infusion) (A); and the (R)- and (S)-salbutamol level in skeletal muscle and plasma (ng/g or ng/mL) at 1 h interval (B), from the *in vivo* perfusion model

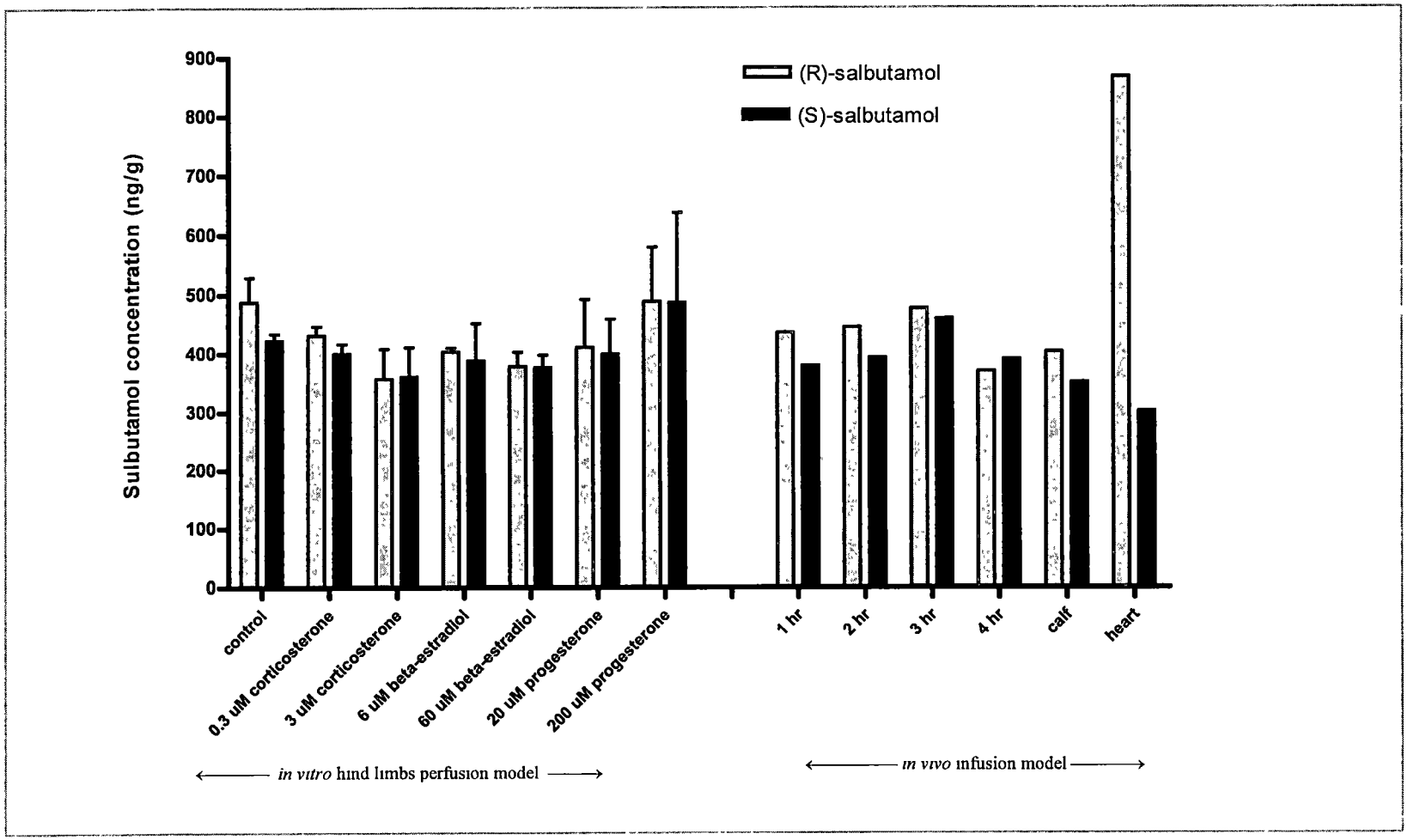


Figure 4.6 (R)- and (S)- salbutamol level from skeletal muscle and heart muscle. Salbutamol enantiomer levels from the *in vitro* hindlimb perfusion model were presented as mean (SEM) levels from right and left leg muscle.

4.5 Discussion

This study is the first report of salbutamol enantiomer uptake into mammalian tissue. The distribution of *rac*-salbutamol between plasma and brain was similar to the work by Malucelli et al in chickens fed salbutamol,[281] with K_m=0.91 at slaughter increasing to 3.7 after a withdrawal period of three days largely due to rapidly falling plasma levels. Unlike other studies, however, a significant uptake of *rac*-salbutamol was observed in skeletal muscle one hour after the last dose in the oral dosing model with K_m=3.80. This is significantly higher than the near equal distribution of K_m=0.91 reported by Malucelli et al[281] but it must be remembered that the results from this current model are from extremely high chronic dosing at 10 mg/kg twice daily for five days. In addition, using the *in vivo* infusion rat model has shown a similar pattern in the cardiac muscle samples, but not skeletal muscle, four hours after the infusion. The uptake is consistent with an active transport mechanism given the concentration gradient observed and ionisation at physiological pH would normally be expected to result in low membrane permeability of salbutamol.

The second major finding of this study was the enantioselective disposition into (rat) muscle, potentially greater in heart muscle than in skeletal muscle, which was not observed in plasma or brain. Previous studies have demonstrated that the plasma pharmacokinetics of salbutamol are enantioselective resulting in greater bioavailability and longer half life of (S)-salbutamol.[231] It is not surprising that tissue distribution of salbutamol also exhibits enantioselective behaviour. It is not uncommon to find enantioselective disposition of various drugs into tissue but enantioselective disposition of salbutamol into muscle has not been reported

previously. The difference between muscle (R)-salbutamol and (S)-salbutamol suggests that the active transport mechanisms involved are enantioselective favouring (R)-salbutamol in muscles. However, given that the *in vivo* infusion model observed enantioselective distribution of salbutamol in cardiac muscle within 4 h of infusion but not in skeletal muscle, the mechanisms involved may be different and/or with different capacity between the two tissues.

Caccia and Fong [307] found blood-brain-barrier penetration but with low partitioning with K_b~0.05 and 100-fold higher levels in the pituitary but this current study found no difference in disposition between whole brain (including pituitary) and partial brain. The pituitary could not be analysed separately given the small size.

Although it is not yet clear if enantioselective uptake of (R)-salbutamol into heart muscle was intensified after chronic dosing or the level observed represents a steady state, cardiac anabolic effects of β-agonist have been reported in both animal model and human subjects.[247,281,314] Given that studies have identified most of salbutamol's adverse effects were delivered by the (R)-salbutamol,[233,246-248] the results of this study have significant relevance with regard to the safety of β-agonist use, where significant effects on heart rate and QT interval were reported.[233,247,250,251] There is particular concern among COPD patients and elderly asthma patients, where underlining cardiovascular co-morbidity are common.[92,251] Despite the finding from Chapter 2 with acute DEM salbutamol use, this observation warrants additional study into long term cardiac effects when a relatively high dose of salbutamol is used chronically.

OCT proteins have been described in intestine, brain, cardiac muscle, liver, kidney, skeletal muscle, lung and brain,[270,315] and stereoselective binding has been suggested with the OCT system.[299,300] Valenzuela et al [316] have demonstrated a simultaneous passive diffusion and active absorption of salbutamol, together with an active capacity-limited efflux mechanism. The saturable efflux mechanism could explain the high K_m at high dose which hasn't been observed in other studies at lower doses. The result here suggest that the OCT proteins are unlikely to be the primary mechanism of immediate salbutamol uptake as observed in the *in vivo* oral dosing model, given that there was very little difference in immediate salbutamol distribution despite some relatively high doses of OCT blockers. However, given that a similar result was observed in the *in vitro* perfusion model to those of the *in vivo* perfusion model, but failed to replicate the stereoselective skeletal muscle uptake of salbutamol observed in the oral dosing model, the involvement of OCT systems remains unclear. The *in vitro* perfusion model was designed to only examine the involvement of OCT uptake system. Therefore, the involvement of other mechanism(s), such as an efflux mechanism or protein binding mechanism, in the stereoselective distribution of salbutamol in tissue can not be excluded. In addition, the bio-physiologial differences between the neonatal mice model and adult rat model, and associated effects on salbutamol enantiomer distribution have not yet been evaluated.

Some recent *in vitro* studies have found that certain genetic variations at human OCT1 were associated with alteration in the function of the transport protein.[270,303,304] Given that the expression of these cellular transport systems varies among tissue and their role in distribution of various xenobiotics, there is a reasonable likelihood of significant individual variation in salbutamol tissue distribution.[270,303,304] Although the

use of experimental animals (both the mice and rat models) have descended from the same genetic background and has largely circumvented this problem, clinical applications and future studies involving the general population will very likely encounter this issue, as it has been observed in other studies presented in this thesis.

Given concerns regarding extrapulmonary cardiac adverse effects of salbutamol, the potential for dose-dependent saturable efflux transport and tissue's retention capacity, plasma level alone may not truly reflect accumulation in certain tissues. More animal pharmacokinetic studies are warranted in this area.

In conclusion, the uptake observed in both skeletal and cardiac muscle is consistent with an enantioselective transport mechanism and therefore chiral assays are warranted in disposition studies of salbutamol. Enantioselective skeletal muscle and heart muscle uptake has relevance for extrapulmonary adverse effects of (R)-salbutamol in airways disease including cardiac effects, its use as a repartitioning agent to increase muscle mass and function in animals and athletes, and mechanistic elucidation of potential therapeutic pathways in muscle wasting diseases such as spinal muscular atrophy.

CHAPTER 5

Collection, Quantification and Quality of Genetic Material

5.1 Abstract

Background: This study was designed to evaluate a non-intrusive and participant friendly method of collecting a reasonable quantity of genetic material of sufficient quality for a typical pharmacogenetics study.

Method: Genetic materia (saliva) was collected form volunteers using a novel lysis gel DNA collecting kit, and DNA was extracted using a modified salt out method. Absorption spectroscopy and fluorescent a binding assay were used for quantity assessment. Gel electrophoresis, PCR and PCR down stream analysis (SNP) were employed for assessment of DNA quality.

Results: The lysis gel DNA collection kit was shown to collect a mean quantity of 185 μg DNA from 5 mL of saliva, which was suitable for typical PCR and down stream analysis.

Discussion: The kit was shown to be a cost effective and participant friendly method of collecting genetic material which was compatible with conventional methods of DNA sampling and suitable for pharmacogenetic studies.

5.2 Introduction

Genetic studies are a fast developing area of medical research. The latest developments in polymerase chain reaction (PCR) and genotyping techniques have dramatically improved the efficacy of genetic analysis. Genetic studies usually require a reliable method of collecting genetic materials such as a blood sample, which is usually painful, invasive and requires training in phlebotomy.[317-319] Many genetic studies, in particularly large scale studies involving subjects from the wider community, such as epidemiological studies, require a relatively inexpensive and participant-friendly method of collecting genetic materials, to achieve optimal subject recruitment.[317-320] Sample size is a particularly important aspect of many pharmacogenetic and pharmacogenomic studies and inconvenient sampling methods have the potential to reduce sample populations and recruitment rates. However, some of the commonly used methods such as hair samples and oral swap samples have experienced limitations including a low quantity yield, reduced stability during storage and are vulnerable to being muddled or lost . [317-319]. Recent studies have identified saliva as a good source of genetic material, due to the cell shredding nature of the mucus membrane and the stickiness of saliva,[317,318] and the method of collecting genetic material remotely was also successfully evaluated by Etter et al.[320]

The aim of this study was to develop a relatively inexpensive, reliable, hazardless, non-invasive and participant-friendly method of collecting genetic material by saliva sample obtained remotely from participants (e.g. through the postal service), without the need for face-to-face participant-researcher contact. In order to demonstrate that the genetic materials collected would meet the requirements of usual genetic

studies,[317,318,321] proof of concept quantity and quality assessments were performed. The performance was also validated against some of the commonly used methods, including finger prick blood sample, buccal swap sample and a commercially available DNA collecting kit. DNA samples extracted from the buffy coat (white blood cells) of venous blood samples, collected using K3EDTA tube and venous puncture from a related study (Chapter 8), were used as reference for optimal performance standards for this new approach. This study also aimed to validate the technique in the various genetic analyses required for the following pharmacogenetic studies. Unless stated otherwise, all experimental process and analyses were performed at the School of Pharmacy laboratory and the CSL laboratory (University of Tasmania).

5.3 Methods

5.3.1 Lysis gel DNA collecting kit

A modified lysis solution, validated by Quinque et al[317], was used as a saliva sample stabiliser to stop the degradation of the genetic material. The modified solution (pH 8.0) consisted of 200 mM Tris, 20 mM EDTA, 200 mM of sucrose, 400 mM of NaCl and 4% SDS and was contained in a water soluble gel (4% tragacanth gel) that served as the carrying medium.

In brief, 4 g of tragacanth power, 12.5 mL of glycerol and approximately 60 mL of water were added to form a mixture. The mixture was heated to the boil with constant stirring and then allowed to cool at room temperature to form the gel base. The lysis solution was prepared by dissolving 1.21 g Tris, 0.74 g EDTA, 3.42 g sucrose and 1.17 g NaCl in approximately 20 mL of warm water, and was then added to the warm gel base. Water was added to adjust to a final weight of 100 g. The combined lysis gel was prepared by stirring the mixture vigorously.

The lysis gel DNA collecting kit was prepared by accurately dispensing an aliquot of 0.25 g lysis gel into a DNA-free plastic tube (15 mL IWAKI™ plastic cryogenic tubes; Asahi Glass Co., Ltd, Osaka, Japan), to which 100 μL of 10% SDS solution was added. The SDS was added separately due to incompatibility between SDS and the tragacanth gel base, which produced foam on mixing. The tube was centrifuged at 3000 rpm (~1200 *g*) for 5 min to ensure the gel was located at the bottom of the tube.

Finally, the tube was positioned upright for 24 h which allowed the SDS solution to imbed on the top of the gel.

Unless otherwise specified, all chemicals used in this chapter were laboratory grade chemical.

5.3.2 Samples collection

5.3.2.1 Subjects

Five volunteers (two male and three females) provided samples for the evaluation of the lysis gel DNA collecting tube. Each subject was instructed to rinse their mouth with water before depositing approximately 1.5 mL of saliva into the tube using a visual level calibration mark, and then told to cap and gently shake the tube to dissolve the gel.

Genetic material was also collected from volunteers using the following methods for comparison:

1. Buccal cell samples collected by brushing the inside of the cheeks for 30 sec with a sterile cotton swab;
2. Blood samples collected by finger-prick blood and transferred into an Eppendorf™ tube; and
3. Saliva samples collected using Oragene™ DNA self-collection Kit (DNA Genotek Inc, Ontario, Canada), according to the manufacture's instructions.

5.3.2.2 DNA extraction

DNA was extracted from the stabilised saliva samples (collected in the lysis gel DNA collecting tube), using a modified salt out method developed by Miller et al. [322] and Nasiri et al. [323]. In brief, 1 mL of the stabilised saliva sample was transferred into a clean Eppendorf™ tube, then 300 µL of 1 mg/mL proteinase K solution (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW Australia) and 75 µL of 10% SDS solution was added and the resulting mixture was vortex mixed. The mixture was then incubated at 53°C for a minimum of 8 h (or overnight). A volume of 250 µL saturated NaCl solution (~5.5 M) was added and the sample was then incubated in ice for 10 min, before being centrifuged at 12000 *g* for 10 min. The supernatant was extracted into a new Eppendorf™ tube, 1 mL of ethanol was added and the sample was gently mixed and incubated for a further 3 min. The mixture was centrifuged at 12000 *g* for 1 min, and the supernatant was carefully removed and discarded, without disturbing the pellet (extracted DNA). Distilled water (750 µL) was added to the pellet and was incubated at room temperature for at least 2 hto re-hydrate the extracted DNA. The extracted DNA sample was then analysed immediately or stored at -20°C until analysis. DNA samples intended for long term storage (> 2 years) were re-hydrated in Tris-Cl buffer (10 mM, pH 9.0), instead of distilled water, to avoid acid hydrolysis.

Genetic material from the comparative samples using buccal swap samples, blood samples and buffy coat samples was extracted using a QIAamp DNA blood mini kit (QIAGEN Australia Pty Ltd., Doncaster, VIC), following the manufacturer's instructions. In brief, each sample was mixed with proteinase and buffer AL before being incubated at 56°C for 10 min. An equal volume of ethanol was then added, and the sample was transferred into the extraction cartridge. The sample was eluted (with

centrifugal force or with vacuum pressure) and the cartridge was washed with buffer AW1 and AW2. Distilled water was added to the cartridge which was then incubated for 15 min (to de-hydrate the extracted DNA), and the DNA sample was eluted into a clean Eppendorf™ tube.

Genetic material (saliva sample) collected in the Oragene™ DNA self-collection kits was extracted according to the manufacturer's instructions. In brief, the sample was first incubated at 50°C for 1 h, and an aliquot was transferred into a clean Eppendorf™ tube. Oragene™ purifier solution was added, and the sample was incubated in ice for 10 min before being centrifuged at 12000 *g* for 3 min. The supernatant was extracted and an equal volume of ethanol was added, before being centrifuged at 12000 *g* for 1 min. The supernatant was discarded and distilled water was added to re-hydrate the pallet (extracted DNA).

5.3.3 Quality Evaluation

5.3.3.1 Stability assessment

The stability and bacterial contamination of saliva samples collected with the lysis gel DNA collecting tube was examined using a bacteria culture method with multipurpose agar plates. The stabilised saliva samples were kept at room temperature for up to 5 days. An agar plate was prepared each day on day 0, 2 and 5, by swabbing the surface with the stabilised saliva sample using a sterile cotton tip swab. The agar plates were incubated at 37°C for 24 h, and evaluated by visual examination. The stability of the assay was evaluated for 5 days, based on the knowledge that sample would be delivered within this period of time, if posted within Australia.

5.3.3.2 Quantity assessment

The quantity of DNA extracted from a unit sample, collected with the lysis gel DNA collecting tube, was evaluated with two commonly employed quantification methods as outlined by Killeen [324] and Gallagher [325] and a comparison was made with other methods of DNA collection as described in Section 5.3.2.1.

5.3.3.2.1 Absorption spectroscopy quantification

The concentrations of extracted DNA samples were examined with absorption spectroscopy analysis using a Shimadzu UV-Mini 1240 spectrophotometer (Shimadzu Australia Manufacturing Pty Ltd, Rydalmere NSW, Australia). DNA concentration was determined by the 260 nm UV light absorbance (A_{260}) of nitrogenous bases of nucleic acid. The spectrophotometer was first calibrated to a zero reading with distilled water (or the selected buffer which the DNA sample was stored, such as Tris-HCl buffer) and the DNA sample was loaded directly into cuvette and the measurement taken. Appropriate dilution was made to achieve accurate measurement (signal reading between 0.5 and 2 units), or to adjust the volume required for measurement. The cuvette was cleaned with distilled water (or the selected buffer used for calibration) between measurements.

DNA concentration (expressed as pure double-stranded DNA) was calculated from the absorption spectroscopy measurement; in which one unit of A260 (adjusted from background 320 nm absorbance (A_{320})) corresponded to 50 μg/mL of DNA. However, a 1 mm cuvette, instead of a 1 cm cuvette, was used in this assessment due to the

relative small volume of DNA solution available and the DNA concentration was calculated with the revised equation:

[DNA] (μg/mL) = 500 x dilution factor x Δ (measured A_{260} - measured A_{320})

The quality of the extracted DNA sample was estimated with a protein contamination factor in the samples, Δ (A_{260} - A_{320}) / Δ (A_{280} – A_{320}) ratio. A a ratio between 1.8 and 2.0 indicated a quality de-proteinised sample.

5.3.3.2.2 Fluorescent binding assay quantification

The concentration of the DNA sample was also examined with a fluorescent binding assay using the Hoefer TKO 100 mini-fluorometer method (fluoro assay solution A, Hoefer Inc.,Holliston, MA USA), and using a BIARAD Versa Fluor ™ Fluorometer. The fluorescent assay solution was freshly prepared before each analysis, and consisted of 0.01% (v/v) fluorescent dye (bis-benzimidazole (Hoechst H33258)) in 1x TNE buffer solution (0.2 M NaCl, 10 mM Tris-Cl, 1 mM EDTA at pH 7.4). The fluorometer was first calibrated using a blank assay and a reference 100 μg/mL calf thymus DNA standard (Hoefer Inc.,Holliston, MA USA) for 0 and 100 signals respectively (LLoQ: 4) so the signal reading directly corresponded to the measured DNA concentration. In brief, 2 μL of each DNA sample (or reference DNA solution) was added to 2 mL of the fluorescent assay solution and mixed gently in the cuvette using a pipette, before the measurement was taken. The cuvette was cleaned with MiliQ water (or distilled water) between measurements.

5.3.3.3 Quality assessment, PCR and down stream analysis

The quality of DNA samples was examined using DNA fragment assessment, and its application in polymerase chain reaction (PCR) and down stream analysis (SNP analysis). Previously validated PCR and SNP analyses (Section 7.3.2.2 and Section 8.3.4.2) were used for the quality assessment.

5.3.3.3.1 Fragment size assessment

The estimated sizes of the DNA fragments in the sample were evaluated by gel electrophoresis, using Horizon® 58 Horizontal Gel Electrophoresis Apparatus (Biometra biomedizinische Analytik GmbH, Goettingen, Germany). In brief, 50-100 ng of the DNA sample (5 μL of 10-20 ng/μL DNA samples), were mixed with 2 μL of the sample loading buffer (40% sucrose, 0.25% bromophenol blue and 0.25% xylene cyanol). This was loaded onto a 1% agarose gel containing ethidium bromide as the fluorecent dye. The gel was submerged in 1x Tris-borate-EDTA (TBE) buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) and electrophoresis was performed at 4 V/cm for 1 h. The fragment size references used were Lambda DNA Hind III Digest (New England Biolabs Inc, Ipswich, MA, USA) and DMW-100L ladder (GeneWorks Pty Ltd, Hindmarsh SA, Australia), with a maximum fragment size of 23,130 nucleotide (nt) and 1000 nt respectively. The gel was examined by visual inspection under UV light after the electrophoresis.

5.3.3.3.2 PCR analysis

The application of DNA samples in PCR was analysed with a hybrid PCR analysis (primer pair: ccccttacacaggatgtccatatta (foward) and gcgtgtcctaatctcgtgagcatt

(reverse)), located on the C-FOS gene and a product size of 162 nt. In brief, each 25 μL PCR reaction contained 2.5 μL of 10x buffer (670mM Tris pH 8.8, 166 mM ammonium sulfate, 4.5% Triton X-100 and 2mg/mL gelatin), 1.5 mM MgCl2, 500 μM dNTPs, 400 nM of each primer, 1.0 unit of Taq Polymerase and 30-100 ng of DNA template. The PCR reactions were run on a MJ Research PTC-200 Thermal Cycler (MJ Research, Inc., MA, USA) using the following conditions: 3 min at 95°C followed by 35 cycles of 95°C for 30 sec, 59°C for 35 sec and 72°C for 50 sec. After 35 cycles there was a 5 min extension step at 72°C followed by an 11°C hold. The PCR product was evaluated with gel electrophoresis as described in section 5.3.3.3.1

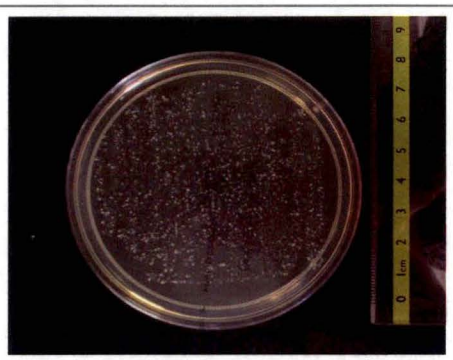
5.3.3.3.3 PCR down stream analysis

The application of PCR product to down stream analysis was evaluated by SNP analyses. Six previously validated and performed SNP analyses, performed by the Australian Genome Research Facility (AGRF; Queensland, Australia; part of the Australian Commonwealth Government's Major National Research Facility Program) were used for this assessment. The AGRF used a Sequenom SNP genotype system for the analysis. SNP assays were designed using the Sequenom MassArray Assay Design© Version 2.0.0.1.6 software. The program requires 50 bases either side of the SNP site to design the forward, reverse and extension primers with sequences from the particular gene being analysed. The PCR and extension reactions were be run on Applied Biosystems (Foster City, Ca, USA) GeneAmp PCR system 9700© 384 well thermocyclers. The reaction was then spotted onto a chip using a Samsung 24 pin main head Nanodispenser©. Chips were fired using a Bruker Daltronics Autoflex©

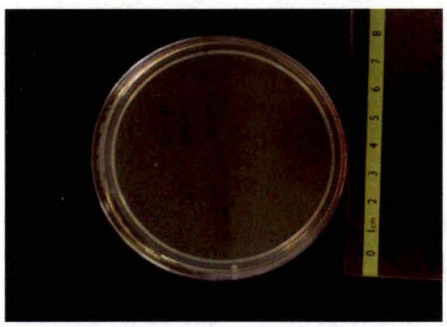
mass spectrometer (Billerica, MA, USA), using a matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry system

5.3 Results

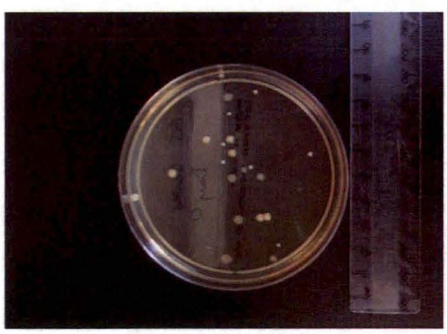
The stability of saliva samples collected using the lysis gel DNA collecting tubes showed minimal bacteria growth after five days when stored at ambient temperature (Figure 5.1).



A. Untreated saliva sample



B. Stabilised saliva sample at day 0



C. Stabilised saliva sample at day 5

Figure 5.1 Microbiological evaluation of saliva sample collected with lysis gel DNA collecting tube.

The quantity of DNA extracted from the stabilised saliva sample was 32–67 μg/mL and 8-75 μg/mL, measured with absorption spectroscopy and fluorescent binding assay respectively. The DNA yield was found to be comparable with samples collected using other various methods. The results of the DNA quantity evaluation is summarised in Table 5.1.

Table 5.1 Quantity of DNA extracted from various matrices and methods

	Amount of DNA yield Mean (±SD) μg/mL	
	Photospectrometer	**Electro-fluoro assay**
Buccal cells sample	1 (±0.2)* †	-
Blood sample	9 (±5)[◆] †	-
Saliva sample (1 mL), Oragene™ DNA self-collection Kit	12 (±4)	12 (±7)
Saliva sample (1 mL), lysis gel DNA collecting tube	37 (±16)	28 (±27)
Buffy coat from 1 mL of blood sample	8 (±4)	14 (±8)

* Measured as μg of DNA yield per swab;
† Lower concentration below detection limit
◆ Mean (±SD) quantity of blood sample collected per finger-pricking was 140 (±45) mg

The quality of DNA samples extracted both immediately and after 5 days stored at ambient temperature was found to be compatible with DNA samples extracted from the buffy coat, without significant degradation and fragmentation (Figure 5.2). The PCR analysis and all six down stream SNP analyses were successfully performed on all samples, and the results were compatible irrespective of the method of DNA collection and extraction (Figure 5.3). The storage period and condition of the stabilised samples, collected using the lysis gel DNA collecting tube, did not show any significant effect on the SNPs analysis or the amount of DNA yield.

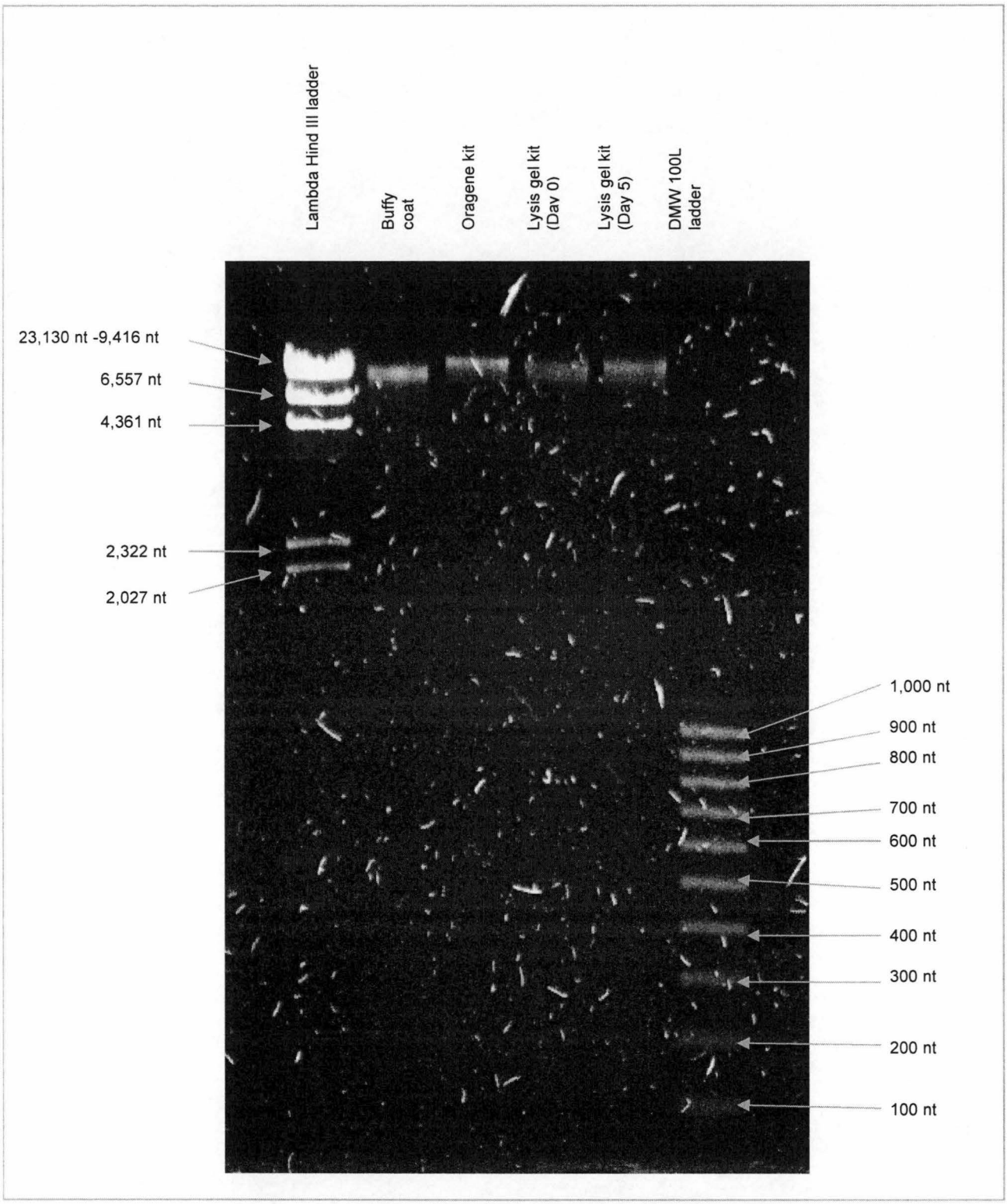


Figure 5.2 1% Agarose gel electrophoresis of raw DNA samples

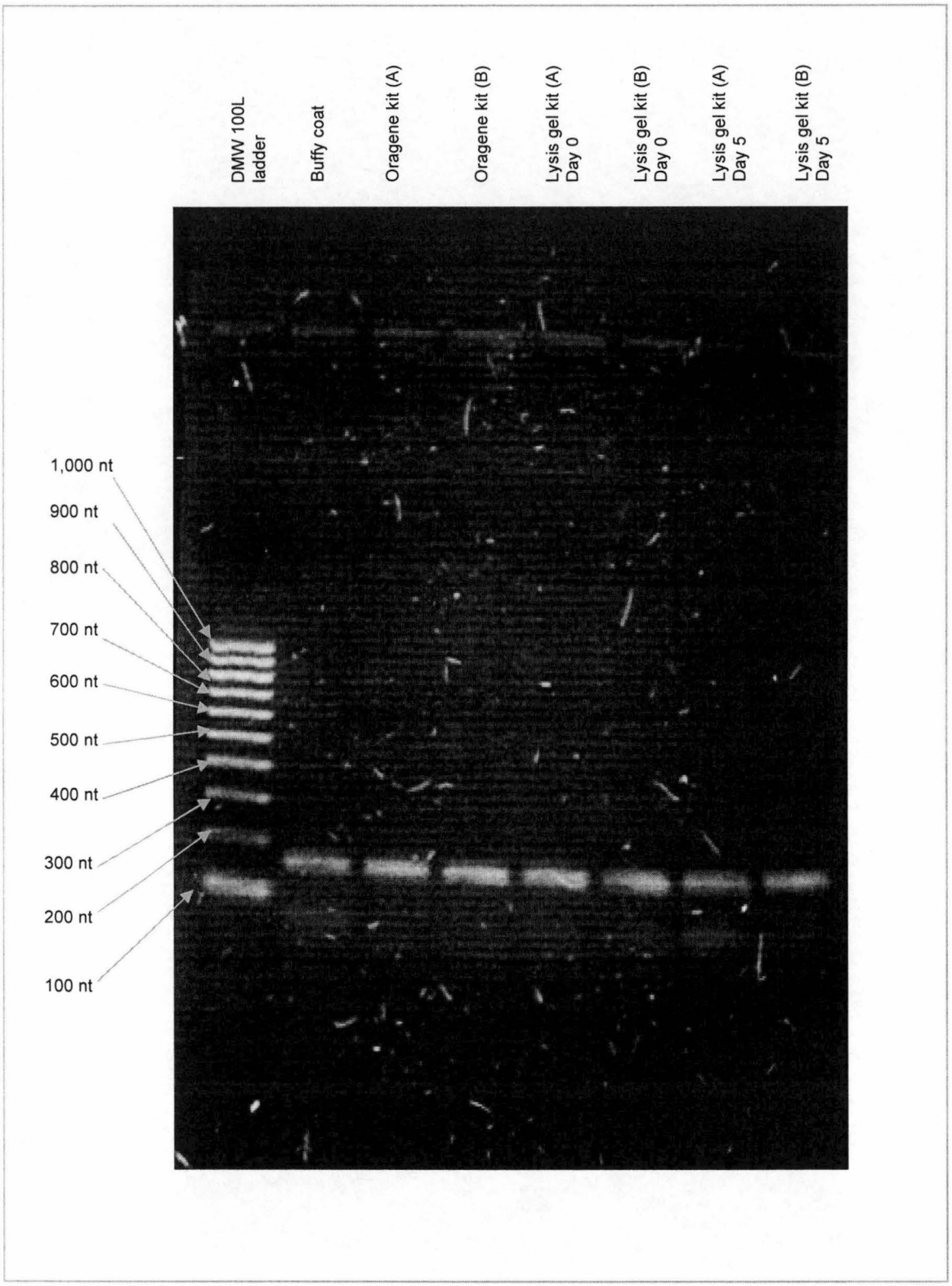


Figure 5.3 1% Agarose gel electrophoresis of PCR product (PCR product size: 162 bp)

5.5 Discussion

The lysis gel DNA collecting tube was shown to be capable of collecting a DNA sample of sufficient quantity and quality required for most pharmacogenetic studies. However, unlike DNA samples obtained from sterile biological samples such as a venous blood, a significant amount of bacterial DNA contamination was expected from genetic material collected from the oral cavity and a degree of contamination which was largely dependent on the individual subject.[317,319,326] In this study, although the stability assessment indicated that most of the micro-organisms were suppressed and/or killed by the lysis solution, contamination of bacteria DNA in saliva samples cannot be avoided, and therefore may not meet the quality requirement of some studies.[317,319]

Unlike the study conducted by Ng et al. [327], which investigated untreated saliva samples, the quality and quantity of DNA samples extracted from the lysis gel DNA collecting tube was not found to be affected by the storage conditions.

In conclusion, the water soluble gel was shown to provide an ideal medium for carrying the lysis compounds, which dissolved and formed a lysis solution when it came in contact with the aqueous sample. The 4% tragacanth gel provided suitable rheological properties (plastic barrier) as well as adhesion to the container wall, which ensured the gel remained in the designated position despite substantial physical agitation. This device was a simple and inexpensive container to release a lysis solution with relative safety and reliability. This lysis gel DNA collecting tube developed in our laboratory is an effective, participant-friendly and relatively

inexpensive method of collecting DNA samples suitable for large population based pharmacogenetic studies.

Chapter 6

Pharmacogenetic determinant of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients, SULT 1A3 SNP (rs1201735)

6.1 Abstract

Background: Salbutamol is a bronchodilator widely used in asthma and COPD that is metabolised by human catecholamine sulfotransferase (SULT 1A3). Studies have indicated that genetic variations, including single nucleotide polymorphisms (SNPs), may influence SULT 1A3 activity. The study was designed to investigate the effect of SULT1A3 SNPs on the pharmacokinetics of salbutamol enantiomers.

Method: SULT1A3 SNPs were determined from a sample of patients with asthma. Patients were recruited through the Royal Hobart Hospital Respiratory Clinic and the wider community through media advertising. DNA was extracted from saliva from each subject and analysed for common SULT1A3 SNPs. Patients identified with homozygote SULT1A3 SNPs (rs1201735) were invited to participate in a study to determine basic pharmacokinetic parameters of both (R)- and (S)-salbutamol enantiomers, along with matched homozygote wild-type controls. Subjects were given a 400 μg dose of inhaled salbutamol via a large volume spacer and blood samples were collected at pre-dose, 15, 30, 60, 120 and 240 min after the dose. Salbutamol was extracted using a solid-phase extraction method from plasma. Plasma levels of (R)- and (S)-salbutamol were determined by an LC-MS/MS assay with a lower limit of quantification of 20 pg/mL.

Results: SNP genotyping was performed on 25 subjects, which identified 10 subjects with wild-type homozygote (AA) and 9 subjects with mutant homozygote (GG) at SULT1A3 SNP (rs1201735). Thirteen asthmatic subjects participated in the pharmacokinetic investigation, which included four mutant SNP homozygotes and nine wild-type SNP homozygotes. Observed C_{max} of (R)- and (S)-salbutamol [mean (±SD)] from the thirteen subjects was 0.64 (±0.30) ng/mL and 1.32 ±(0.98) ng/mL,

respectively. The mean $t_{1/2}$ of (R)- and (S)-salbutamol from the thirteen subjects was 2.94 (±1.17) h and 7.86 (±6.14) h, respectively. There were no significant differences in pharmacokinetic parameters ($t_{1/2}$, C_{max}, R:S ratio) between mutant SNP genotype and wild-type genotypes for either enantiomer.

Discussion: The pharmacokinetics of salbutamol enantiomers was found to be highly variable, but even with the small numbers involved it seems unlikely that the major SULT1A3 SNP tested has a significant effect on these levels.

6.2 Introduction

Studies have demonstrated that the pharmacokinetics of salbutamol are stereoselective.[230] When salbutamol is given in the form of an inhalation, only a small fraction (~10%) of the dose is delivered to the airway, but this produces the majority of its therapeutic effects. Most of the remaining dose is swallowed,[231] and up to three quarters of this is metabolised by extensive first pass hepatic metabolism, the majority of which is carried out by the enzyme human catecholamine sulfotransferase (SULT 1A3).[231,328]. Salbutamol is eliminated from the circulation as both unchanged drug and metabolite.[231] The absorption, metabolism and renal tubular excretion of salbutamol, are complex and shown to exhibit stereoselective behavior.[231,232]

The human cytosolic sulfotransferase (SULT) is a family of cytochrome P450 (CYP) like enzymes consisting of 11 known members.[329-331] The enzyme SULT 1A3 was found responsible for the metabolism (sulphate conjugation) of endogenous amines and structurally related drugs.[329] The common function of SULT sulfonation of endogenous and exogenous chemicals, involves transferring a sulfonate group (SO^{3-}) from a universal donor (3'-phosphoadenosine 5'-phosphosulfate).[329,331] The process also involves a number of other enzymes and cellular proteins, in particular sulfurylase, APS kinase, organic transport molecules and multi-drug resistant protein.[329] Unlike some members of the SULT family, the SULT 1A3-substrate relationship was found to be highly selective and largely determined by hydrogen-bonding group and lipophilicity of the substrate,[331,332] Like the CYP system, the enzyme's activity were also found to be affected by a number of factors, including dietary chemicals and genetic variations,[329,331] and difference in enzyme activity has

been observed between different individuals.[255,330] It has been suggested some common features among the SULT members, such as the substrate binding site, are critical for the enzymes's activity,[330-333] and genetic variations as well as alteration of the amino acid sequence or structure were found to effect the enzyme activities.[331,334,335]

Recent studies have found a number of functional SNPs in SULT1A3, including four non-synonymous SNPs and up to five SNPs considered common (≥ 1%) in Caucasian populations.[254,255,335] Hildebrandt and colleagues also discovered a gene duplication of the SULT1A3 (named SULT1A4) nearby, with a 99.8% identical sequence.[254] The discovery suggested that the SULT 1A3/1A4 enzymes could be encoded from 4 instead of 2 genetic alleles, however the difference in biological activities (if any) and regulation of the two enzymes are yet to be determined.[254]

There are data to suggest that small genetic variations, such as SNPs, have the potential to influence the activity of an enzyme, such as SULT 1A3, [334,336,337] including the alteration of amino acid sequences and protein structure, gene splitting, affecting the gene/protein expression and isoform formation.[163] With SULT1A3, an *in vitro* study of the non-synonymous SNP (codon 105) has observed up to a 72% reduction of the enzymes activity among the genotype Asn/Asn compared to the genotype Lys/Lys, and a greater enzyme degradation rate (up to 71%) among the Asn/Asn genotype.[255] Recent studies have also found that some of the other SNPs were related to the reduction of the enzyme's activity.[335] However, the *in vivo* effects of the SNP(s), including the impact on salbutamol metabolism remains unknown.

This study was designed to investigate the impact of SULT1A3 SNPs on the pharmacokinetics of salbutamol enantiomers in people with chronic asthma after a single dose of inhaled *rac*-salbutamol.

6.3 Methods

The study design was a two-group comparison and was approved by the State Human Research and Ethics Committee. Three SULT1A3 SNPs were selected for the study.

The main outcome measure were maximum plasma concentration (C_{max}), half life ($t_{1/2}$), and area under curve (AUC) over the 4 hperiod for both enantiomers. Secondary outcome measures were the pharmacodynamic parameters of FEV1 and REF.

Unless otherwise specified, all chemicals used in this chapter were laboratory grade chemical.

6.3.1 Subject recruitment and sample collection

Subjects were chronic asthma patients recruited through the Respiratory Unit (Royal Hobart Hospital), advertisements in print and radio media and newsletters of the State Asthma Foundation from Greater Hobart, Tasmania, Australia.. After providing written informed consent, a DNA sample was collected for genetic analysis from all participants. Saliva samples were collected remotely using an Oragene^{TM} DNA self-collection Kit (DNA Genotek Inc, Ontario, Canada), mailed to patients with written instructions and a return postage package. DNA was extracted in the laboratory following the manufacturer's instructions upon return of the collection kit in the mail. The gel collection approach described in Chapter 5 was not used as it was not validated in time for the commencement of the study.

Demographic and asthma information was collected prior to the pharmacokinetic investigation. Disease control was assessed by spirometry (Vitalograph® COMPACT spirometer, Vitalograph Ltd., Ennis, Co Clare, Ireland) and an asthma score (Asthma Control Test™, QualityMetrix Inc, GSK Pty Ltd. VIC, Australia)

6.3.2 Genetic analysis

The selection criteria for the SNPs to be examined in this study were SULT1A3 SNPs identified in Caucasian population with mutant SNP frequency of ≥5% and non-synonymous SNP as identified by Thomae at al. [255]. The SNPs selected for this study were SULT1A3 SNPs located at codon 105, codon 702 and intron 7 (113)

SNP analyses were performed by the AGRF as described in Section 5.3.3.3.3. AGRF was responsible for the primer design and SNP analyses using a Sequenom SNP genotype system, using a Bruker Daltronics Autoflex© mass spectrometer (Billerica, MA, USA). SNP assays were designed using the Sequenom MassArray Assay Design© Version 2.0.0.1.6 software.

6.3.3 Drug administration and sample collection

Case (mutant SNP, GG genotype; SULT1A3 SNP codon 105, rs1201735) and controls (wild-type (W/T), AA genotype) subjects were invited to the pharmacokinetic phase of the study. Subjects were instructed to withhold short acting β_2-agonist for at least four hours before and during the study period. Subjects were

excluded from the study if it was medically determined that short-acting β_2-agonists were likely to be required during this sampling period.

Each subject was administered 400 μg *rac*-salbutamol (4 x 100 μg, puffs, each separated by 30 seconds), delivered by a CFC-free pressurised metered dose inhaler (Ventolin®, GSK Australia Pty Ltd. VIC, Australia) with a large volume spacer device. Optimal technique was ensured by direct observation and subjects were instructed to rinse the mouth with water after completion. An intravenous cannula was inserted into the arm of each subject for blood sample collection. Blood samples (2 ml) were collected in Vacuette® EDTA K3 tubes (Greiner Bio-One, Kremsmuenster, Austria), and spirometry performed pre-dosing and at 15, 30, 60, 120 and 240 min after salbutamol. Plasma was separated immediately from each blood sample by centrifugation and stored at -20°C until analysis.

Pharmacodynamic response was measured using FEV1 and PEF spirometry at each blood sampling time.

6.3.4 Analysis of plasma salbutamol enantiomer concentrations

Salbutamol analyses were performed by the previously reported method, in Chapter 3,[262] using a state-of-the-art LTQ Orbitrap™ hybrid mass spectrometer, with deuterated salbutamol (D3-salbutamol) as the internal standard. In brief, a working solution (100 ng/mL) containing 1 ng of salbutamol-D_3 (3-hydroxymethyl-D_2, α-D_1; Medical Isotopes, Inc. Pelham, NH, USA) was first added to each plasma sample

(250 μL), vortex mixed and then let stand at room temperature for 30 min. Salbutamol was extracted from the plasma sample using a solid-phase extraction method with Oasis™ HLB extraction cartridges (Waters Corporation, Milford, Massachusetts, UAS). The salbutamol was then analysed by a chiral LC-MS/MS assay, using an advanced LTQ Orbitrap™ Hybrid Mass Spectrometer with a detection limit of 0.008 ng/mL and a lower limit of quantification of 20 pg/mL for each salbutamol enantiomer. The percentage salbutamol enantiomer recovery for the sample batch was 63 (±2.8) (mean (±SD)) with a reproducibility RSD of 4.2% at the level of 0.50 ng/mL, and a linear calibration curve over the range 0.20 – 5.00 ng/mL (R^2=0.999).

6.3.5 Statistical analysis

Based on the finding by Schmekel et al (n=6) [230] after 400 μg of inhaled salbutamol, the observed AUC, Cmax and tmax mean (95% confidence interval) were 7.0 (5.5-8.5) ng/mL/h, 2.0 (1.5-2.5) ng/mL and 38 (25-50) min respectively. Given a sample size of 20 cases and 20 controls, it was predicted the study could observe differences in the means AUC, Cmax and tmax of 1.63 ng/mL/h, 0.54 ng/ml and 13.6 min for respectively (2-sided α = 0.05, β=0.20). Based on the allele frequency of the SULT1A3 nucleotide 105 SNP of 10.8%,[255] 20 cases would be obtained by screening about 200 patients. The enzyme activity with a common African-American SULT1A3 SNP was shown to decrease enzyme activity by over 70 percent.[255] If an effect of this magnitude is observed, it would be expected to have marked effects on pharmacokinetic parameters in excess of the minimum detectable differences.

Although the sample size was relatively small, comparisons between case and control were made using a two-tailed unpaired t-test. These parameters have been shown to be consistent with a parametric model [230,238]. Salbutamol levels less than the detection limit were treated as 0.02 ng/mL for statistical purposes. Difference in pharmacodynamics parameters (FEV1 and PEF) between groups were also assessed using a two-tailed unpaired t-test. AUC was determined using GraphPad PRISM version 4 software (GraphPad Software, Inc. CA USA). All analyses were performed using SPSS 14.0 for Windows (SPSS Australasia Pty. Ltd. Chatswood, NSW, Australia). Results with $p<0.05$ were considered statistically significant.

6.4 Results

Thirty subjects agreed to participate, but only 25 provided DNA samples. Five subjects withdrawn at this phase of the study due to personal reasons, timing/time required to take part in the pharmacokinetic phase of the study or unable to be contacted.

All SNP analyses were successfully performed in >90% of the samples. The allele frequencies and heterozygote frequency of SNPs at various locations are summarised in Table 6.1.

Table 6.1 Allele frequency of SNPs at SULT1A3 genes

	Allele Frequency				Homozygote Frequency	
	W/T		SNP		W/T	SNP
SULT1A3 Codon 105 (*rs1201735*) (N=23) [a]	A	0.52	G	0.48	0.43	0.39
SULT1A3 Codon 702 (N=24) [b]	G	1.00	T	0.00	1.00	0.00
SULT1A3 Intron 7 (113) (N=23) [a]	G	0.98	T	0.02	0.96	0.00

[a] Number of SNP analysis failed n=2

[b] Number of SNP analysis failed n=1

Nineteen subjects were found to carry homozygote genotypes [9 mutant SNPs (GG) and 10 W/T (AA) at SULT1A3 codon 105 (*rs1201735*)], and were invited to participate in the next pharmacokinetic phase. Unfortunately, six subjects subsequently withdrew before the laboratory visit (due to personal reasons, relocation, unrelated health issue

or was unable to be contacted). Thus, thirteen subjects only completed the pharmacokinetic phase, with four of the subjects carrying the mutant SNP genotype (GG) and nine subjects carrying the W/T genotype (AA). Basic demographic information on the subjects is summarised in Table 6.2. The groups were generally similar, but there were no females or smokers in the case group.

Table 6.2 Demographic information of case and control subjects who completed the pharmacokinetic phase of the study

	Case	Control
	Median (range)	
Age (years)	40 (19-47)	57 (35-84)
Gender	0 F; 4 M	6 F; 3 M
Smoking history	0 smoker; 0 ex-smoker	1 smoker; 4 ex-smoker
Height (cm)	177 (170-186)	167 (151-179)
Weight (kg)	83 (73-103)	88 (54-109)
Asthma control assessment *	1 Good; 3 Fair	7 Fair; 1 Poor; 1 Missing

*Asthma symptom control as defined in Asthma Management Handbook 2006 (NAC) [7]

SNPs at other locations were found to have a low SNP allele frequency and/or a low SNP homozygote frequency in our sample population (Table 6.1). Based on sample size power calculations demonstrating the need for very large sample size, pharmacokinetic analyses were not performed on these SNPs.

Salbutamol enantiomer analyses were performed successfully from all samples. A large variation in salbutamol concentration was observed between subjects, with higher concentrations of (S)-salbutamol than (R)-salbutamol in all subjects. The observed C_{max}, calculated half-life and AUC of salbutamol enantiomers were all greater for (S)-salbutamol than (R)-salbutamol (Table 6.3). There were no significant

differences in pharmacokinetic parameters (C_{max}, $t_{1/2}$ and AUC) between case and control for either enantiomer (Table 6.4).

There were no significant differences in pharmacodynamic response, between case and control, after the inhaled salbutamol dose (Table 6.5).

Table 6.3 Pharmacokinetic properties of salbutamol enantiomers in all subjects

	(R)-salbutamol Mean (±SD)	(S)-salbutamol Mean (±SD)
Baseline level (ng/mL)	0.13 (±0.14)	0.41 (±0.91)
Observed plasma Cmax (ng/mL) *	0.64 (±0.30)	1.32 (±0.98)
Calculated half-life (h) *	2.94 (±1.17)	7.86 (±6.14)
AUC (240 min) †	56 (±27)	153 (±78)

* $p<0.05$
† $p<0.01$

Table 6.4 Pharmacokinetic properties of salbutamol enantiomers in case and control subjects.

	Control (N = 9) Mean (±SD)	Case (N = 4) Mean (±SD)	Independent *t* test Mean difference [95% C])
Plasma C_{max} (ng/mL)			
(R)-salbutamol	0.68 (±0.26)	0.57 (±0.26)	0.11 [-0.29 - 0.50]
(S)-salbutamol	1.46 (±0.50)	1.00 (±0.50)	0.46 [-0.54 - 1.46]
Calculated half life (h)			
(R)-salbutamol	3.07 (±1.38)	2.64 (±0.45)	0.44 [-0.70 – 1.58]
(S)-salbutamol	7.23 (±5.24)	9.28 (±7.69)	-2.05 [-13.41 – 9.31]
AUC (0-240 min)			
(R)-salbutamol	56 (±30)	57 (±24)	-1.06 [-38.04 – 35.93]
(S)-salbutamol	151 (±90)	157 (±55)	-5.75 [-97.23 – 87.72]

Table 6.5 Lung function test by spirometry (mean (±SD)) before and after inhaled salbutamol dose

Lung function	Case (n=4)	Control (n=9)
Baseline		
% Predicted PEF	119 (±18)	102 (±31)
% Predicted FEV_1	102 (±5)	80 (±31)
15 min post-salbutamol		
% Predicted PEF	126 (±13)	104 (±33)
% Predicted FEV_1	105 (±8)	84 (±31)
240 min post-salbutamol		
% Predicted PEF	128 (±13)	102 (±35)
% Predicted FEV_1	104 (±7)	80 (±33)

6.5 Discussion

The results show that individuals with asthma exhibit major differences in salbutamol pharmacokinetics when the drug is delivered via inhalation using a spacer device (Refer to Appendix X). Although showing a similar pattern in the concentration-time curve, the study observed a significant difference between the two enantiomers, with much higher peak concentrations, total dose (determined by AUC) and longer half-life of (S)-salbutamol compared to (R)-salbutamol. This observation of stereoselectivity is in accordance with previously published data although at a lower dose.[230,238] The observation of high (S)-salbutamol levels also suggest that despite the use of a spacer device and mou`th rinsing method after inhalation, a substantial amount of the delivered dose is being swallowed and absorbed, as demonstrated by Ward et al [232].

Although the number of participants was small, the pharmacogenetic investigation showed that SULT1A3 SNPs (*rs1201735*) was very unlikely to have influenced the pharmacokinetics of salbutamol enantiomers among the study subjects. The observations cannot conclusively dismiss the hereditary influence of the SULT1A3 SNP on the pharmacokinetic parameters of salbutamol enantiomers but the effect does not appear to be significant from a clinical perspective, at least at low-moderate doses. In addition, it is difficult to categorically determine the influence of the SNPs, where the observed outcomes are likely to be affected by a combination of various genetic variations (e.g. haplotype). However, subjects with GG genotypes seemed to express less variation in the concentration time curve of salbutamol, than the W/T genotype (Figure 6.1). The effect of heterozygote genotype was not examined in the study as it is likely to be intermediate and much less predictable, as experienced by other

studies.[179] In addition, we would suspect less consistency in influence among heterozygote genotypes given that duplicate genes were identified for SULT1A3.[254]

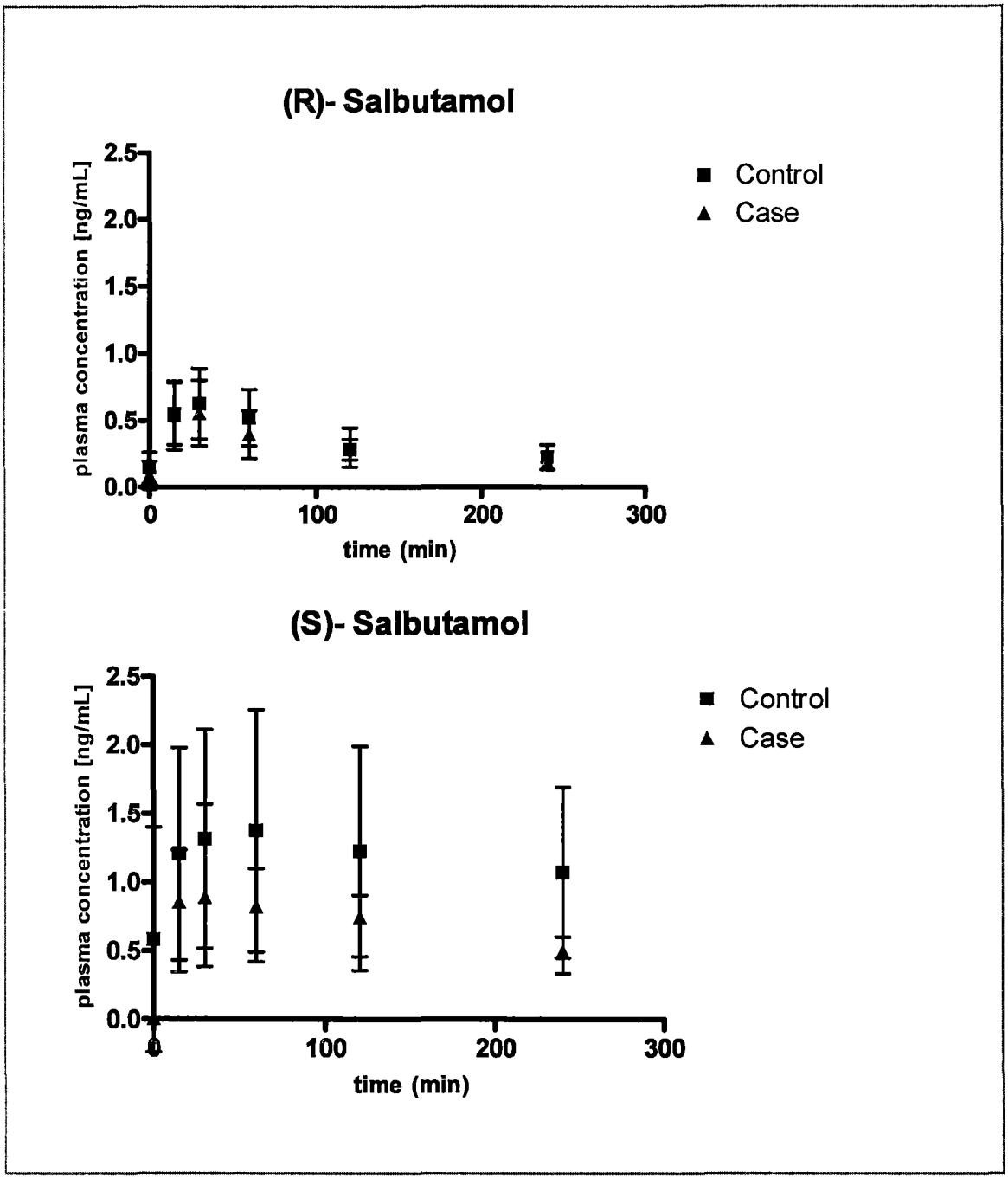


Figure 6.1 Concentration time curve of plasma (R)- and (S)-salbutamol (mean ± 95%CI) between case and control subjects

The study also found no significant pharmacodynamic response (determined by spirometry) after the single inhaled dose, and no relationship with circulating

salbutamol enantiomer levels. Due to the relatively well controlled state of the disease (determined by Asthma control assessment), this observation is not a surprising outcome. Given that previous pharmacogenetic studies have indicated that patients with β_2-adrenoceptor (ADRB2) SNPs who use short acting β_2-agonists on a regular basis experience a desensitization and deterioration of lung function[162,179], background ADRB2 SNPs were examined, with no substantial differences. Moreover, it has previously been well demonstrated that the respiratory effects of inhaled salbutamol, and most anti-asthma medications, are not determined by their levels found in the circulation but are related to local airway deposition.[338-340] It is the adverse effects of salbutamol that are most relevant to blood levels.[238,338,341]

It has been suggested that the influence of individual SNPs, in particular non-translating SNPs and synonymous SNPs, may be difficult to identify and that there is a high frequency of type I errors, i.e. effects found to be present when they are not. This impediment may be due to a number of other factors, including both genetic and non-genetic factors,[342] however, modifying the method of analysis, e.g. haplotype analysis, may allow studies to overcome some of these difficulties.[343,344] Investigations have observed that some genotypes are more likely to co-exist with each others, known as haplotype relationship, in particularly SNPs located at common proximal regions with each other.[343,344] It is believed that, although the individual influence of the majority of SNPs is relatively minor, the collective effect of multiple SNPs would have greater clinical significance and consistency.[343-345] Although it requires a larger sample population, haplotype analysis is more likely to identify the consistent effects of genetic variations such as SNPs, and it is therefore recommended for future studies.

Despite three separate rounds of recruitment process with different media and targeted population, it is clear from our study that laboratory-based pharmacokinetic studies are difficult to perform due to: 1) low participation rates, 2) the need to have prolonged studies which is both inconvenient and requires well controlled asthma to participate. The difficulties in recruiting the target number of subjects significantly reduced the power of this study. It is difficult to know the exact reason(s) of the low participation rate, but reluctance of the community to be involved in 'genetic studies' is commonly encountered in genetic research.[346,347]

In conclusion, the study has shown that the SNP (rs1201735) in the SULT1A3 enzyme is unlikely to affect the pharmacokinetic parameters of either (R)- or (S)-salbutamol in great magnitude among mild asthmatic subjects.

CHAPTER 7

Montelukast pharmacogenetics in a clinical setting as an adjunct therapy among patients using corticosteroids

7.1 Abstract

Background: Leukotrienes are a family of mediators important in the airway inflammation of asthma. Montelukast is a leukotriene receptor antagonist shown to be effective therapy in asthma. The effects of anti-leukotriene agents, such as montelukast, have been reported to be influenced by genetic variations in key pathway enzymes. This study was designed to investigate the effect of relevant common genetic variations on treatment outcomes of montelukast in the clinical management of severe asthma.

Method: Participants were recruited through the Royal Hobart Hospital from Pharmacy dispensing records. Asthma treatment outcomes following montelukast, categorised as responder or non-responder, were determined according to the Hospital prescribing protocol based on changes in lung function and asthma symptoms. DNA was extracted from saliva samples and analysed for genetic variation at the ALOX5 promoter region using a novel PCR assay as well as 7 single nucleotide polymorphisms (SNPs) in genes of several enzymes, namely ALOX5, LTC4S, cysLTR1 and LTA4H.

Results: Sixty-six participants were recruited (52 responders and 14 non-responders). None of the genetic variations were found to have a relationship with the treatment outcome categorisation.

Discussion: The common genetic variations investigated do not influence treatment outcomes of montelukast therapy in severe asthma in patients with concomitant corticosteroid treatment. There is currently a very limited role in genetic testing to pre-determine the likely efficacy of montelukast among severe asthma patients.

7.2 Introduction

Asthma is a chronic inflammatory airway disease, in which leukotrienes have been suggested to play an important role in the complex inflammatory responses, both during acute exacerbations and during stable but active disease.[104,107,113,348] Leukotrienes are produced by a number of inflammatory and structured airway cells. Several enzymes and receptors have been found to be essential for the production as well as the actions of leukotrienes. [104,107,112,113,348]

Leukotriene receptor antagonists, including montelukast, zaprilukast and pranlukast, comprise a relatively new group of medications used in the treatment of asthma.[104,106,112] They act to reduce leukotriene-mediated airway inflammation by blocking the type 1 cysteinyl leukotriene receptor.[104,106]

Although different methods have been used for measuring treatment outcome, studies have regularly found that these medications are only effective in 50-70% of patients.[107,113,349] Guidelines suggest they should be used as add on medication for uncontrolled asthma in patients maximally treated with inhaled corticosteroids and LABA. It has been suggested that genetic variations at key enzymes and receptors may be a factor in individual treatment response.[173,176,178,350,351] Examples include the 5-lipoxygenase gene (ALOX5) and cys-leukotriene-C_4-synthase gene (LTC4S) where naturally occurring genetic variations have been identified.[176,205,352,353]

5-Lipoxygenase is an important enzyme that is involved in the early steps of leukotriene production. The 5' upstream promoter region of ALOX5 contains a

number of transcription factor binding sites, including the zinc finger (Sp1/Egr-1) binding motif, which is involved in mediating gene transcription.[206,352,353] The binding motif usually consists of 5 copies of a repeated sequence (GGGCGG), but variations with 3, 4 and 6 copies of the repeated sequences have been reported. .[206,352-354] These variations have been found to affect gene transcription, perhaps due to impaired binding to transcription factors, and subsequent decreased production of leukotrienes.[353,354] This genetic mutation was found to be associated with variable treatment outcome with anti-LTs, and is believed to be related to impaired production in leukotrienes.[173,178,206,353,355]

Several genetic variations, such as single nucleotide polymorphisms (SNP), are also found in various genes that encode the enzymes and receptors involved in the leukotriene pathway.[176,205,206] Some studies have reported a relationship between these SNPs and the effect of anti-leukotriene treatment in asthma,[175,205,356] however the findings are not consistent among studies.[207] Most of the genetic variations being investigated were located on ALOX5, LTC4S, Leukotriene A4 Hydrolase (LTA4H) and Cys-Leukotriene Receptor-1 (cysLTR1).[175,178,206,207,357,358] These genetic variations were believed to influence the expression and function of the corresponding enzymes or receptor, as well as affecting the underlining inflammatory nature of the disease.[178,205,206,356]

In spite of guideline recommendations on the use of anti-leukotriene medications, most pharmacogenetic studies have included mild to moderate asthmatic patients, subgroups of asthma, such as aspirin-induced, or anti-leukotrienes used as monotherapy.

Montelukast is a restricted use medication at the Royal Hobart Hospital (RHH), based on a strict protocol adapted from national and international treatment guidelines. Asthma patients who fulfill the prescription criteria (Table 7.1) are initiated on a 4 week trial of montelukast. At the end of the trial, only patients who show improvement in asthma control by 15% continue receiving treatment. Measurements of asthma control include lung function (FEV_1 and PEF), and an asthma diary (asthma symptoms and rescue medication use).

Table 7.1 Criteria for receiving trial treatment of montelukast at RHH

A. Non-exercise-induced asthma
Poor control of asthmatic symptoms despite at least 3 months of high-dose inhaled corticosteroid* and long acting β_2-agonist or theophylline
* High dose of inhaled corticosteroid is defined as one of the following:
1600 μg budesonide per day
2000 μg beclomethasone per day
1000 μg fluticasone per day
Persistent asthma symptoms but cannot receive other "add-on" agents due to adverse drug effects
B. Exercise-induced asthma
Persistent asthma symptoms that cannot be controlled by standard therapies in maximal doses

7.3 Methods

7.3.1 Subject recruitment and sample collection

The study was retrospective in design. Potential participants were identified from the dispensing records of the RHH Pharmacy Department, between 2000 and 2006, and all subjects prescribed montelukast were invited to take part in the study. Subjects were recruited by letter and telephone calls. Written informed consent was obtained and the study was approved by the State Human Research and Ethics Committee.

DNA samples were collected from each subject's saliva using an Oragene^{TM} DNA self-collection Kit (DNA Genotek Inc, Ontario, Canada). Kits were sent to patients through the post with return postage and written instructions. DNA was extracted following the manufacturer's instructions, at the School of Pharmacy laboratory, and stored in TAE buffer at -20°C until analysis.

Patients were categorised into 'responders' and 'non-responders', based on a 15% improvement in one parameter (FEV_1, PEF and an asthma diary for symptoms and rescue medication use) over the initial treatment period. Improvement determined by asthma diary was measured as the percentage reduction (15%) in the number of episodes of asthma symptoms and/or the number of doses of rescue medication required, over the treatment period. The treatment outcome was then evaluated against several genetic variations that were found to be associated with anti-LTs treatment outcomes from previous studies. The target genetic variations in this investigation were polymorphisms at the promoter region of ALOX5 (microsatellite),

as well as seven common SNPs located at the following genes: ALOX5, LTC4S, LTA4H and cysLTR1.

7.3.2 Microsatellite analysis

Microsatellite analysis was designed, validated and performed at the School of Pharmacy laboratory and the CSL laboratory,University of Tasmania.

7.3.2.1 Primer design

Forward and reverse primers were designed using the Primer 3 (v. 0.4.0) program designed by Rozen and Skaletsky, [359] and were further modified using the Amplify 3 (Version 3.1 For MacOSX) PCR simulation program (Amplify 3© Bill Engels, 2005, University of Wisconsin).

Forward primer (5' to 3'): *gagagaacgagtgaacgaa*

Reverse primer (5' to 3'): *gtccaggtgtccgcatcta*

Product size: 303 bp.

7.3.2.2 Microsatellite chemical premix and PCR reaction

Each 25 μL PCR reaction contained 2.5 μL of 10x buffer (670mM Tris pH 8.8, 166 mM ammonium sulfate, 4.5% Triton X-100 and 2mg/mL gelatin), 1.5 mM $MgCl_2$, 500 μM dNTPs, 400 nM of each Primer, 1.0 unit of *Taq* Polymerase and 30-100 ng of DNA template. The PCR reactions were run on an MJ Research PTC-200 Thermal Cycler (MJ Research, Inc., MA, USA) using the following conditions: 3 min at 95°C followed by 35 cycles of 95°C for 30 sec, 56.6°C for 35 sec and 72°C for

50 sec. After 35 cycles there was a 5 min extension step at 72°C followed by an 11°C hold

In order to determine an effective PCR reaction for the analysis, combination of various chemical premix (tinkering with primers combination and/or concentration of chemical ingredients) and various annealing temperatures for the reaction (guided by the melting temperature of each primer pair) was investigated, using the build-in temperature gradient function on the thermal cycler. The PCR products were then examined by gel electrophoreses and visually analysed under UV light as described in Chapter 5 (Section 5.3.3.3.1 Fragment size assessment).

7.3.2.3 Microsatellite fragment identification

The ALOX5 promoter region was identified by sequencing the resulting PCR product from five of the samples. The PCR products were first purified using a MO BIO UltraClean™ PCR Purification Kit (MO BIO Laboratories, Inc, CA, USA) following the manufacturer's instructions. The purified PCR products were sequenced using a Beckman Coulter GenomeLab™ DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (Beckman Coulter Inc. Fullerton, CA, USA) according to the kit instructions. In brief, a chemical premix consisting with ~50 fmol of purified template, buffer solution, the forward primer, DTCS mix and water, was run under the same PCR conditions as described above. After the reaction, a desalting procedure was carried out using an ethanol precipitation method, where stop solution (containing sodium acetate, EDTA and glycogen) was added to the sequencing PCR product; follow by ice-cold 95% ethanol. The mixture was centrifuged and supernatant was discarded. The palette (containing sequencing extension products) was washed twice

with ice-cold 70% ethanol, vacuum dried and re-dissolved in sample loading solution. The sequencing extension products were separated on a Beckman Coulter® CEQ8000 Genetic Analysis System and scored by eye.

7.3.2.4 Microsatellite fragment analysis

Fluorescently labeled PCR products containing the ALOX5 promoter region were separated in the presence of an internal DNA size standard (DNA Size Standard kit - 400, Beckman Coulter Inc. Fullerton, CA, USA) using a Beckman Coulter® CEQ8000 Genetic Analysis System. The resulting chromatograms were scored using the Beckman Coulter Fragment Analysis Software (Fragment Analysis Algorithm Version 3.2.42, Software version 8.0.52). The 5-repeat fragment and 4-repeat fragment were scored as 300 bp and 294 bp respectively.

7.3.3 Single nucleotide polymorphism (SNP) analysis

SNP analysis was performed by the Australian Genome Research Facility Ltd (AGRF, Queensland, Australia, part of the Australian Commonwealth Government's Major National Research Facility Program). The AGRF was responsible for the primer design and analysis using the Sequenom SNP genotype system. SNP assays were designed using the Sequenom MassArray Assay Design© Version 2.0.0.1.6 software. The PCR and extension reactions were run on an Applied Biosystems (Foster City, Ca, USA) GeneAmp PCR system 9700© 384 well thermocycler. The reactions were spotted onto a chip using a Samsung 24 pin main head Nanodispenser©. Chips were fired using a Bruker Daltronics Autoflex© mass

spectrometer (Billerica, MA, USA) with a matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry system.

7.3.4 Statistical analysis

According to the RHH Pharmacy Department dispensing records, in excess of 200 patients had been prescribed montelukast since the year 2000. With an estimated participation rate of 50%, 100 subjects (50 from each group) were anticipated. Given a wild-type (homozygote) genotype prevalence of 60% and the significant difference in FEV_1 improvement (18% and -1.2% for WT and mutant genotype respectively),[173] study power was 85% to detect a 50% difference in genotypes between responders and non-responders (α=0.05).

Comparisons between responder and non-responder groups were examined for each genetic variant, using two-tailed chi-square tests with $p<0.05$ considered statistically significant. All analyses were performed using SPSS 14.0 for Windows (SPSS Australasia Pty. Ltd. Chatswood, NSW, Australia) statistic software.

7.4 Results

Microsatellite fragment analysis was successfully designed and identified with sequencing method as described in Section 7.3.2.3, chromatogram of the sequence analysis is shown in Figure 7.1.

Of 198 asthmatics who were prescribed a trial of montelukast over the six year study period, eighty-nine participants were recruited to the study, and 71 provided a DNA sample. Fifty-two subjects were identified as 'responders' and fourteen as 'non-responders (according to the criteria in table 7.1), while five subjects were excluded due to ambiguity in clinical documentation and could not be clearly categorised. Demographic information on the subjects is summarised in Table 7.2. No differences were found between the demographics of the two groups of subjects.

Table 7.2 Subject demographic information

	Responder	**Non-responder**
Number of Participants (% of total invited)	52 (40%)	14 (25%)
Gender	21 (M) 31 (F)	6 (M) 8 (F)
Age in years(median [range])	57 [20-87]	61 [31-85]
Smoking history (pack years)	13 (25%)	4 (29%)
Concurrent diseases:		
COPD	7 (13%)	1 (7%)
Auto-immune	6 (12%)	4 (29%)
Aspirin intolerant asthma	2 (4%)	0 (0%)

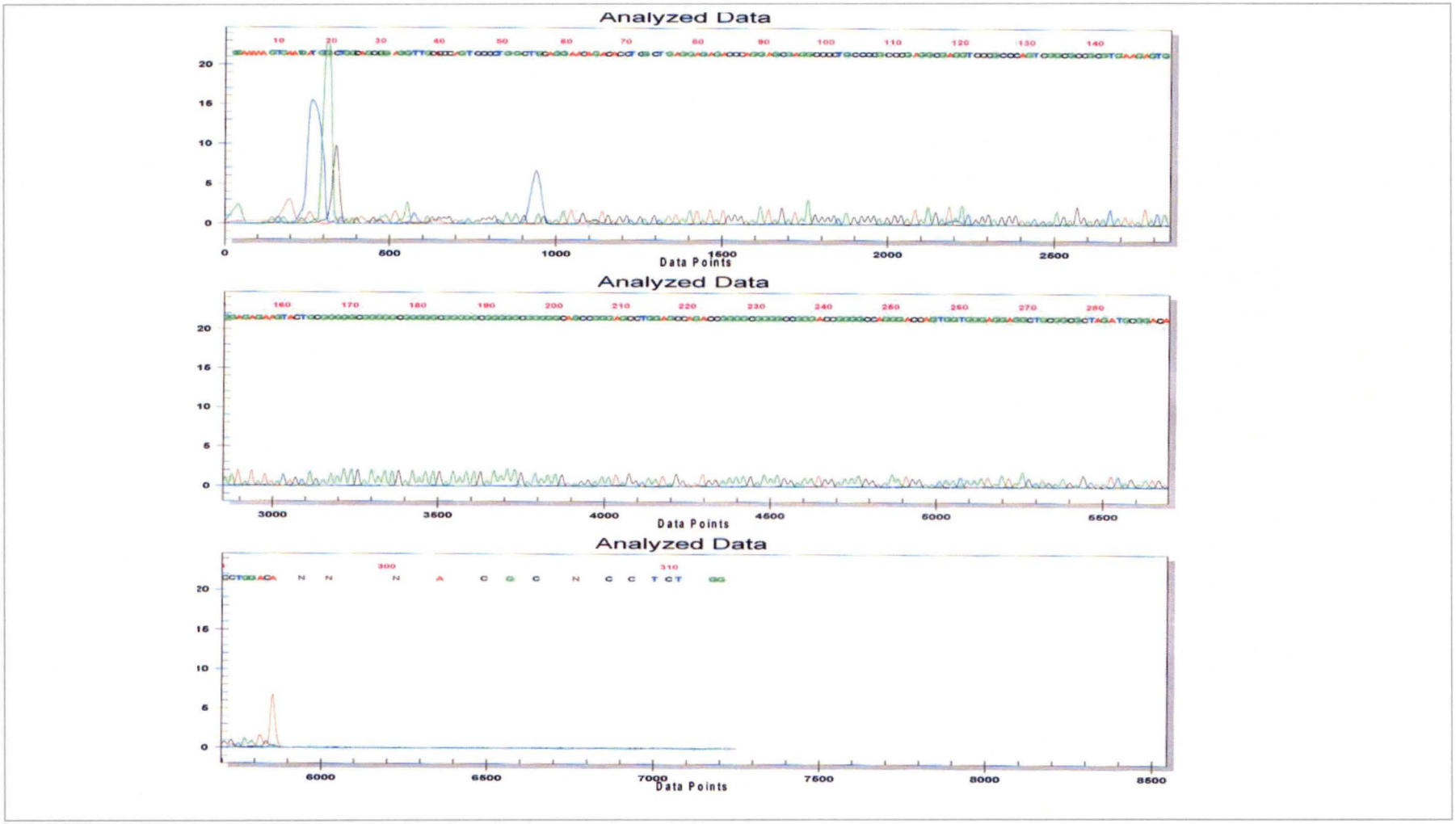


Figure 7.1 Chromatogram of ALOX5 microsatellite analysis (from top to bottom: heterozygote, 4-repeat-mutant homozygote and 5-repeat-wild-type homozygote)

7.4.1 Polymorphisms at the ALOX5 gene promoter region

Sequencing of the PCR products containing the ALOX5 promoter region proved difficult due to the high GC content of the sequence flanking the tandemly repeated motif. Our attempts to use previously published primers designed to amplify the ALOX5 promoter region [360] resulted in a weak PCR product coupled with numerous non-specific PCR artifacts, despite several attempts at re-optimising our PCR conditions. Nonetheless, the assay we developed and have reported in this study can successfully amplify a PCR product free of such artifacts (Figure 7.2).

Microsatellite analysis of the ALOX5 gene promoter region was successfully performed in 68 out of the 71 DNA samples (including 5 subjects who were later excluded as they were unable to be categorised). The wild-type motif (5 repeats) and 4-repeats motif were detected at the promoter region but no sample tested positive for the 3-repeat motif or 6-repeat motif. Overall, 36 samples were identified as wild-type homozygotes, 9 samples were 4-repeat motif homozygotes and 19 samples were heterozygotes. There was no association between the lack of response to montelukast and the genotype of ALOX5 (Table 7.3).

Table 7.3 Distribution of ALOX5 genotypes between montelukast treatment responders and non-responders.

	Responder N (%)* (N= 52)	Non-responder N (%) (N= 14)
Wild-type homozygote (5-repeats)	27 (52)	9 (64)
Heterozygote (5-repeat and 4-repeat)	14 (27)	4 (29)
Mutant homozygote (4-repeats)	9 (17)	-
Failed	2 (4)	1 (7)

7.4.2 SNPs at enzymes and receptor genes

Five SNP analyses were successfully performed in over 95% of the samples, and the results are summarised in Table 7.4. Two SNP analyses were excluded due to low rates of successful analysis (80% success rate for SNP rs2228064 and 90% success rate for rs3776944), with only the G nucleotide identified at both locations. No associations were identified between any of the SNP genotypes and response or non-response to treatment.

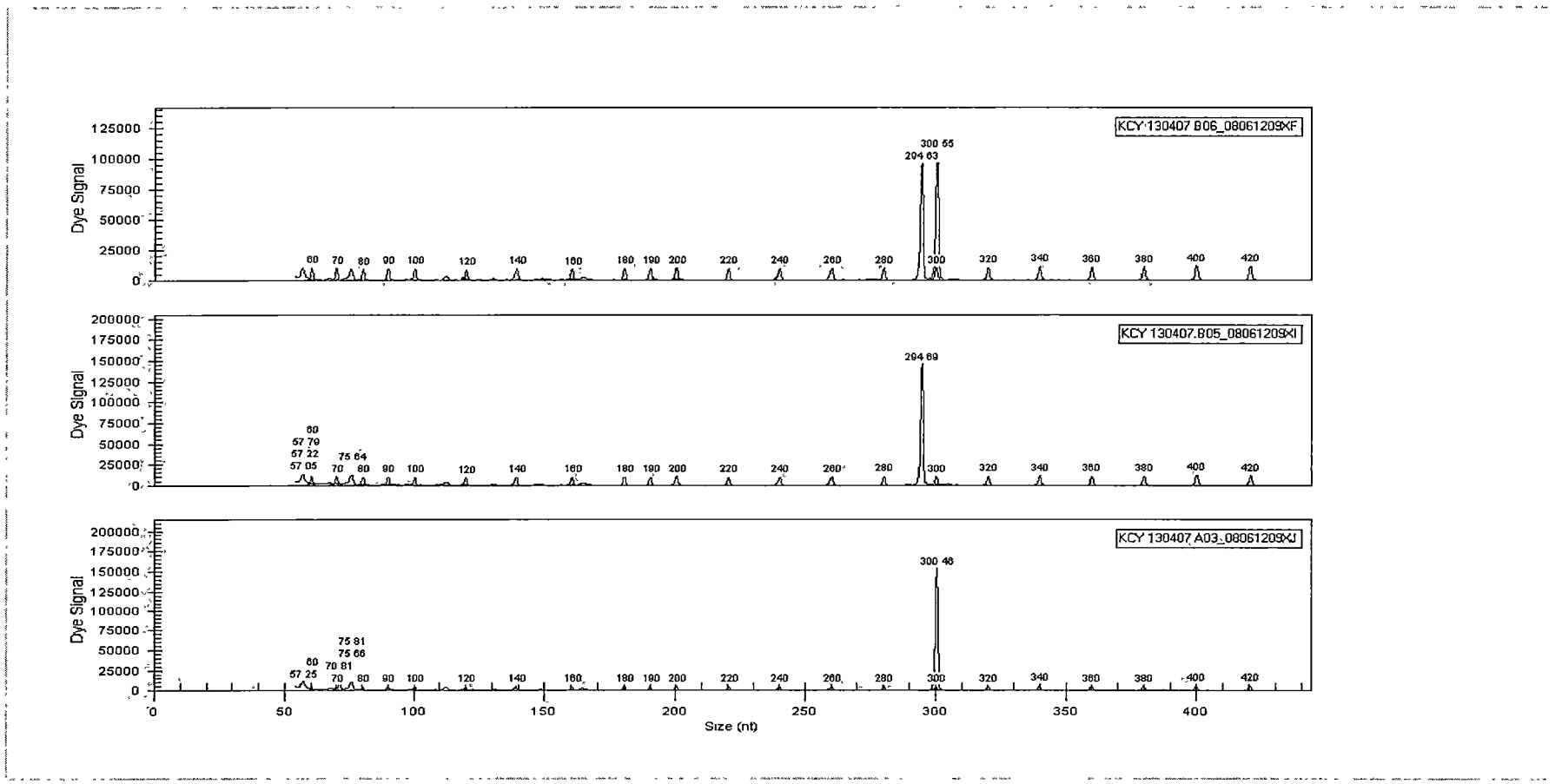


Figure 7.2 Chromatogram of ALOX5 microsatellite analysis (from top to bottom: heterozygote, 4-repeat-mutant homozygote and 5-repeat-wild-type homozygote)

Table 7.4 Distribution of SNPs at various enzymes and receptors between montelukast treatment responders and non-responders

Gene (location)	Responder N (%) (N= 52)	Non-responder N (%) (N= 14)	P value (Chi-Square test)
5- Lipoxygenase			
ALOX5 (21 C→T)			0.41
C	30 (58)	9 (64)	
T	6 (12)	-	
CT	16 (31)	5 (36)	
Failed	-	-	
ALOX5 (1728 A→G) (*rs2229136*)			0.35
A	45 (87)	14 (100)	
G	2 (4)	-	
AG	5 (10)	-	
Failed	-	-	
Leukotriene C_4 Synthase			
LTC4S (-444 A→C) (*rs730012*)			0.93
A	23 (44)	7 (50)	
C	8 (15)	2 (14)	
AC	21 (40)	5 (36)	
Failed	-	-	
Leukotriene A_4 Hydrolase			
LTA4H (-9226) (*rs2660845*)			0.62
A	5 (10)	-	
T	27 (52)	6 (43)	
G	1 (2)	1 (7)	
C	2 (4)	1 (7)	
TC	16 (31)	6 (43)	
AG	1 (2)	-	
Failed	-	-	
Cys-Leukotriene Receptor-1			
cysLTR1 (927 T→C) (*rs320995*)			0.68
T	38 (73)	9 (64)	
C	7 (13)	3 (21)	
TC	5 (10)	2 (14)	
Failed	2 (4)	-	

7.5 Discussion

The study did not show any significant relationship between response or non-response to montelukast, and any of the various genetic polymorphisms investigated. The findings of this study were limited by the uneven participation between the two groups of subjects (40% among responders and 25% among non-responders), which largely reduced the power of this study. This was thought to be due to a lack of interest among patients who did not benefit from the treatment. In addition, the observed frequencies of some of the polymorphisms in this study vary from previously published data.

Previous studies that found association between genetic variations and treatment outcomes were often performed on mild to moderate asthmatics subjects and often used anti-LTs as the primary preventive medication for asthma.[173,178,205,206,361] However, the results presented here with classifications based on asthma guideline recommendations, do not concur with some of the previous studies..[173,178,355,357] The results of this study are more in line with some studies that involved the greater population or used anti-LTs inconjunction with other medications among subjects with more severe disease.[358,362] However, it is difficult to directly compare the findings between studies due to the difference in population groups and diffences in measurements used to determine treatment outcome.

Taken as an audit of clinical outcomes, the study found that montelukast is effective in improving asthma control for over two-thirds of the severe asthma patients, despite the wide range of demographics and concomitant high dose inhaled corticosteroid

therapy, where limited data are currently available in the literature. However, due to the retrospective nature of the study, further investigations into outcomes in addition to the categorization of "responder/non-responder" could not be made.

Corticosteroids are known to suppress inflammation through a wide range of mechanisms, while the effect on leukotriene-mediated inflammation is relatively weak.[348,363] When montelukast is used in addition to corticosteroid therapy resulting in improvement in clinical control, it is reasonable to suggest that airway inflammation may not be fully controlled by the corticosteroids, probably due to a significant contribution by leukotrienes.[348] Our study, unfortunately, suggests that genetic screening for leukotriene pathway enzymes is not going to be useful in identifying which patients are most likely to benefit.

Although leukotrienes play an important role in mediating inflammation in asthma,[104,348] it is important to recognise that other inflammatory pathways also contribute to the disease, and indeed seems to be considerably more important.[73] It is suggested the reason some asthma patients fail to respond to anti-leukotriene treatment maybe due to the dominance of non-leukotriene mediated inflammation in the airways.[173,178,352] The current study was based on the hypothesis that any unresponsive subgroup of asthma patients include individuals with genetic variations leading to a reduced production or function of leukotrienes. However, due to the complex pathway of leukotriene production and function, multiple factors (both genetic and non-genetic) may contribute to the overall outcome of treatment response to leukotriene receptor antagonists and contribute to our negative finding. In addition, leukotrienes also mediate inflammatory responses by binding to other receptors, such

as the type-2 cys-LTs receptor, that are not suppressed by leukotriene receptor antagonists.[348,364] As a result it is perhaps not surprising that studies have been unable to identify who will respond to leukotriene receptor antagonists or not consistently.[173,178,205,207,354,355,357,361,365]

Previous experience has suggested that the influence of individual SNPs is difficult to identify and reproduce, as they may be affected by variety of factors.[147,164,366] In addition, the production of leukotrienes will also be affected by the expression and therefore the translation of various genes, independent of the genetic variation. However, modifying the method of analysis may allow studies to overcome some of these difficulties.[176,367,368] These analyses include leukotrienes level analysis, haplotype analysis and genetic expression analysis (for example histone acetylation, deacetylation and methylation),[176,367,368] Although it requires a larger sample population, haplotype analysis is more likely to identify the consistent effects of multiple genetic variations such as SNPs,[147,369] and is recommended for future studies.

The ALOX5 promoter region is widely known to be notoriously difficult to genotype, often produceing artefacts and a relatively weak signal. Sequencing the PCR products containing the ALOX5 promoter region also proved to be difficult due to the high GC content of the sequence flanking the tandemly repeated motif, Numerious modifications of the PCR condition were required in this study to achieve a high-quality product that is suitable for the genotype analysis. Nonetheless, the assay reported in this study can successfully produce a PCR product that is free of any significant artefact (Figure 7.3).

In conclusion, the result of this study suggests that there is currently a very limited role in genetic testing to pre-determine the likely efficacy of montelukast among severe asthma patients with concomitant corticosteroid treatment who remain uncontrolled in spite of maximal inhaled corticosteroids and long-acting β_2-agonist, as per international treatment guidelines. Future advances in analytical methods, such as haplotype analysis, may prove to be more informative.

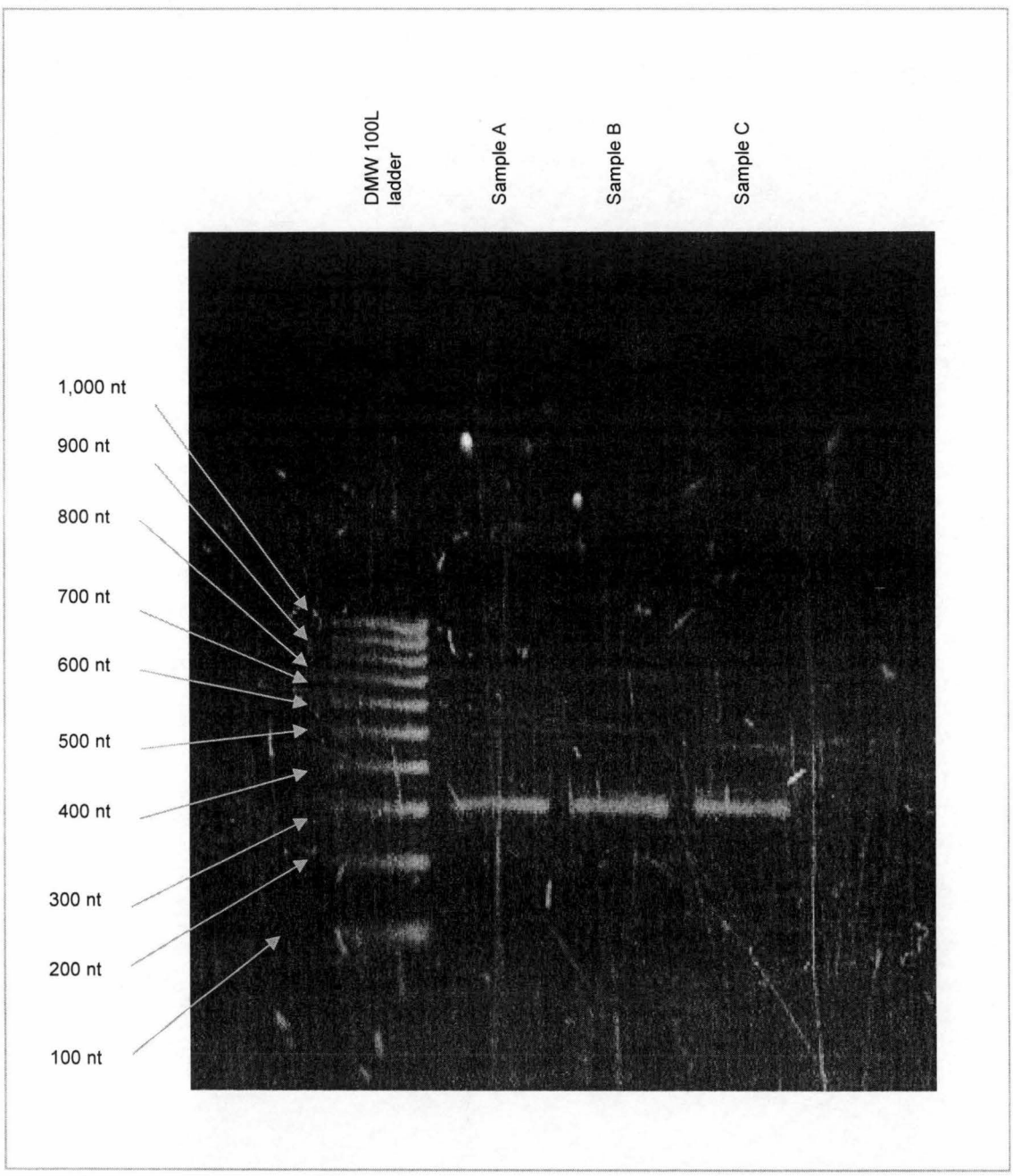


Figure 7.3 1% Agarose gel electrophoresis of PCR product (ALOX5 PCR product size: 303 bp)

Chapter 8

Relationship between genetic variations at AP-1 and steroid insensitive asthma

8.1 Abstract

Background: Corticosteroids, a group of potent anti-inflammatories, are the corner stone of current anti-asthma therapy. However, there are suggestions that some inflammatory pathways (e.g. activator protein-1 (AP-1)) are not efficiently suppressed by existing corticosteroid therapy. This study was designed to investigate the relationship of some common genetic variations in AP-1 with treatment responses to corticosteroids in asthma.

Method: Asthma treatment outcomes following corticosteroids, categorised as steroid-insensitive asthma or steroid-sensitive asthma, were determined according to the American Thoracic Society guidelines (2000). Blood and induced sputum samples were collected from subjects for differential cell analysis. DNA was extracted from the blood samples and analysed for single nucleotide polymorphisms (SNP) at AP-1 related genes. Haplotype analysis was also performed on 16 SNP on c-Jun-N-terminal-kinase gene.

Result: Twenty subjects were recruited (7 steroid insensitive asthma sufferers and 13 steroid sensitive asthma sufferers). No significant difference in genetic variations or differential cell analyses were observed between the two groups.

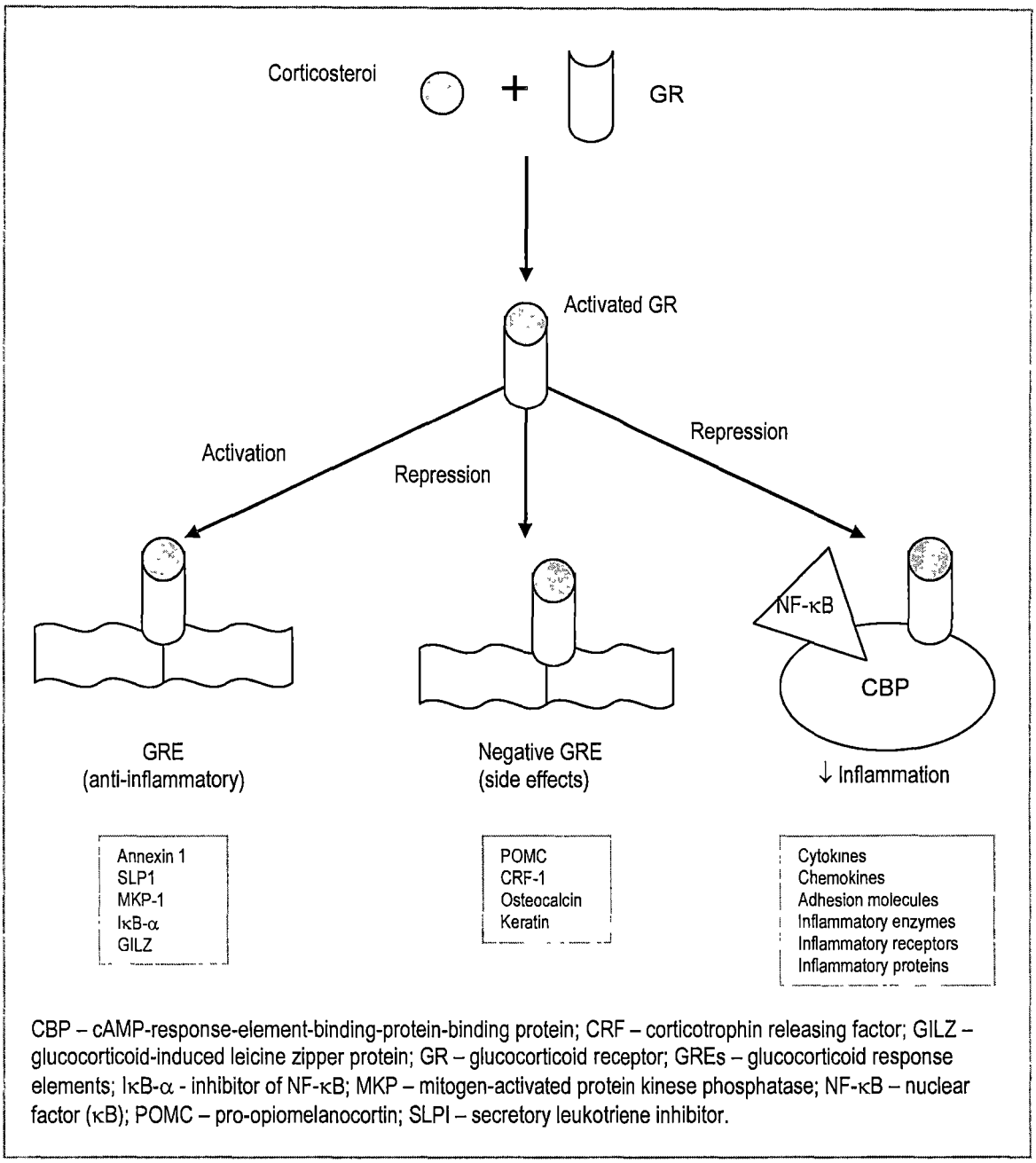
Discussion: The common genetic variations at AP-1 and c-Jun-N-termial kinase gene were not related to asthma treatment outcomes with corticosteroids. There was also no significant difference in airway inflammation between the two groups of subjects, indicated by sputum eosinophil counts (from differential cell analyses).

8.2 Introduction

Most patients with asthma are found to have mild or moderate symptoms and can be controlled by existing treatments. However, a subgroup of asthmatics (about 5%) have more troublesome disease and are usually unable to obtain adequate disease control, despite aggressive treatment.[94,97,100] This subgroup is commonly referred to as having refractory asthma, steroid insensitive asthma or steroid resistant asthma, and there is limited understanding of this severe disease.[94,97,100]

Corticosteroids are the corner stone of current anti-asthma treatment, and are routinely used as preventative therapy with substantial efficacy.[8,94-96,102] Corticosteroids exert their therapeutic effects (glucocorticoid effects) by binding to and activating the glucocorticoid receptor (GR).[93-98] It is believed that the activated GR exerts anti-inflammatory effects through a variety of genomic and non-genomic pathways, including promotion and inhibition of gene transcriptions, interaction with other transcription factors and alteration of circulating mRNA activities (Figure 8.1).[93-99]

Although having a wide range of anti-inflammatory activities, corticosteroids are found not to suppress all the inflammatory pathways that occur in asthma.[94,95,97] The outstanding inflammation is believed to play a key role in the reduced therapeutic responses of conventional corticosteroids in some asthmatic patients, that is those with refractory asthma.[94,95,97] These patients usually require a much higher dose of corticosteroid(s) to achieve disease control similar to that of 'regular' asthmatics, and there is evidence that genetic variations are likely to be an important factor.[97,208]



CBP – cAMP-response-element-binding-protein-binding protein; CRF – corticotrophin releasing factor; GILZ – glucocorticoid-induced leicine zipper protein; GR – glucocorticoid receptor; GREs – glucocorticoid response elements; IκB-α - inhibitor of NF-κB; MKP – mitogen-activated protein kinese phosphatase; NF-κB – nuclear factor (κB); POMC – pro-opiomelanocortin; SLPI – secretory leukotriene inhibitor.

Figure 8.1 The anti-inflammatory action of corticosteroids in asthma (Adapted from Barnes 2006 [93])

Genetic variations, in particularly variations at the GR, have been the target of many recent studies that have explored refractory asthma. Some studies have observed linkage relationship between genetic variations and the disease, for example polymorphisms at the α-GR gene.[94,95,97,180] In addition, various genes and proteins related to inflammation have been found to be related to refractory asthma, including

histone deacetylase, mitogen-activated protein kinases and transcription factors such as activator protein-1 (AP-1).[95,97,208,370]

Some recent studies have observed elevation in AP-1 activity and reduction in the interaction between AP-1 and activated GR, among patients with refractory asthma.[371-373] AP-1 is an array of dimeric basic region-leucin zipper proteins compiled from a Jun (c-Jun, JunB and JunD) and a Fos (c-Fos, FosB, Fra1 and Fra2) subunit.[371,372,374] Over-expression of the c-Fos subunit and increased activity of the c-Jun subunit have been demonstrated in refractory asthma.[371,372] In addition, the c-Jun-N-terminal-kinase (JNK), a mitogen-activated protein kinase that regulates the expression and activation of Jun and Fos subunits, has also been found to be related to the abnormal AP-1 activity among refractory asthmatics.[372,375] Recently, several polymorphisms have been identified in genes that encode JNK as well as a number of AP-1 subunits,[208,376] but to the author's knowledge, there is no publication to date examine the effect of these polymorphisms in relation to refractory asthma.

The study was designed to investigate the effect of common single nucleotide polymorphisms (SNPs) on AP-1 related genes, in relation to refractory asthma.

8.3 Method

The study was group-comparison in design. Subjects were categorised into one of the two study groups, steroid insensitive asthma (SIA) and steroid sensitive asthma (SSA). The definition of refractory asthma by the American Thoracic Society (Table 8.1) [100] was used for the classification of SIA. Subjects who did not fulfill either of the major characteristics of the definition were classified as SSA. The study was approved by the State Human Research and Ethics Committee.

8.3.1 Subjects

Potential participants were asthmatic patients from Southern Tasmania. An invitation letter was first sent to all respiratory physicians in Southern Tasmania, and potential subjects were identified. Potential subjects who expressed interest in the study were referred to the researchers, who then invited them to take part in the study. Written informed consent was obtained from each subject before the initiation of the study.

Participating subjects were invited to the Centre for Clinical Research, Royal Hobart Hospital (RHH), for sample and data collection.

Table 8.1 Definition of refractory asthma of American Thoracic Society (2000) [100]

ATS criteria of refractory asthma*† as follow:

Major Characteristics

In order to achieve control to a level of mild–moderate persistent asthma:

1. Treatment with continuous or near continuous (50% of year) oral corticosteroids
2. Requirement for treatment with high-dose inhaled corticosteroids:

Drug	Dose (mcg/day)
Beclomethasone dipropionate	1,260
Budesonide	1,200
Flunisolide	2,000
Fluticasone propionate	880
Triamcinolone acetonide	2,000

Minor Characteristics

1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting beta-agonist, theophylline, or leukotriene antagonist
2. Asthma symptoms requiring short-acting beta-agonist use on a daily or near daily basis
3. Persistent airway obstruction
4. FEV 1 <80% predicted
5. diurnal PEF variability >20%
6. One or more urgent care visits for asthma per year
7. Three or more oral steroid "bursts" per year
8. Prompt deterioration with 25% reduction in oral or inhaled corticosteroid dose
9. Near fatal asthma event in the past

* Requires that other conditions have been excluded, exacerbating factors treated, and patient felt to be generally adherent.

† Definition of refractory asthma requires one or both major criteria and two minor criteria.

8.3.2 Measurements

The measurements were single nucleotide polymorphisms (SNPs) on AP-1 related genes. The AP-1 related genes examined in this study were: c-Jun N-terminal kinase 1 gene (JNK1/MAPK8; OMIM 601158), c-Jun gene (JUN; OMIM 165160), c-Fos gene (FOS; OMIM 164810) and corticotropin releasing hormone receptor 1 gene (CRHR1; OMIM 122561). The SNPs identified for this study were divided into two groups, SNP-analysis-I (for haplotype analysis) and SNP-analysis-II (for particular SNP analysis)

SNP-analysis-I included 16 SNPs located on JNK1 (RS10857561, RS10857564, RS3827680, RS10857565, RS9888128 RS11101318, RS11597435, RS10508901, RS2289805, RS17780725, RS12358297, RS10508903, RS11101320, RS7086275, RS4838590 and RS9284). The SNPs were identified using the *PubMed SNP* online database (March 2008) [377], with both the wild-type and SNP homozygote frequencies $\geq 5\%$.

SNP-analysis-II included SNPs located mostly at the exons or the 5'upstream promoter regions of AP-1 related genes. The SNPs identified for this study were: rs4647001 (JUN; 5' upstream nucleotide –237), rs11688 (JUN; synonymous SNP; nucleotide 751), rs9989 (JUN; non-synonymous SNP; nucleotide 890), rs2234706 (FOS; 5' upstream nucleotide –237), rs4645852 (FOS; 5'upstrean nucleotide -83), rs45542032 (JNK1; non-synonymous SNP; nucleotide 1116), rs1876828 (CRHR1; intron 3) and rs242941 (CRHR1; intron 13). The SNPs were identified from *Ensembl* online database (August 2006, revised in September 2008) [378], with validated SNP frequency.

Differential white cell count was employed as a biomarker of airway inflammation. The differential white cell counts were performed from both peripheral blood samples and induced sputum samples. Sputum samples from 12 non-asthmatic individuals were used for comparison.

8.3.3 Sample collection

Peripheral blood samples were collected from subjects by a research nurse, with venous puncture and Vacuette® EDTA K3 tubes (Greiner Bio-One, Kremsmuenster, Austria), for full blood examination and DNA extraction. DNA samples were extracted from the buffy coat with a QIAamp DNA blood mini kit (QIAGEN Australia Pty Ltd., Doncaster, VIC) following the manufacturer's instruction, as outlined in Chapter 5 (Section 5.3.2.2). The extracted DNA samples were stored at -20°C until analysis.

Sputum samples were induced according to the protocol from the RHH Respiratory Unit, by a research nurse, as outlined by Reid et al. [379,380] In brief, subjects were dosed with 400 μg salbutamol via a MDI and spacer, and FEV1 was measured pre- and post-salbutamol dose (Vitalograph® COMPACT spirometer, Vitalograph Ltd., Ennis, Co Clare, Ireland). The procedure was terminated if the subject's measured FEV1 was <0.8 L post-salbutamol dose. Fifteen min after the salbutamol dose, subjects were instructed to inhale nebulised hypertonic saline (4.5%) by mouth for 5 min, then to rinse and gargle the mouth with water to discard saliva, and attempt to expectorate sputum. If no sputum was produced, the procedure was repeated for a maximum of

six times if necessary. The procedure was terminated if an adequate amount of sputum sample was expectorated (≥ 2.5 mL of visible airway plug), the maximum of six expectoration attempts were reached or the subject started to feel uncomfortable. FEV1 was measured at the end of the procedure or when the subject felt uncomfortable, and salbutamol was administered if FEV1 was ≤ 80% of the pre-induction value.

8.3.4 Sample analysis

8.3.4.1 Differential white cell count

The differential white cell count from peripheral blood samples was determined with full blood examination, performed by the RHH Pathology Department in according to the RHH resident protocol.

Sputum samples were processed according to the protocol from the RHH Respiratory Unit, outlined by Reid et al [379,380] and Fahy.[381] In brief, dithiothreitol 0.1% (Sputalysin Calbiochem Ltd, San Diego, CA, USA) was added to the sputum (4:1 ratio v/v), gently mixed and incubated in a 38°C water bath for 30 min. The sample was then centrifuged at 250 *g* for 10 min, and the cell pellet resuspended with PBS to the original volume of sputum. The total cell count was performed and the resuspended sample was spun (Shandon Cytospin III, Runcorn, UK; 82 *g*) for 10 min. Cytospots were stained with Haem Kwik Stains (CliniPure; HD Scientific Supplies Pty Ltd., VIC, Australia), and a differential cell count was performed by a resident serologist at the RHH, blinded to the subject's details. At least 200 non-squamous cells were

counted from each slide, and samples containing <80% squamous cells were considered adequate.

8.3.4.2 SNP analysis

The SNP determination was performed in two separate analyses. SNP determination for SNP-analysis-I was performed by AGRF, as outlined in Chapter 5 (Section 5.3.3.3.3). SNP-analysis-II was determined using a GenomeLab™ SNPStart Primer Extension Kit (Beckman Coulter Inc., Gladesville, Australia) at the CSL laboratory.

The GenomeLab™ SNPStart Primer Extension kit used PCR products created by hybridisation of unlabelled locus primer pairs and DNA templates, followed by single-base extension of a fluorescent dye labelled terminators. The extended fragments were analysed with a Beckman Coulter® CEQ 8000/8800 Genetic Analysis System (Beckman Coulter Inc. Fullerton, CA, USA).

PCR product was first prepared for each SNP, as outlined in Chapter 8 (Section 8.4.2.1 and Section 8.4.2.2). In brief, primers for each targeted SNP were designed, and PCR reactions were run with automated thermal cycler (MJ Research, Inc., MA, USA). The locus primer pairs, interrogation primers and selected annealing temperatures for the SNPs are listed in Table 8.2.

Table 8.2 Primers and annealing temperatures for SNP-analysis-II

SNP	Primer (5'-3')	Product size	Annealing temperature
rs4647001	Hybrid primers:	186 bp	59°C
	L: cgggcaatacaaatctctcggcttcta		
	R: ataccgcccttaaggtggctctgtgaa		
	Int primer 1: ax36-tcggagtgttctcaacgtgggggcc *	63 bp	
	Int primer 2: ax18-taagtttaggggcggtctcccgagagt	46 bp	
rs11688	Hybrid primers:	209 bp	65°C
	L: cctgaaggaggagcctcagacagtgc		
	R: ccagctccgagttctgagctttcaaggttt		
	Int primer: ax18-ctgtcccccatcgacatggagtccca	45 bp	
rs9989	Hybrid primers:	193 bp	59°C
	L: agaatcgcccggctggaggaaaaagtg		
	R: ctcagcccccgacggtctctcttcaaa		
	Int primer: ax42-aagctcagaactcggagctggcgtcca *	70 bp	
rs2234706	Hybrid primers:	162 bp	59°C
	L: ccccttacacaggatgtccatatta		
	R:gcgtgtcctaatctcgtgagcatt		
	Int Primer 1: ax30- gggagccatccccgaaacccc *	52 bp	
	Int primer 2: ax6-agaggtctcgtgggcccccccaagatg	33 bp	
rs4645852	Hybrid primers:	223 bp	59°C
	L: gctcacgagattaggacacgcgccaa		
	R: cgccggctcagtcttggcttctcagtt		
	Int primer 1: ax24-aagcgcccaggcccgcgcgccacccctctgg *	56 bp	
	Int primer 2: ax14-cgtcacgggctcaaccacggtggc *	39 bp	
rs45542032	Hybrid primers:	150 bp	59°C
	L: gtcattgtaaggacactgtttgaagta		
	R: ggctcttagactttgaaaagttcatt		
	Int primer: aggaagttatggacttggaggagaga	28 bp	
rs1876828	Hybrid primers:	169 bp	65°C
	L: ctgtgaggcctgttgggactggcgat		
	R: gcttctgagagcaaaggaggggctgt		
	Int primer: ax6-ccagggctgcctctctccctccctg	32 bp	
rs242941	Hybrid primers:	191 bp	65°C
	L: cctgagtccagcagagaaagggagccaat		
	R: tttgagccccagcgtccccaggttaat		
	Int primer: ax12-gggccaggaaccatgaaccagcgcg	38 bp	

* Genotyping failed in > 50% of the reactions

Excess PCR reagents were removed with an enzymatic reaction. The enzyme premix for each 10 μL PCR product contained 2 unit Exonuclease I (Exo), 1.9 μL of 10x Exo buffer, 1 unit Antarctic Phosphatase (AP), 1.48 μL of 10x AP buffer and 0.78 μL of miliQ water. The enzymes and corresponding buffers were obtained from New

England Biolabs Inc (Ipswich, MA, USA).The mixture was incubated at 37°C for 60 min, follow by enzyme deactivation at 75°C for 15 min.

Chemical premixes and reactions for single-base extension reactions were prepared, and adjustments were made, in accordance with the manufacturer's instructions. Each SNP analysis was first validated with a single-plex reaction (one SNP analysis per reaction), and number of SNP analyses (with product size ≥6 bp) were combined to create a multiplex reaction (multiple SNP analyses per reaction). The chemical premix for single-plex reactions and multiplex reactions are summarised in Table 8.3. The reactions were run under the following conditions: 90°C for 10 sec then 45°C for 20 sec for 30 cycles, followed by an 11°C hold. Samples were re-analysed with single-plex reaction(s) if necessary.

Table 8.3 Chemical premix for single-base extension reaction (SNP-analysis-II)

	Single-Plex	**Multiplex A**	**Multiplex B**
		rs45542032; rs2234706; rs4647001	**rs1876828; rs242941; rs11688**
SNPStart Master Mix (μL)	1.0	1.0	1.0
Interrogation Primers (nM)	50	17; 17; 42	32; 32; 26
PCR products (μL)	2.5	0.7; 0.7; 2.1	0.8; 0.8; 1.6
MiliQ water (μL)	5.5	4.0	4.0
Total volume (μL)	10	10	10

The excess dye labelled terminators and reagents were removed with an enzymatic reaction, consisting of 0.7 unit of AP and 1.9 μL of 1x AP buffer for each 10 μL

single-base extension product. The enzymatic reaction was run at 37°C for 30 min, followed by enzyme deactivation at 65°C for 15 min.

The reaction products were loaded onto a Beckman Coulter® CEQ Genetic Analysis System for automated separation, detection and genotyping. A size standard (GenomeLab™ DNA Size Standard 80 Kit, Beckman Coulter Inc. Fullerton, CA, USA) was used as the internal standard for genotyping analysis.

8.3.5 Statistical analysis

SIA is a relatively uncommon form of asthma, with a prevalence of about 5% (estimated 40 patients in Southern Tasmania)[100] Although relatively uncommon, the abnormal relationship between AP-1 and SIA has been observed in studies with relatively small sample size.[371-373].

Based on the frequency of mutation of 10% at nucleotide -277 of the c-Fos gene and 5% at nucleotide 1116 of the JNK gene among normal population, as reported on the GenBank database,[377] with α=0.05 (two tailed) and β=0.20, 20 subjects from each group would allow detection of an odds ratio of 5 and 10 for AP-1 and JNK SNPs respectively. Hance, a detection of 10 subjects or more, with the mutated gene(s), among the SIA subjects will produce a significant result.

Comparisons between SIA and SSA groups were examined for differential cell counts with two-tailed student *t*-test, and genetic variants with two-tailed chi-square test, with $p<0.05$ considered statistically significant. Haplotype was identified and analysed

using Haploview 4.1 program (Broad Institute, Cambridge, MA, USA). Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Australasia Pty. Ltd. Chatswood, NSW, Australia) statistics software and the Haploview program.

8.4 Results

Twenty subjects were recruited, aged between 27 and 78 years, with 7 subjects classified as SIA and 13 subjects as SSA. The demographics of subjects are summarised in Table 8.4.

Table 8.4 Subject demographics

	SIA (n=7)	SSA (n=13)
Mean age (±SD)	52 (±9)	62 (±14)
Gender	2 M; 5 F	7 M; 6 F
Smoking history		
Current smoker	4	3
Ex-smoker	0	2
Co-morbidity of COPD	1	1
Post bronchodilator predicted LFT		
% PEF (mean (±SD))	99 (±23)	109 (±16)
(change from baseline)	9%	9%
% FEV1 (mean (±SD))	79 (±27)	92 (±15)
(change from baseline)	15%	6%

Differential white cell counts from the peripheral blood samples and the induced sputum samples were performed for all samples, and expressed as the percentage of total white cells counted (Table 8.5). However, three of the results (from induced sputum samples) were excluded due to sample degradation and/or excessive squamous cell contamination. The differential white cell counts from sputum samples showed a greater percentage of eosinophils compared to non-asthmatic subjects (mean difference [95%CI] = 4.7 [0.5:8.9]), but no major difference was observed in other cell types. The comparison between SIA and SSA found significant differences in lymphocyte count, but not in other cell types. The differential cell counts from

induced sputum samples did not show a correlation with the differential cell counts performed from peripheral blood samples (r^2 0.01, 0.02, 0.03 and 0.08 for eosinophil, macrophage, neutrophil and lymphocyte respectively).

SNP-analysis-I was successfully performed in all samples. Two SNPs (rs9989 and rs4645852) from SNP-analysis-II were unable to be successfully analysed on a consistent basis and were excluded. The remainder of the SNP-analysis-II were successfully performed in over 95% of the samples. However, multiplex B reaction was successfully performed in <50% of the cases, and most of the SNPs were determined with single-plex analysis.An example chromatogram of SNP-analysis-II was shown in Figure 8.2.

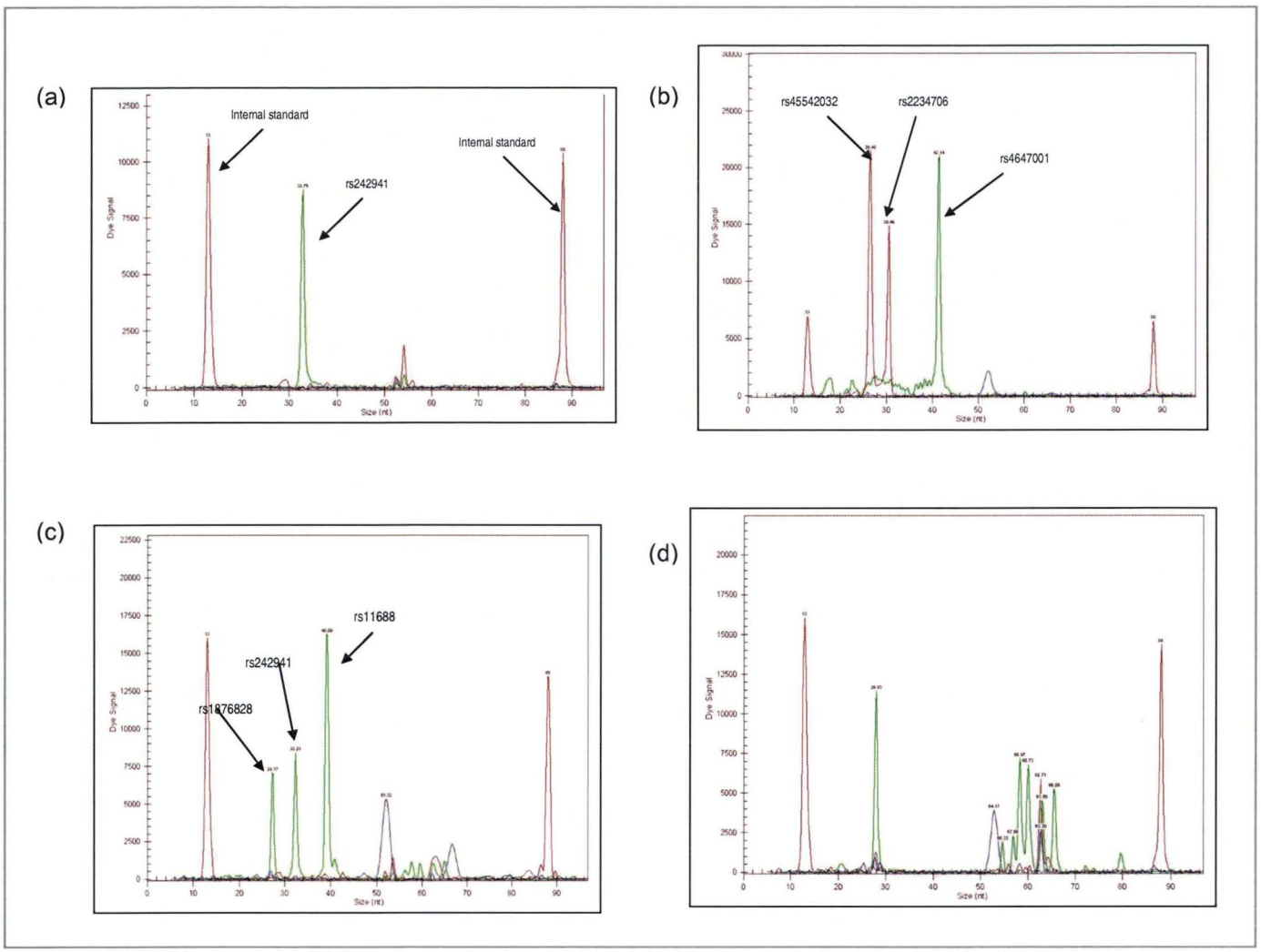


Figure 8.2 Examples of chromatograms of SNP analysis using GenomeLab^{TM} SNPStart Primer Extension Kit. (a) Single-plex (rs242941, product size of 38 bp, internal standard size 13 and 80); (b) Multiplex A (produce size are 28 bp, 33 bp and 46 bp for rs45542032, rs2234706 and rs4647001 respectively); (c) Multiplex B (product size of 32 bp, 38 bp and 45 bp for rs1876828, rs242941 and rs11688 respectively); (d) Single-Plex analysis with signal interference (at 50-70 nt)

Table 8.5 Differential white cell counts from blood sample and sputum sample

	Non-asthmatic (N=12) Mean (±SD)	SIA (N=7) Mean (±SD)	SSA (N=13) Mean (±SD)	SIA vs SSA Mean difference [95% CI]
Blood sample[#]				
WCC (x10^6/mL)		10.2 (±4.2)	6.8 (±1.5)	
% Eosinophil		5.4 (±5.3)	3.8 (±1.5)	1.5 [-1.7: 4.7]
% Macrophages		0.6 (±0.3)	0.8 (±0.1)	-0.2 [-0.4: 0.03]
% Neutrophil		68.2 (±13.8)	58.4 (±8.0)	9.7 [-0.4: 20.0]
% Lymphocytes		19.6 (±7.2)	28.9 (±8.3)	-9.3 [-17.1: -1.4)]*
Sputum sample				
WCC (x10^6/mL) [##]		2.4 (±1.2)	1.7 (±1.6)	
% Eosinophil	0.5 (±1.0)	3.9 (±4.4)	4.9 (±7.4)	-1.0 [-8.6: 6.7]
% Macrophage	44.4 (±25.5)	47.7 (±30.6)	43.1 (±25.0)	4.7 [-25.5: 34.9]
% Neutrophil	32.1 (±21.5)	53.7 (±31.3)	51.6 (±25.1)	2.1 [-28.4: 32.6]
% Lymphocyte	1.5 (±2.0)	1.1 (±0.7)	0.5 (±0.5)	0.6 [0.05: 1.2] *

* $p<0.05$

[#] n=7 for SIA; n=13 for SSA

[##] n=6 for SIA; n=9 for SSA

Haplotype analysis identified 5 major haplotypes on JNK1 (frequency > 5%), where haplotype 1 and 2 were composed of only the major and minor allele of the SNPs respectively. The distribution of haplotypes was not found to be different between the two groups (Table 8.6). SNP-analysis-II found no variations at the following SNP: rs4647001, rs11688, rs2234706, rs45542032, rs1876828 and rs242941, with only wild type genotype being identified.

Table 9.6 The distribution of haplotypes (overall frequency ≥5%) in SIA and SSA.

	Haplotype frequency			Chi Square	P value
	Overall	SIA	SSA		
Haplotype 1					
GCGGAGGCTGACGACG	48%	43%	50%	0.186	0.67
Haplotype 2					
AGAAGAAATACAATAT	18%	14%	19%	0.154	0.69
Haplotype 3					
GCGGAGGCGGACGACG	15%	21%	12%	0.698	0.40
Haplotype 4					
ACAGGAGATGAAATAT	10%	7%	12%	0.195	0.66
Haplotype 5					
GCAGGAGCTGAAATAT	5%	7%	4%	0.208	0.65

8.5 Discussion

Asthma is a chronic airway inflammatory disease that is commonly characterised by a peculiar type of inflammation, which features airway eosinophilic infiltration.[382-384] It has also been suggested that the pathophysiology of airway inflammation in refractory asthma is different from that in general asthma.[94,100] The eosinophil count from bronchoalveolar lavage has been found to reflect the airway inflammation,[383-390] and a reduction in sputum eosinophils was found to be correlated with improvement in asthma control from corticosteroid therapy.[383,384,391] Although some studies have suggested that elevated sputum eosinophil and/or neutrophil counts can act as an indicator for refractory asthma,[100,382,384,391,392] this study did not observe significant differences in sputum eosinophil count between the two groups. Although differences in lymphocyte counts were observed, their relationship with airway inflammation and/or asthma is not clear from previous studies and remains largely unknown. At face value, these findings suggest that there is no major difference in airway inflammation between the two groups. However, some studies have suggested that sputum eosinophil counts do not fully represent the airway inflammation and/or correlate with clinical symptoms, because they do not distinguish the mature inflammatory cells and the activities they are embarked upon.[383,384,386,392]

The present study did not find significant differences in genetic variations or haplotypes between the two groups. The results suggest that these SNPs did not have a major linkage relationship with treatment response to corticosteroids. However, given the small sample size and high wild-type SNP frequency, the probability of a type II error is difficult to discount. Due to the suboptimal participation rate (less than

half the required number of subjects were recruited), the power of this study was significantly reduced. The lack of difference in airway inflammation, measured by differential white cell count, should also be taken into consideration when interpreting the results. Other than genetic and pathophysiological factors, airway remodelling due to persistent asthma is also known to affect the treatment response to corticosteroids.[73,96,100] Airway remodelling is believed to be influenced by a number of factors, including extended period(s) of under-treatment and/or poor compliance with treatment, anytime during the onset of the disease.[73,100]

The results presented here have partly reflected the lack of understanding and difficulties in distinguishing refractory asthma from the milder form of steroid sensitive asthma. It is suggested that future studies investigating refractory asthma include a wide-range of measurements to determine airway inflammation, including the combination of sputum eosinophil count with other inflammatory markers,[383,386,390,393] such as exhaled nitric oxide and/or eosinophil cationic protein (ECP). Given the number of genetic variations and statistical analyses being performed, haplotype analysis is preferred as it reduces the probability of a type I error. Haplotype analysis is also found to provide more reliable information regarding genetic influence on disease status and clinical outcomes.[147,164,366] In addition, analyses that indicate the activities of AP-1 and/or related inflammation protein (eg. gene expression analysis, mRNA analysis or protein and transcription factors analysis) will also be informative in determining the real-time relationship between the genetic variations and the pathophysiology of airway inflammation.[367,368]

Unlike some previous studies, this study did not observe a relationship between peripheral eosinophil count and asthma/SIA, or a correlation with sputum eosinophil count. This observation is consistent with studies that have examined various airway inflammation markers.[386,389,394] Localised inflammation and corticosteroid use were believed to play an important role for the lack of relationship. Nonetheless, the results suggest that measurements from peripheral blood samples have minimal value in the prediction and/or evaluation of airway inflammation among patients with athma.

The experience of this study also suggests that single-base primer extension methods of SNP analysis, as described in Section 8.3.4.2, are not the most effective methods. During the analysis, many samples were required to be re-analysed, with single-plex or multiplex reactions, due to suboptimal and ambiguous results. Continuous adjustment of chemical premix(s) was also required due to interference with the analysed signals. Figures 8.2 (chromatograph (b) and (d)) demonstrate the chromatogram with suboptimal results (signal interference from 50-70 nt).

In conclusion, there were no linkage relationship observed between refractory asthma and the selected SNPs on AP-1 related genes. However, the study also observed no significant difference in airway inflammation determined by the mean differences in sputum eosinophil count. This study highlights the lack of understanding and difficulties in distinguishing refractory asthma from milder forms of asthma (steroid sensitive).

Chapter 9

Conclusion

This project was set up to investigate some pharmacogenetics aspects of asthma therapy discovered in recent times, with the intention of applying these findings into future clinical practice. The pharmacokinetic and pharmacodynamic pathways of a drug often involve numbers of important enzymes, receptors and various other proteins, where changes in these proteins (eg. genetic variation) has the potential to affect its function as well as the eventual clinical outcome.

In the studies presented here, we observed some large differences in salbutamol enantiomer levels among patients presenting with airway disease exacerbation (between 0.0-24.8 and 0.0-57.3 ng/mL for (R)- and (S)-salbutamol respectively), but failed to show a significant relationship with some of the potential cardiovascular adverse effects (in particular QTc interval). The study also observed stereoselective tissue distribution of salbutamol in animal models (K_m =5.69 and 1.69; K_h =4.07 and 0.48, for (R)- and (S) salbutamol respectively), which suggest that the measurement of salbutamol enantiomers in the circulation may not represent the tissue disposition and predict the extra-pulmonary side effects. Variations in the pharmacokinetics of salbutamol enantiomers (after single inhaled dose of *rac*-salbutamol) were not found to be the related to the genetic variations (SNP) identified on the SULT 1A3 enzyme. Given that some previous studies have raised concerns about the extra-pulmonary effects of β_2-agonists, future studies that investigate genetic variations that are relevant to salbutamol enantiomer tissue disposition and β_1-adrenoceptors are recommended

The pharmacogenetics of montelukast and corticosteroids were also examined, however, the genetic variations identified from a small selection of genes involved in

pharmacokinetic or pharmacodynamic pathways, were not found to have a significant relationship with the clinical outcome of either montelukast (ALOX5, LTC4S, LTA4H and cysLTR1) or corticosteroids (AP-1 related proteins: JNK1, JUN, FOS and CRHR1) in the management of asthma.

The complex pathway of pharmacokinetics and pharmacodynamics, as well as the diversity in both genetic and non-genetic factors within the general population is thought to have played a significant role in the study outcomes, where wide variations in both the pharmacokinetics and pharmacodynamics parameters were observed among the participants. These variations were often greater than anticipated (based on previously published data), and we believe that the strict subject selection criteria in some previous studies, may be an important factor.

However, small sample sizes remain the primary limitation of the work presented here, which have significantly reduced the power of these investigations. One of the real concerns is the much lower than anticipated participation rate among our targeted population, and we believe that was mostly contributed to by the general perceptions and lack of informed knowledge regarding genetic studies in the community. There is no reliable indication to confirm the barrier(s) of the potential subject's taking part in these studies. Therefore, other than engaging in a much larger targeted population or multiple populations, it is difficult to overcome similar problem in future studies.

The studies also found that the outcomes observed in many pharmacogenetic studies were often difficult to be replicated. This was mainly due to the different frequencies of genetic variations found between populations, as well as the measurements of

treatment outcome. From the experience of this project, we suggest future research to consider a much larger sample size (eg. double the calculated minimum sample size) in the attempt to overcome the isuue of lack in power, as constantly encountered in this project.

In recent years, there has been in explosion in the knowledge of genes and genetic variations identified at the major pathways of anti-asthma medications and many have been found to be related to the disease in isolated study populations.[58] The heterogeneous nature of genetic effects encountered during the study was a reflection of the complexity of pharmacogenetics and pharmacogenomics of anti-asthma medications. Given the large number of genetic targets and the variables in pharmacokinetics and pharmacodynamics of anti-asthma medications (demonstrated by the investigations of salbutamol enantiomers), the extent of pharmacogenetic and pharmacogenomic influence in anti-asthma medications will not be fully realised in the immediate future, and more extensive studies are required.

In summary, studies in this thesis have found no overwhelming pharmacogenetic influence, in common anti-asthma medications (salbutamol, montelukast and corticosteroids). We have not found any result that would lead to changes in current clinical practice of asthma management. Given that a large number of genetic variations may contribute to the eventual variation in clinical outcome, a much greater number of genetic variations and participants would be required in future pharmacogenetic studies involving the general population. In addition the difficulties of subject recruitment in a 'genetic' asthma study also need to be carefully considered despite the widespread nature of the disease.

References

1. Asthma (Fact Sheet No307) [homepage on the Internet]. Geneva: World Health Organization, [updated Aug 2006, cited 28 Aug 2006]. Available from: http://www.who.int/mediacentre/factsheets/fs307/en/print.html

2. Smyth RL. Asthma: a major pediatric health issue. *Respir Res* 2002;3 Suppl 1:S3-7.

3. Global strategy for asthma management and prevention 2007 [guideline on the internet]: Global Initiative for Asthma (GINA), WHO Initiative. [updated 2007, cited 9 Nov 2008]. Available from: www.ginasthma.org

4. Berend N, Madden R, editors. Asthma in Australia 2005. In: *Asthma series 2.* Canberra: Australian Centre for Asthma Monitoring, 2005. Available from: http://www.aihw.gov.au/publications/acm/aia05/aia05.pdf

5. Woolcock AJ, Bastiampillai SA, Marks GB, Keena VA. The burden of asthma in Australia. *Med J Aust* 2001;175(3):141-145.

6. Asthma Foundation of Australia, Asthma [updated: 18 Aug 2004, cited 9 Dec 2005] Available from: http://www.asthmaaustralia.org.au/asthma.html

7. Asthma management handbook 2006. sixth ed. South Melbourne, Australia: National Asthma Council Australia Ltd, 2006.

8. Tattersfield AE, Knox AJ, Britton JR, Hall IP. Asthma. *Lancet* 2002;360(9342):1313-1322.

9. Poulos LM, Toelle BG, Marks GB. The burden of asthma in children: an Australian perspective. *Paediatr Respir Rev* 2005;6(1):20-27.

10. Ampon RD, Williamson M, Correll PK, Marks GB. Impact of asthma on self-reported health status and quality of life: a population based study of Australians aged 18-64. *Thorax* 2005;60(9):735-739.

11. Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC). *Eur Respir J* 1998;12(2):315-335.

12. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 1998;351(9111):1225-1232.

13. Pearce N, Sunyer J, Cheng S, Chinn S, Bjorksten B, Burr M, et al. Comparison of asthma prevalence in the ISAAC and the ECRHS. ISAAC Steering Committee and the European Community Respiratory Health Survey. International Study of Asthma and Allergies in Childhood. *Eur Respir J* 2000;16(3):420-426.

14. Keller MB, Lowenstein SR. Epidemiology of asthma. *Semin. Respir. Crit. Care Med.* 2002;23(4):317-329.

15. Heinrich J, Richter K, Frye C, Meyer I, Wolke G, Wjst M, et al. [European Community Respiratory Health Survey in Adults (ECRHS)]. *Pneumologie* 2002;56(5):297-303.

16. Glasgow NJ, Goodchild EA, Yates R, Ponsonby AL. Respiratory health in Aboriginal and Torres Strait Islander children in the Australian Capital Territory. *J Paediatr Child Health* 2003;39(7):534-539.

17. Couzos S, Davis S. Inequities in Aboriginal health--access to the Asthma 3+ Visit Plan. *Aust Fam Physician* 2005;34(10):837-840.

18. Downs SH, Marks GB, Sporik R, Belosouva EG, Car NG, Peat JK. Continued increase in the prevalence of asthma and atopy. *Arch Dis Child* 2001;84(1):20-23.

19. Taylor R, Comino E, Bauman A. Asthma mortality in Australia 1920-94: Age, period, and cohort effects. *J Epidemiol Community Health* 1997;51(4):408-411.

20. Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. *Immunol. Rev.* 2006;212:238-255.

21. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 2008;372(9643):1107-1119.

22. Toelle BG, Ng K, Belousova E, Salome CM, Peat JK, Marks GB. Prevalence of asthma and allergy in schoolchildren in Belmont, Australia: three cross sectional surveys over 20 years. *Bmj* 2004;328(7436):386-387.

23. Marks GB, Correll PK, Williamson M. Asthma in Australia 2005. *Med J Aust* 2005;183(9):445-446.

24. Comino EJ. What explains falling asthma mortality? *Med J Aust* 2004;180(11):595.

25. Pearce N, Douwes J. The global epidemiology of asthma in children. *Int J Tuberc Lung Dis* 2006;10(2):125-132.

26. Maddox L, Schwartz DA. The pathophysiology of asthma. *Annu Rev Med* 2002;53:477-498.

27. Lemanske RF, Busse WW. Asthma: Factors underlying inception, exacerbation, and disease progression. *J Allergy Clin Immunol* 2006;117(2):S456-S461.

28. Schaub B, Lauener R, von Mutius E. The many faces of the hygiene hypothesis. *J Allergy Clin Immunol* 2006;117(5):969-977.

29. Romagnani S. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* 2004;112(3):352-363.

30. National Heart L, and Blood Institute, USA, National Asthma Education and Prevention Program U. Expert penal report 3: Guideline for the diagnosis and management of asthma (full report 2007): National Heart, Lung, and Blood Institute, 2007. Available from: http:www.nhlbi.nih.gov/guidelines/asthma/asthgdln.pdf

31. Del Prete G. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy* 1992;47(5):450-455.

32. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003;111(3):450-463.

33. Busse WW, Lemanske RF. Advances in immunology - Asthma. *N Engl J Med* 2001;344(5):350-362.

34. Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol* 2003;15(6):627-633.

35. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103(2):175-183.

36. Holt PG, Upham JW, Sly PD. Contemporaneous maturation of immunologic and respiratory functions during early childhood: implications for development of asthma prevention strategies. *J Allergy Clin Immunol* 2005;116(1):16-24.

37. Seroogy CM, Gern JE. The role of T regulatory cells in asthma. *J Allergy Clin Immunol* 2005;116(5):996-999.

38. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006;117(6):1277-1284.

39. Umetsu DT, Akbari O, Dekruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;112(3):480-487.

40. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 2001;409(6822):928-933.

41. Warrier MR, Hershey GK. Asthma genetics: personalizing medicine. *J Asthma* 2008;45(4):257-264.

42. Borish L, Culp JA. Asthma: a syndrome composed of heterogeneous diseases. *Ann Allergy Asthma Immunol* 2008;101(1):1-8.

43. Drake KA, Galanter JM, Burchard EG. Race, ethnicity and social class and the complex etiologies of asthma. *Pharmacogenomics* 2008;9(4):453-462.

44. Zhang J, Pare PD, Sandford AJ. Recent advances in asthma genetics. *Respir Res* 2008;9:4.

45. Vercelli D. Advances in asthma and allergy genetics in 2007. *J Allergy Clin Immunol* 2008;122(2):267-271.

46. Holgate ST, Yang Y, Haitchi HM, Powell RM, Holloway JW, Yoshisue H, et al. The genetics of asthma: ADAM33 as an example of a susceptibility gene. *Proc Am Thorac Soc* 2006;3(5):440-443.

47. Holgate ST, Davies DE, Powell RM, Howarth PH, Haitchi HM, Holloway JW. Local genetic and environmental factors in asthma disease pathogenesis: chronicity and persistence mechanisms. *Eur Respir J* 2007;29(4):793-803.

48. Scirica CV, Celedon JC. Genetics of asthma: potential implications for reducing asthma disparities. *Chest* 2007;132(5 Suppl):770S-781S.

49. Weiss ST, Litonjua AA, Lange C, Lazarus R, Liggett SB, Bleecker ER, et al. Overview of the pharmacogenetics of asthma treatment. *Pharmacogenomics J* 2006;6(5):311-326.

50. Blumenthal MN. The role of genetics in the development of asthma and atopy. *Curr Opin Allergy Clin Immunol* 2005;5(2):141-145.

51. Denham S, Koppelman GH, Blakey J, Wjst M, Ferreira MA, Hall IP, et al. Meta-analysis of genome-wide linkage studies of asthma and related traits. *Respir Res* 2008;9:38.

52. Yang IA, Fong KM, Zimmerman PV, Holgate ST, Holloway JW. Genetic susceptibility to the respiratory effects of air pollution. *Thorax* 2008;63(6):555-563.

53. Szefler SJ, Apter A. Advances in pediatric and adult asthma. *J Allergy Clin Immunol* 2005;115(3):470-477.

54. Choudhry S, Ung N, Avila PC, Ziv E, Nazario S, Casal J, et al. Pharmacogenetic differences in response to albuterol between Puerto Ricans and Mexicans with asthma. *Am J Respir Crit Care Med* 2005;171(6):563-570.

55. Additional Mechanisms of Asthma Pathophysiology Targeting in Search of New Treatment. 98th International conference of the American Thoracic Society; 2002 May 17 - 22; Atlanta, Georgia.

56. Woitsch B, Carr D, Stachel D, Schmid I, Weiland SK, Fritzsch C, et al. A comprehensive analysis of interleukin-4 receptor polymorphisms and their association with atopy and IgE regulation in childhood. *Int Arch Allergy Immunol* 2004;135(4):319-324.

57. Fields S. Proteomics. Proteomics in genomeland. *Science* 2001;291(5507):1221-1224.

58. Hall IP, Sayers I. Pharmacogenetics and asthma: false hope or new dawn? *Eur Respir J* 2007;29(6):1239-1245.

59. Borger P, Tamm M, Black JL, Roth M. Asthma: is it due to an abnormal airway smooth muscle cell? *Am J Respir Crit Care Med* 2006;174(4):367-372.

60. Panettieri Jr RA, Kotlikoff MI, Gerthoffer WT, Hershenson MB, Woodruff PG, Hall IP, et al. Airway Smooth Muscle in Bronchial Tone, Inflammation and Remodeling: Basic to Clinical Relevance. *Am J Respir Crit Care Med* 2007;177(3):248-252.

61. An SS, Bai TR, Bates JH, Black JL, Brown RH, Brusasco V, et al. Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. *Eur Respir J* 2007;29(5):834-860.

62. Jordan D. Central nervous pathways and control of the airways. *Respir Physiol* 2001;125(1-2):67-81.

63. Canning BJ. Reflex regulation of airway smooth muscle tone. *J Appl Physiol* 2006;101(3):971-985.

64. Halayko AJ, Tran T, Ji SY, Yamasaki A, Gosens R. Airway smooth muscle phenotype and function: interactions with current asthma therapies. *Curr Drug Targets* 2006;7(5):525-540.

65. Canning BJ, Fischer A. Neural regulation of airway smooth muscle tone. *Respir Physiol* 2001;125(1-2):113-127.

66. Richardson JB. Nerve supply to the lungs. *Am Rev Respir Dis* 1979;119(5):785-802.

67. Mazzone SB, Canning BJ. Evidence for differential reflex regulation of cholinergic and noncholinergic parasympathetic nerves innervating the airways. *Am J Respir Crit Care Med* 2002;165(8):1076-1083.

68. de Jongste JC, Jongejan RC, Kerrebijn KF. Control Of Airway Caliber By Autonomic Nerves In Asthma And In Chronic Obstructive Pulmonary-Disease. *Am Rev Respir Dis* 1991;143(6):1421-1426.

69. Fahy JV, Corry DB, Boushey HA. Airway inflammation and remodeling in asthma. *Curr Opin Pulm Med* 2000;6(1):15-20.

70. Widdicombe JG. Overview of neural pathways in allergy and asthma. *Pulm Pharmacol Ther* 2003;16(1):23-30.

71. Saetta M, Turato G. Airway pathology in asthma. *Eur Respir J Suppl* 2001;34:18s-23s.

72. Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy* 2008;38(6):872-897.

73. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161(5):1720-1745.

74. Halayko AJ, Amrani Y. Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma. *Respir Physiol Neurobiol* 2003;137(2-3):209-222.

75. Pascual RM, Peters SP. Airway remodeling contributes to the progressive loss of lung function in asthma: an overview. *J Allergy Clin Immunol* 2005;116(3):477-486.

76. Lazaar AL, Panettieri RA, Jr. Airway smooth muscle: a modulator of airway remodeling in asthma. *J Allergy Clin Immunol* 2005;116(3):488-495.

77. Hanania NA. Targeting airway inflammation in asthma: current and future therapies. *Chest* 2008;133(4):989-998.

78. Asthma in adults and schoolchildren. The General Practioner in Asthma Group, the British Association of Accident and Emergency Medicine, the British

Paediatric Respiratory Society and the Royal College of Paediatrics and Child Health. *Thorax* 1997;52 Suppl 1:S2-8, S11-15, S20-11.

79. British guideline on the management of asthma. *Thorax* 2003;58 Suppl 1:i1-94.

80. King CS, Moores LK. Clinical asthma syndromes and important asthma mimics. *Respir Care* 2008;53(5):568-580.

81. Myers TR. Guidelines for asthma management: a review and comparison of 5 current guidelines. *Respir Care* 2008;53(6):751-767.

82. Bousquet J, Clark TJ, Hurd S, Khaltaev N, Lenfant C, O'Byrne P, et al. GINA guidelines on asthma and beyond. *Allergy* 2007;62(2):102-112.

83. Silverman R. Treatment of acute asthma. A new look at the old and at the new. *Clin Chest Med* 2000;21(2):361-379.

84. Moore WC. Update in asthma 2007. *Am J Respir Crit Care Med* 2008;177(10):1068-1073.

85. Apter AJ, Szefler SJ. Advances in adult and pediatric asthma. *J Allergy Clin Immunol* 2006;117(3):512-518.

86. Szefler SJ. Facing the challenges of childhood asthma: what changes are necessary? *J Allergy Clin Immunol* 2005;115(4):685-688.

87. Skorodin MS. Pharmacotherapy For Asthma And Chronic Obstructive Pulmonary-Disease - Current Thinking, Practices, And Controversies. *Arch Intern Med* 1993;153(7):814-828.

88. Moore WC, Peters SP. Update in asthma 2006. *Am J Respir Crit Care Med* 2007;175(7):649-654.

89. Sears MR, Lotvall J. Past, present and future - beta(2)-adrenoceptor agonists in asthma management. *Respir Med* 2005;99(2):152-170.

90. Barnes PJ. Drugs for asthma. *Br J Pharmacol* 2006;147 Suppl 1:S297-303.

91. Donohue JF. Safety and efficacy of beta agonists. *Respir Care* 2008;53(5):618-622.

92. Cazzola M, Matera MG, Donner CF. Inhaled beta2-adrenoceptor agonists: cardiovascular safety in patients with obstructive lung disease. *Drugs* 2005;65(12):1595-1610.

93. Barnes PJ, Adcock IM. How do corticosteroids work in asthma? *Ann. Intern. Med.* 2003;139(5):359-370.

94. Barnes PJ, Greening AP, Crompton GK. Glucocorticoid Resistance In Asthma. *Am J Respir Crit Care Med* 1995;152(6):S125-S140.

95. Barnes PJ. Corticosteroids: The drugs to beat. *Eur J Pharmacol* 2006;533(1-3):2-14.

96. Barnes PJ, Pedersen S, Busse WW. Efficacy and safety of inhaled corticosteroids - New developments. *Am J Respir Crit Care Med* 1998;157(3):S1-S53.

97. Ito K, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol* 2006;117(3):522-543.

98. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids - New mechanisms for old drugs. *N Engl J Med* 2005;353(16):1711-1723.

99. Adcock IM. Molecular mechanisms of glucocorticosteroid actions. *Pulm Pharmacol Ther* 2000;13(3):115-126.

100. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000;162(6):2341-2351.

101. Barnes NC. The properties of inhaled corticosteroids: similarities and differences. *Prim Care Respir J* 2007;16(3):149-154.

102. Caramori G, Adcock I. Pharmacology of airway inflammation in asthma and COPD. *Pulm Pharmacol Ther* 2003;16(5):247-277.

103. Randhawa I, Klaustermeyer WB. Oral corticosteroid-dependent asthma: a 30-year review. *Ann Allergy Asthma Immunol* 2007;99(4):291-302.

104. Drazen JM, Israel E, O'Byrne PM. Treatment of asthma with drugs modifying the leukotriene pathway. *N Engl J Med* 1999;340(3):197-206.

105. Holgate ST, Bradding P, Sampson AP. Leukotriene antagonists and synthesis inhibitors: New directions in asthma therapy. *J Allergy Clin Immunol* 1996;98(1):1-13.

106. Lipworth BJ. Leukotriene-receptor antagonists. *Lancet* 1999;353(9146):57-62.

107. Jarvis B, Markham A. Montelukast - A review of its therapeutic potential in persistent asthma. *Drugs* 2000;59(4):891-928.

108. Dunn CJ, Goa KL. Zafirlukast - An update of its pharmacology and therapeutic efficacy in asthma. *Drugs* 2001;61(2):285-315.

109. Liu MC, Dube LM, Lancaster J. Acute and chronic effects of a 5-lipoxygenase inhibitor in asthma: A 6-month randomized multicenter trial *J Allergy Clin Immunol* 1996;98(5):859-871.

110. Sims EJ, Jackson CM, Lipworth BJ. Add-on therapy with montelukast or formoterol in patients with the glycine-16 beta(2)-receptor genotype. *Br J Clin Pharmacol* 2003;56(1):104-111.

111. Salvi SS, Krishna MT, Sampson AP, Holgate ST. The anti-inflammatory effects of leukotriene-modifying drugs and their use in asthma. *Chest* 2001;119(5):1533-1546.

112. Centanni S, Santus P. Antileukotrienes in clinical development for asthma. *Expert Opin Investig Drugs* 2002;11(1):49-58.

113. Smith LJ. Comparative efficacy of inhaled corticosteroids and antileukotriene drugs in asthma. *Biodrugs* 2001;15(4):239-249.

114. Rodrigo GJ, Rodrigo C. The role of anticholinergics in acute asthma treatment - An evidence-based evaluation. *Chest* 2002;121(6):1977-1987.

115. Aaron SD. The use of ipratropium bromide for the management of acute asthma exacerbation in adults and children: A systematic review. *J Asthma* 2001;38(7):521-530.

116. Beakes DE. The use of anticholinergics in asthma. *J Asthma* 1997;34(5):357-368.

117. Lin RY, Pesola GR, Bakalchuk L, Morgan JP, Heyl GT, Freyberg CW, et al. Superiority of ipratropium plus albuterol over albuterol alone in the emergency department management of adult asthma: A randomized clinical trial. *Ann Emerg Med* 1998;31(2):208-213.

118. Rodrigo G, Rodrigo C, Burschtin O. A meta-analysis of the effects of ipratropium bromide in adults with acute asthma. *Am J Med* 1999;107(4):363-370.

119. Nakano Y, Enomoto N, Kawamoto A, Hirai R, Chida K. Efficacy of adding multiple doses of oxitropium bromide to salbutamol delivered by means of a metered-dose inhaler with a spacer device in adults with acute severe asthma. *J Allergy Clin Immunol* 2000;106(3):472-478.

120. Rodrigo GJ, Castro-Rodriguez JA. Anticholinergics in the treatment of children and adults with acute asthma: a systematic review with meta-analysis. *Thorax* 2005;60(9):740-746.

121. Qureshi F, Pestian J, Davis P, Zaritsky A. Effect of nebulized ipratropium on the hospitalization rates of children with asthma. *N Engl J Med* 1998;339(15):1030-1035.

122. Vassallo R, Lipsky JJ. Theophylline: Recent advances in the understanding of its mode of action and uses in clinical practice. *Mayo Clin Proc* 1998;73(4):346-354.

123. Barnes PJ, Adcock IM, Ito K. Histone acetylation and deacetylation: importance in inflammatory lung diseases. *Eur Resp J* 2005;25(3):552-563.

124. Weinberger M, Hendeles L. Drug therapy - Theophylline in asthma. *N Engl J Med* 1996;334(21):1380-1388.

125. Vianna EO, Martin RJ. Bronchodilators and corticosteroids in the treatment of asthma. *Drugs Of Today* 1998;34(3):203-223.

126. Hansel TT, Tennant RC, Tan AJ, Higgins LA, Neighbour H, Erin EM, et al. Theophylline: Mechanism of action and use in asthma and chronic obstructive pulmonary disease. *Drugs Today* 2004;40(1):55-69.

127. Jacobson GA, Peterson GM. Prescribing trends for anti-asthma drugs in Tasmania following the National Asthma Campaign. *J Clin Pharm Ther* 1996;21(5):317-324.

128. Plosker GL, Keam SJ. Omalizumab: a review of its use in the treatment of allergic asthma. *Biodrugs* 2008;22(3):189-204.

129. Adcock IM, Caramori G, Chung KF. New targets for drug development in asthma. *Lancet* 2008;372(9643):1073-1087.

130. Boulet LP, Drollmann A, Magyar P, Timar M, Knight A, Engelstatter R, et al. Comparative efficacy of once-daily ciclesonide and budesonide in the treatment of persistent asthma. *Respir Med* 2006;100(5):785-794.

131. Manning P, Gibson PG, Lasserson TJ. Ciclesonide versus other inhaled steroids for chronic asthma in children and adults. *Cochrane Database Syst Rev* 2008(2):CD007031.

132. Adachi M, Ishihara K, Inoue H, Kudo K, Takahashi K, Morita Y, et al. Efficacy and safety of once-daily inhaled ciclesonide in adults with mild to moderate asthma: a double-blind, placebo-controlled study. *Respirology* 2007;12(4):566-572.

133. Erin EM, Zacharasiewicz AS, Nicholson GC, Tan AJ, Neighbour H, Engelstatter R, et al. Rapid effect of inhaled ciclesonide in asthma: a randomized, placebo-controlled study. *Chest* 2008;134(4):740-745.

134. Spina D. Theophylline and PDE4 inhibitors in asthma. *Curr Opin Pulm Med* 2003;9(1):57-64.

135. Spina D. Phosphodiesterase-4 inhibitors in the treatment of inflammatory lung disease. *Drugs* 2003;63(23):2575-2594.

136. Shaheen SO, Sterne JA, Thompson RL, Songhurst CE, Margetts BM, Burney PG. Dietary antioxidants and asthma in adults: population-based case-control study. *Am J Respir Crit Care Med* 2001;164(10 Pt 1):1823-1828.

137. Gibson PG, Powell H, Coughlan J, Wilson AJ, Abramson M, Haywood P, et al. Self-management education and regular practitioner review for adults with asthma. *Cochrane Database Syst Rev* 2003(1):CD001117.

138. McCann DC, McWhirter J, Coleman H, Calvert M, Warner JO. A controlled trial of a school-based intervention to improve asthma management. *Eur Respir J* 2006;27(5):921-928.

139. Slader CA, Reddel HK, Spencer LM, Belousova EG, Armour CL, Bosnic-Anticevich SZ, et al. Double blind randomised controlled trial of two different

breathing techniques in the management of asthma. *Thorax* 2006;61(8):651-656.

140. Singh V, Wisniewski A, Britton J, Tattersfield A. Effect of yoga breathing exercises (pranayama) on airway reactivity in subjects with asthma. *Lancet* 1990;335(8702):1381-1383.

141. Warner JO. Non pharmacological management of asthma: little change in the evidence base over 5 yr. *Pediatr Allergy Immunol* 2007;18(2):91.

142. Nagarathna R, Nagendra HR. Yoga for bronchial asthma: a controlled study. *Br Med J (Clin Res Ed)* 1985;291(6502):1077-1079.

143. Israel E. Genetics and the variability of treatment response in asthma. *J Allergy Clin Immunol* 2005;115(4):S532-S538.

144. Palmer LJ, Silverman ES, Weiss ST, Drazen JM. Pharmacogenetics of asthma. *Am J Respir Crit Care Med* 2002;165(7):861-866.

145. Evans WE, McLeod HL. Pharmacogenomics--drug disposition, drug targets, and side effects. *N Engl J Med* 2003;348(6):538-549.

146. Eichelbaum M, Ingelman-Sundberg M, Evans WE. Pharmacogenomics and individualized drug therapy. *Annu Rev Med* 2006;57:119-137.

147. Weinshilboum RM, Wang L. Pharmacogenetics and pharmacogenomics: development, science, and translation. *Annu Rev Genomics Hum Genet* 2006;7:223-245.

148. Evans WE, Relling MV. Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* 1999;286(5439):487-491.

149. Weinshilboum R. Inheritance and drug response. *N Engl J Med* 2003;348(6):529-537.

150. Kidd RS, Curry TB, Gallagher S, Edeki T, Blaisdell J, Goldstein JA. Identification of a null allele of CYP2C9 in an African-American exhibiting toxicity to phenytoin. *Pharmacogenetics* 2001;11(9):803-808.

151. Soga Y, Nishimura F, Ohtsuka Y, Araki H, Iwamoto Y, Naruishi H, et al. CYP2C polymorphisms, phenytoin metabolism and gingival overgrowth in epileptic subjects. *Life Sci* 2004;74(7):827-834.

152. Charlier C, Broly F, Lhermitte M, Pinto E, Ansseau M, Plomteux G. Polymorphisms in the CYP 2D6 gene: association with plasma concentrations of fluoxetine and paroxetine. *Ther Drug Monit* 2003;25(6):738-742.

153. Dalen P, Dahl ML, Bernal Ruiz ML, Nordin J, Bertilsson L. 10-Hydroxylation of nortriptyline in white persons with 0, 1, 2, 3, and 13 functional CYP2D6 genes. *Clin Pharmacol Ther* 1998;63(4):444-452.

154. Furuta T, Shirai N, Xiao F, El-Omar EM, Rabkin CS, Sugimura H, et al. Polymorphism of interleukin-1beta affects the eradication rates of Helicobacter pylori by triple therapy. *Clin Gastroenterol Hepatol* 2004;2(1):22-30.

155. Schwab M, Schaeffeler E, Klotz U, Treiber G. CYP2C19 polymorphism is a major predictor of treatment failure in white patients by use of lansoprazole-based quadruple therapy for eradication of Helicobacter pylori. *Clin Pharmacol Ther* 2004;76(3):201-209.

156. Brinkmann U, Roots I, Eichelbaum M. Pharmacogenetics of the human drug-transporter gene MDR1: impact of polymorphisms on pharmacotherapy. *Drug Discov Today* 2001;6(16):835-839.

157. Choo EF, Leake B, Wandel C, Imamura H, Wood AJ, Wilkinson GR, et al. Pharmacological inhibition of P-glycoprotein transport enhances the

distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metab Dispos* 2000;28(6):655-660.

158. Lombardi VR, Fernandez-Novoa L, Etcheverria I, Seoane S, Cacabelos R. Association between APOE epsilon4 allele and increased expression of CD95 on T cells from patients with Alzheimer's disease. *Methods Find Exp Clin Pharmacol* 2004;26(7):523-529.

159. Poirier J, Delisle MC, Quirion R, Aubert I, Farlow M, Lahiri D, et al. Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. *Proc Natl Acad Sci U S A* 1995;92(26):12260-12264.

160. Mukae S, Aoki S, Itoh S, Iwata T, Ueda H, Katagiri T. Bradykinin B(2) receptor gene polymorphism is associated with angiotensin-converting enzyme inhibitor-related cough. *Hypertension* 2000;36(1):127-131.

161. Mukae S, Itoh S, Aoki S, Iwata T, Nishio K, Sato R, et al. Association of polymorphisms of the renin-angiotensin system and bradykinin B2 receptor with ACE-inhibitor-related cough. *J Hum Hypertens* 2002;16(12):857-863.

162. Israel E, Chinchilli VM, Ford JG, Boushey HA, Cherniack R, Craig TJ, et al. Use of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. *Lancet* 2004;364(9444):1505-1512.

163. Roden DM, Altman RB, Benowitz NL, Flockhart DA, Giacomini KM, Johnson JA, et al. Pharmacogenomics: challenges and opportunities. *Ann Intern Med* 2006;145(10):749-757.

164. Giacomini KM, Brett CM, Altman RB, Benowitz NL, Dolan ME, Flockhart DA, et al. The pharmacogenetics research network: from SNP discovery to clinical drug response. *Clin Pharmacol Ther* 2007;81(3):328-345.

165. O'Shaughnessy KM. HapMap, pharmacogenomics, and the goal of personalized prescribing. *Br J Clin Pharmacol* 2006;61(6):783-786.

166. Rettie AE, Wienkers LC, Gonzalez FJ, Trager WF, Korzekwa KR. Impaired (S)-warfarin metabolism catalysed by the R144C allelic variant of CYP2C9. *Pharmacogenetics* 1994;4(1):39-42.

167. Takahashi H, Kashima T, Nomoto S, Iwade K, Tainaka H, Shimizu T, et al. Comparisons between in-vitro and in-vivo metabolism of (S)-warfarin: catalytic activities of cDNA-expressed CYP2C9, its Leu359 variant and their mixture versus unbound clearance in patients with the corresponding CYP2C9 genotypes. *Pharmacogenetics* 1998;8(5):365-373.

168. Bodin L, Horellou MH, Flaujac C, Loriot MA, Samama MM. A vitamin K epoxide reductase complex subunit-1 (VKORC1) mutation in a patient with vitamin K antagonist resistance. *J Thromb Haemost* 2005;3(7):1533-1535.

169. Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, et al. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* 2005;352(22):2285-2293.

170. Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, et al. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 2004;427(6974):537-541.

171. Green SA, Turki J, Bejarano P, Hall IP, Liggett SB. Influence of Beta(2)-Adrenergic Receptor Genotypes on Signal-Transduction in Human Airway Smooth-Muscle Cells. *Am J Respir Cell Mol Biol* 1995;13(1):25-33.

172. Moore PE, Laporte JD, Abraham JH, Schwartzman IN, Yandava CN, Silverman ES, et al. Polymorphism of the beta(2)-adrenergic receptor gene and desensitization in human airway smooth muscle. *Am J Respir Crit Care Med* 2000;162(6):2117-2124.

173. Drazen JM, Yandava CN, Dube L, Szczerback N, Hippensteel R, Pillari A, et al. Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nat Genet* 1999;22(2):168-170.

174. Hines RN, McCarver DG. Pharmacogenomics and the future of drug therapy. *Pediatr Clin N Am* 2006;53(4):591-619.

175. Mastalerz L, Nizankowska E, Sanak M, Mejza F, Pierzchalska M, Bazan-Socha S, et al. Clinical and genetic features underlying the response of patients with bronchial asthma to treatment with a leukotriene receptor antagonist. *Eur J Clin Invest* 2002;32(12):949-955.

176. Choi JH, Park HS, Oh HB, Lee JH, Suh YJ, Park CS, et al. Leukotriene-related gene polymorphisms in ASA-intolerant asthma: an association with a haplotype of 5-lipoxygenase. *Hum Genet* 2004;114(4):337-344.

177. Litonjua AA, Lasky-Su J, Schneiter K, Tantisira KG, Lazarus R, Klanderman B, et al. ARG1 is a novel bronchodilator response gene: screening and replication in four asthma cohorts. *Am J Respir Crit Care Med* 2008;178(7):688-694.

178. Lima JJ, Zhang S, Grant A, Shao L, Tantisira KG, Allayee H, et al. Influence of leukotriene pathway polymorphisms on response to montelukast in asthma. *Am J Respir Crit Care Med* 2006;173(4):379-385.

179. Israel E, Drazen JM, Liggett SB, Boushey HA, Cherniack RM, Chinchilli VM, et al. The effect of polymorphisms of the beta(2)-adrenergic receptor on the

response to regular use of albuterol in asthma. *Am J Respir Crit Care Med* 2000;162(1):75-80.

180. Charmandari E, Kino T, Ichijo T, Zachman K, Alatsatianos A, Chrousos GP. Functional characterization of the natural human glucocorticoid receptor (hGR) mutants hGR alpha R477H and hGR alpha G679S associated with generalized glucocorticoid resistance. *J Clin Endocrinol Metab* 2006;91(4):1535-1543.

181. Giuliani R, Durbecq V, Di Leo A, Paesmans M, Larsimont D, Leroy JY, et al. Phosphorylated HER-2 tyrosine kinase and Her-2/neu gene amplification as predictive factors of response to trastuzumab in patients with HER-2 overexpressing metastatic breast cancer (MBC). *Eur J Cancer* 2007;43(4):725-735.

182. Reihsaus E, Innis M, Macintyre N, Liggett SB. Mutations in the Gene Encoding for the Beta-2-Adrenergic Receptor in Normal and Asthmatic Subjects. *Am J Respir Cell Mol Biol* 1993;8(3):334-339.

183. Liggett SB. Polymorphisms of the beta(2)-adrenergic receptor and asthma. *Am J Respir Crit Care Med* 1997;156(4):S156-S162.

184. Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, et al. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interactions. *Chem Res Toxicol* 1992;5(1):54-59.

185. Bertilsson L, Lou YQ, Du YL, Liu Y, Kuang TY, Liao XM, et al. Pronounced differences between native Chinese and Swedish populations in the polymorphic hydroxylations of debrisoquin and S-mephenytoin. *Clin Pharmacol Ther* 1992;51(4):388-397.

186. Johansson I, Lundqvist E, Bertilsson L, Dahl ML, Sjoqvist F, Ingelman-Sundberg M. Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. *Proc Natl Acad Sci U S A* 1993;90(24):11825-11829.

187. Pharmacogenomics and its role in drug safety (FDA Drug Safety Newslatter). [Internet] US. Food and Drug administration, US. department of Health & Human Services [updated 12 Jun 2008, cited 6 Sep 2009]. Available from: http://www.fda.gov/Drugs/DrugSafety/DrugSafetyNewsletter/ucm119991.htm

188. Viani GA, Afonso SL, Stefano EJ, De Fendi LI, Soares FV. Adjuvant trastuzumab in the treatment of her-2-positive early breast cancer: a meta-analysis of published randomized trials. *BMC Cancer* 2007;7:153.

189. Drysdale CM, McGraw DW, Stack CB, Stephens JC, Judson RS, Nandabalan K, et al. Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. *Proc Natl Acad Sci U S A* 2000;97(19):10483-10488.

190. Dewar JC, Hall IP. Personalised prescribing for asthma--is pharmacogenetics the answer? *J Pharm Pharmacol* 2003;55(3):279-289.

191. Glassroth J. The role of long-acting beta-agonists in the management of asthma: analysis, meta-analysis, and more analysis. *Ann Intern Med* 2006;144(12):936-937.

192. Small KM, McGraw DW, Liggett SB. Pharmacology and physiology of human adrenergic receptor polymorphisms. *Annu Rev Pharmacol Toxicol* 2003;43:381-411.

193. Brodde OE, Leineweber K. beta(2)-adrenoceptor gene polymorphisms. *Pharmacogenet Genomics* 2005;15(5):267-275.

194. Kelly HW. What is new with the beta(2)-agonists: Issues in the management of asthma. *Ann Pharmacother* 2005;39(5):931-938.

195. Chong LK, Chowdry J, Ghahramani P, Peachell PT. Influence of genetic polymorphisms in the beta2-adrenoceptor on desensitization in human lung mast cells. *Pharmacogenetics* 2000;10(2):153-162.

196. Taylor DR, Drazen JM, Herbison GP, Yandava CN, Hancox RJ, Town GI. Asthma exacerbations during long term beta agonist use: influence of beta(2) adrenoceptor polymorphism. *Thorax* 2000;55(9):762-767.

197. Martinez FD, Graves PE, Baldini M, Solomon S, Erickson R. Association between genetic polymorphisms of the beta2-adrenoceptor and response to albuterol in children with and without a history of wheezing. *J Clin Invest* 1997;100(12):3184-3188.

198. Liggett SB. The pharmacogenetics of beta2-adrenergic receptors: relevance to asthma. *J Allergy Clin Immunol* 2000;105(2 Pt 2):S487-492.

199. Martinez FD. Safety of long-acting beta agonists - An urgent need to clear the air. *N Engl J Med* 2005;353(25):2637-2639.

200. Tattersfield AE, Harrison TW. beta-adrenoceptor polymorphisms - Focus moves to long-acting beta-agonists. *Am J Respir Crit Care Med* 2006;173(5):473-474.

201. Wechsler ME, Lehman E, Lazarus SC, Lemanske RF, Jr., Boushey HA, Deykin A, et al. beta-Adrenergic receptor polymorphisms and response to salmeterol. *Am J Respir Crit Care Med* 2006;173(5):519-526.

202. Bleecker ER, Postma DS, Lawrance RM, Meyers DA, Ambrose HJ, Goldman M. Effect of ADRB2 polymorphisms on response to longacting beta2-agonist

therapy: a pharmacogenetic analysis of two randomised studies. *Lancet* 2007;370(9605):2118-2125.

203. Bleecker ER, Yancey SW, Baitinger LA, Edwards LD, Klotsman M, Anderson WH, et al. Salmeterol response is not affected by beta2-adrenergic receptor genotype in subjects with persistent asthma. *J Allergy Clin Immunol* 2006;118(4):809-816.

204. Panebra A, Schwarb MR, Swift SM, Weiss ST, Bleecker ER, Hawkins GA, et al. Variable-length poly-C tract polymorphisms of the beta2-adrenergic receptor 3'-UTR alter expression and agonist regulation. *Am J Physiol Lung Cell Mol Physiol* 2008;294(2):L190-195.

205. Sampson AP, Siddiqui S, Buchanan D, Howarth PH, Holgate ST, Holloway JW, et al. Variant LTC(4) synthase allele modifies cysteinyl leukotriene synthesis in eosinophils and predicts clinical response to zafirlukast. *Thorax* 2000;55 Suppl 2:S28-31.

206. In KH, Asano K, Beier D, Grobholz J, Finn PW, Silverman EK, et al. Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription. *J Clin Invest* 1997;99(5):1130-1137.

207. Currie GP, Lima JJ, Sylvester JE, Lee DK, Cockburn WJ, Lipworth BJ. Leukotriene C4 synthase polymorphisms and responsiveness to leukotriene antagonists in asthma. *Br J Clin Pharmacol* 2003;56(4):422-426.

208. Pelaia G, Vatrella A, Gallelli L, Cazzola M, Maselli R, Marsico SA. Potential genetic influences on the response to asthma treatment. *Pulm Pharmacol Ther* 2004;17(5):253-261.

209. Leung DYM, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, et al. Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med* 1997;186(9):1567-1574.

210. Gagliardo R, Chanez P, Vignola AM, Bousquet J, Vachier I, Godard P, et al. Glucocorticoid receptor alpha and beta in glucocorticoid dependent asthma. *Am J Respir Crit Care Med* 2000;162(1):7-13.

211. Tantisira KG, Lake S, Silverman ES, Palmer LJ, Lazarus R, Silverman EK, et al. Corticosteroid pharmacogenetics: association of sequence variants in CRHR1 with improved lung function in asthmatics treated with inhaled corticosteroids. *Hum Mol Genet* 2004;13(13):1353-1359.

212. Poon AH, Tantisira KG, Litonjua AA, Lazarus R, Xu J, Lasky-Su J, et al. Association of corticotropin-releasing hormone receptor-2 genetic variants with acute bronchodilator response in asthma. *Pharmacogenet Genomics* 2008;18(5):373-382.

213. Obase Y, Shimoda T, Kawano T, Saeki S, Tomari SY, Mitsuta-Izaki K, et al. Polymorphisms in the CYP1A2 gene and theophylline metabolism in patients with asthma. *Clin Pharmacol Ther* 2003;73(5):468-474.

214. Hakonarson H, Bjornsdottir US, Halapi E, Bradfield J, Zink F, Mouy M, et al. Profiling of genes expressed in peripheral blood mononuclear cells predicts glucocorticoid sensitivity in asthma patients. *Proc Natl Acad Sci U S A* 2005;102(41):14789-14794.

215. Tantisira KG, Small KM, Litonjua AA, Weiss ST, Liggett SB. Molecular properties and pharmacogenetics of a polymorphism of adenylyl cyclase type 9 in asthma: interaction between beta-agonist and corticosteroid pathways. *Hum Mol Genet* 2005;14(12):1671-1677.

216. Shastry BS. Pharmacogenetics and the concept of individualized medicine. *Pharmacogenomics J* 2006;6(1):16-21.

217. Ross JS, Schenkein DP, Pietrusko R, Rolfe M, Linette GP, Stec J, et al. Targeted therapies for cancer 2004. *Am J Clin Pathol* 2004;122(4):598-609.

218. Table of Valid Genomic Biomarkers in the Context of Approved Drug Labels [Internet] US. Food and Drug administration, US. department of Health & Human Services. [updated 18 Aug 2009, cited 6 Sap 2009]. Available from: http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm

219. Lugogo NL, Ginsburg GS, Que LG. Genetic profiling and tailored therapy in asthma: are we there yet? *Curr Opin Mol Ther* 2007;9(6):528-537.

220. Pharmacogenomics [homepage on the Internet]: Human Genome Project Information. [updated 19 Sep 2008, cited 5 Oct 2008]. Available from: http://www.ornl.gov/sci/techresources/Human_Genome/medicine/pharma.shtml

221. Sestini P, Renzoni E, Robinson S, Poole P, Ram FS. Short-acting beta 2 agonists for stable chronic obstructive pulmonary disease. *Cochrane Database Syst Rev* 2002(4):CD001495.

222. Whale CI, Sovani MP, Mortimer K, Oborne J, Cooper S, Harrison TW, et al. Effects of rac-albuterol on arterial blood gases in patients with stable hypercapnic chronic obstructive pulmonary disease. *Br J Clin Pharmacol* 2006;62(2):153-157.

223. Manning HL. Bronchodilator therapy in chronic obstructive pulmonary disease. *Curr Opin Pulm Med* 2000;6(2):99-103.

224. Emerman CL, Cydulka RK. Effect of different albuterol dosing regimens in the treatment of acute exacerbation of chronic obstructive pulmonary disease. *Ann Emerg Med* 1997;29(4):474-478.

225. Nair S, Thomas E, Pearson SB, Henry MT. A randomized controlled trial to assess the optimal dose and effect of nebulized albuterol in acute exacerbations of COPD. *Chest* 2005;128(1):48-54.

226. Hancox RJ, Aldridge RE, Cowan JO, Flannery EM, Herbison GP, McLachlan CR, et al. Tolerance to beta-agonists during acute bronchoconstriction. *Eur Respir J* 1999;14(2):283-287.

227. Walters EH, Walters J. Inhaled short acting beta2-agonist use in asthma: regular vs as needed treatment. *Cochrane Database Syst Rev* 2000(4):CD001285.

228. Bakale RP, Wald SA, Butler HT, Gao Y, Hong Y, Nie X, et al. Albuterol. A pharmaceutical chemistry review of R-, S-, and RS-albuterol. *Clin Rev Allergy Immunol* 1996;14(1):7-35.

229. Barnes PJ. Treatment with (R)-albuterol has no advantage over racemic albuterol. *Am J Respir Crit Care Med* 2006;174(9):969-972.

230. Schmekel B, Rydberg I, Norlander B, Sjosward KN, Ahlner J, Andersson RG. Stereoselective pharmacokinetics of S-salbutamol after administration of the racemate in healthy volunteers. *Eur Respir J* 1999;13(6):1230-1235.

231. Boulton DW, Fawcett JP. The pharmacokinetics of levosalbutamol: what are the clinical implications? *Clin Pharmacokinet* 2001;40(1):23-40.

232. Ward JK, Dow J, Dallow N, Eynott P, Milleri S, Ventresca GP. Enantiomeric disposition of inhaled, intravenous and oral racemic-salbutamol in man--no evidence of enantioselective lung metabolism. *Br J Clin Pharmacol* 2000;49(1):15-22.

233. Lipworth BJ, Clark DJ, Koch P, Arbeeny C. Pharmacokinetics and extrapulmonary beta 2 adrenoceptor activity of nebulised racemic salbutamol and its R and S isomers in healthy volunteers. *Thorax* 1997;52(10):849-852.

234. Agrawal DK, Ariyarathna K, Kelbe PW. (S)-Albuterol activates pro-constrictory and pro-inflammatory pathways in human bronchial smooth muscle cells. *J Allergy Clin Immunol* 2004;113(3):503-510.

235. Ameredes BT, Calhoun WJ. Modulation of GM-CSF release by enantiomers of beta-agonists in human airway smooth muscle. *J Allergy Clin Immunol* 2005;116(1):65-72.

236. Hoshiko K, Morley J. Exacerbation of airway hyperreactivity by (+/-)salbutamol in sensitized guinea pig. *Jpn J Pharmacol* 1993;63(2):159-163.

237. Johansson F, Rydberg I, Aberg G, Andersson RG. Effects of albuterol enantiomers on in vitro bronchial reactivity. *Clin Rev Allergy Immunol* 1996;14(1):57-64.

238. Boulton DW, Fawcett JP. Pharmacokinetics and pharmacodynamics of single oral doses of albuterol and its enantiomers in humans. *Clin Pharmacol Ther* 1997;62(2):138-144.

239. Nelson HS, Bensch G, Pleskow WW, DiSantostefano R, DeGraw S, Reasner DS, et al. Improved bronchodilation with levalbuterol compared with racemic albuterol in patients with asthma. *J Allergy Clin Immunol* 1998;102(6 Pt 1):943-952.

240. Pleskow WW, Nelson HS, Schaefer K, Claus R, Roach JM. Pairwise comparison of levalbuterol versus racemic albuterol in the treatment of moderate-to-severe asthma. *Allergy Asthma Proc* 2004;25(6):429-436.

241. Carl JC, Myers TR, Kirchner HL, Kercsmar CM. Comparison of racemic albuterol and levalbuterol for treatment of acute asthma. *J Pediatr* 2003;143(6):731-736.

242. Truitt T, Witko J, Halpern M. Levalbuterol compared to racemic albuterol: efficacy and outcomes in patients hospitalized with COPD or asthma. *Chest* 2003;123(1):128-135.

243. O'Riordan TG, Mao W, Palmer LB, Chen JJ. Assessing the effects of racemic and single-enantiomer albuterol on airway secretions in long-term intubated patients. *Chest* 2006;129(1):124-132.

244. Westerhof FJ, Zuidhof AB, Kok L, Meurs H, Zaagsma J. Effects of salbutamol and enantiomers on allergen-induced asthmatic reactions and airway hyperreactivity. *Eur Respir J* 2005;25(5):864-872.

245. Ahrens R, Weinberger M. Levalbuterol and racemic albuterol: are there therapeutic differences? *J Allergy Clin Immunol* 2001;108(5):681-684.

246. Gumbhir-Shah K, Kellerman DJ, DeGraw S, Koch P, Jusko WJ. Pharmacokinetics and pharmacodynamics of cumulative single doses of inhaled salbutamol enantiomers in asthmatic subjects. *Pulm Pharmacol Ther* 1999;12(6):353-362.

247. Lotvall J, Palmqvist M, Arvidsson P, Maloney A, Ventresca GP, Ward J. The therapeutic ratio of R-albuterol is comparable with that of RS-albuterol in asthmatic patients. *J Allergy Clin Immunol* 2001;108(5):726-731.

248. Jantikar A, Brashier B, Maganji M, Raghupathy A, Mahadik P, Gokhale P, et al. Comparison of bronchodilator responses of levosalbutamol and salbutamol given via a pressurized metered dose inhaler: a randomized, double blind, single-dose, crossover study. *Respir Med* 2007;101(4):845-849.

249. Abramson MJ, Bailey MJ, Couper FJ, Driver JS, Drummer OH, Forbes AB, et al. Are asthma medications and management related to deaths from asthma? *Am J Respir Crit Care Med* 2001;163(1):12-18.

250. Handley DA, Tinkelman D, Noonan M, Rollins TE, Snider ME, Caron J. Dose-response evaluation of levalbuterol versus racemic albuterol in patients with asthma. *J Asthma* 2000;37(4):319-327.

251. Salpeter SR, Ormiston TM, Salpeter EE. Cardiovascular effects of beta-agonists in patients with asthma and COPD: a meta-analysis. *Chest* 2004;125(6):2309-2321.

252. Kallergis EM, Manios EG, Kanoupakis EM, Schiza SE, Mavrakis HE, Klapsinos NK, et al. Acute electrophysiologic effects of inhaled salbutamol in humans. *Chest* 2005;127(6):2057-2063.

253. Taylor DR. Pharmacogenetics of beta2-agonist drugs in asthma. *Clin Rev Allergy Immunol* 2006;31(2-3):247-258.

254. Hildebrandt MA, Salavaggione OE, Martin YN, Flynn HC, Jalal S, Wieben ED, et al. Human SULT1A3 pharmacogenetics: gene duplication and functional genomic studies. *Biochem Biophys Res Commun* 2004;321(4):870-878.

255. Thomae BA, Rifki OF, Theobald MA, Eckloff BW, Wieben ED, Weinshilboum RM. Human catecholamine sulfotransferase (SULT1A3) pharmacogenetics: functional genetic polymorphism. *J Neurochem* 2003;87(4):809-819.

256. Taylor MR. Pharmacogenetics of the human beta-adrenergic receptors. *Pharmacogenomics J* 2007;7(1):29-37.

257. Taylor MR, Bristow MR. The emerging pharmacogenomics of the beta-adrenergic receptors. *Congest Heart Fail* 2004;10(6):281-288.

258. Jacobson GA, Chong FV, Wood-Baker R. (R,S)-salbutamol plasma concentrations in severe asthma. *J Clin Pharm Ther* 2003;28(3):235-238.

259. Bell HM, McElnay JC, Hughes CM. Changing profile in the use of anti-asthma drugs. *Pharmacoepidemiol Drug Saf* 1999;8(6):385-394.

260. Rodrigo G, Rodrigo C. Early prediction of poor response in acute asthma patients in the emergency department. *Chest* 1998;114(4):1016-1021.

261. Global Strategy for Diagnosis, Management, and Prevention of COPD (updated 2008) [guideline on the internet]: Global initiative for chronic obstructive lung disease.Available from: http://www.goldcopd.com/Guidelineitem.asp?l1=2&l2=1&intId=2003.

262. Jacobson GA, Chong FV, Davies NW. LC-MS method for the determination of albuterol enantiomers in human plasma using manual solid-phase extraction and a non-deuterated internal standard. *J Pharm Biomed Anal* 2003;31(6):1237-1243.

263. Stein LM, Cole RP. Early administration of corticosteroids in emergency room treatment of acute asthma. *Ann Intern Med* 1990;112(11):822-827.

264. Mallmann F, Fernandes AK, Avila EM, Nogueira FL, Steinhorst AM, Saucedo DZ, et al. Early prediction of poor outcome in patients with acute asthma in the emergency room. *Braz J Med Biol Res* 2002;35(1):39-47.

265. Rosenkranz B, Rouzier R, Kruse M, Dobson C, Ayre G, Horowitz A, et al. Safety and tolerability of high-dose formoterol (via Aerolizer) and salbutamol in patients with chronic obstructive pulmonary disease. *Respir Med* 2006;100(4):666-672.

266. Hussein R, Charles BG, Morris RG, Rasiah RL. Population pharmacokinetics of perhexiline from very sparse, routine monitoring data. *Ther Drug Monit* 2001;23(6):636-643.

267. Devadason SG. Recent advances in aerosol therapy for children with asthma. *J Aerosol Med* 2006;19(1):61-66.

268. Melani AS. Inhalatory therapy training: a priority challenge for the physician. *Acta Biomed* 2007;78(3):233-245.

269. Virchow JC. Guidelines versus clinical practice--which therapy and which device? *Respir Med* 2004;98 Suppl B:S28-34.

270. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* 2007;24(7):1227-1251.

271. Takane H, Shikata E, Otsubo K, Higuchi S, Ieiri I. Polymorphism in human organic cation transporters and metformin action. *Pharmacogenomics* 2008;9(4):415-422.

272. Zair ZM, Eloranta JJ, Stieger B, Kullak-Ublick GA. Pharmacogenetics of OATP (SLC21/SLCO), OAT and OCT (SLC22) and PEPT (SLC15) transporters in the intestine, liver and kidney. *Pharmacogenomics* 2008;9(5):597-624.

273. Kindermann W. Do inhaled beta(2)-agonists have an ergogenic potential in non-asthmatic competitive athletes? *Sports Med* 2007;37(2):95-102.

274. van Baak MA, Mayer LH, Kempinski RE, Hartgens F. Effect of salbutamol on muscle strength and endurance performance in nonasthmatic men. *Med Sci Sports Exerc* 2000;32(7):1300-1306.

275. Fowler EG, Graves MC, Wetzel GT, Spencer MJ. Pilot trial of albuterol in Duchenne and Becker muscular dystrophy. *Neurology* 2004;62(6):1006-1008.

276. Kinali M, Mercuri E, Main M, De Biasia F, Karatza A, Higgins R, et al. Pilot trial of albuterol in spinal muscular atrophy. *Neurology* 2002;59(4):609-610.

277. Kissel JT, McDermott MP, Mendell JR, King WM, Pandya S, Griggs RC, et al. Randomized, double-blind, placebo-controlled trial of albuterol in facioscapulohumeral dystrophy. *Neurology* 2001;57(8):1434-1440.

278. Messina S, Hartley L, Main M, Kinali M, Jungbluth H, Muntoni F, et al. Pilot trial of salbutamol in central core and multi-minicore diseases. *Neuropediatrics* 2004;35(5):262-266.

279. Saux MC, Girault J, Bouquet S, Fourtillan JB, Courtois P. Abstract: [Comparative study of the tissue distribution of two beta-mimetics: clenbuterol and salbutamol in the dog]. *J Pharmacol* 1986;17(4):692-698. (French)

280. Smith DJ. The pharmacokinetics, metabolism, and tissue residues of beta-adrenergic agonists in livestock. *J Anim Sci* 1998;76(1):173-194.

281. Malucelli A, Ellendorff F, Meyer HH. Tissue distribution and residues of clenbuterol, salbutamol, and terbutaline in tissues of treated broiler chickens. *J Anim Sci* 1994;72(6):1555-1560.

282. Joyce KB, Jones AE, Scott RJ, Biddlecombe RA, Pleasance S. Determination of the enantiomers of salbutamol and its 4-O-sulphate metabolites in biological matrices by chiral liquid chromatography tandem mass spectrometry. *Rapid Commun Mass Spectrom* 1998;12(23):1899-1910.

283. Waldeck B. Beta-adrenoceptor agonists and asthma--100 years of development. *Eur J Pharmacol* 2002;445(1-2):1-12.

284. Hamby PL, Stouffer JR, Smith SB. Muscle metabolism and real-time ultrasound measurement of muscle and subcutaneous adipose tissue growth in lambs fed diets containing a beta-agonist. *J Anim Sci* 1986;63(5):1410-1417.

285. McElligott MA, Barreto A, Jr., Chaung LY. Effect of continuous and intermittent clenbuterol feeding on rat growth rate and muscle. *Comp Biochem Physiol C* 1989;92(1):135-138.

286. MacLennan PA, Edwards RH. Effects of clenbuterol and propranolol on muscle mass. Evidence that clenbuterol stimulates muscle beta-adrenoceptors to induce hypertrophy. *Biochem J* 1989;264(2):573-579.

287. Martineau L, Horan MA, Rothwell NJ, Little RA. Salbutamol, a beta 2-adrenoceptor agonist, increases skeletal muscle strength in young men. *Clin Sci (Lond)* 1992;83(5):615-621.

288. Prezelj A, Obreza A, Pecar S. Abuse of clenbuterol and its detection. *Curr Med Chem* 2003;10(4):281-290.

289. Carter WJ, Lynch ME. Comparison of the effects of salbutamol and clenbuterol on skeletal muscle mass and carcass composition in senescent rats. *Metabolism* 1994;43(9):1119-1125.

290. Maltin CA, Reeds PJ, Delday MI, Hay SM, Smith FG, Lobley GE. Inhibition and reversal of denervation-induced atrophy by the beta-agonist growth promoter, clenbuterol. *Biosci Rep* 1986;6(9):811-818.

291. Zeman RJ, Feng Y, Peng H, Etlinger JD. Clenbuterol, a beta(2)-adrenoceptor agonist, improves locomotor and histological outcomes after spinal cord contusion in rats. *Exp Neurol* 1999;159(1):267-273.

292. Zeman RJ, Peng H, Etlinger JD. Clenbuterol retards loss of motor function in motor neuron degeneration mice. *Exp Neurol* 2004;187(2):460-467.

293. Carbo N, Lopez-Soriano J, Tarrago T, Gonzalez O, Llovera M, Lopez-Soriano FJ, et al. Comparative effects of beta2-adrenergic agonists on muscle waste associated with tumour growth. *Cancer Lett* 1997;115(1):113-118.

294. Smith DJ. Total radioactive residues and clenbuterol residues in edible tissues, and the stereochemical composition of clenbuterol in livers of broilers after exposure to three levels of dietary [14C]clenbuterol HCl and three preslaughter withdrawal periods. *J Anim Sci* 1998;76(12):3043-3053.

295. von Deutsch DA, Abukhalaf IK, Wineski LE, Aboul-Enein HY, Pitts SA, Parks BA, et al. Beta-agonist-induced alterations in organ weights and protein content: comparison of racemic clenbuterol and its enantiomers. *Chirality* 2000;12(8):637-648.

296. Ehrhardt C, Kneuer C, Bies C, Lehr CM, Kim KJ, Bakowsky U. Salbutamol is actively absorbed across human bronchial epithelial cell layers. *Pulm Pharmacol Ther* 2005;18(3):165-170.

297. Horvath G, Schmid N, Fragoso MA, Schmid A, Conner GE, Salathe M, et al. Epithelial organic cation transporters ensure pH-dependent drug absorption in the airway. *Am J Respir Cell Mol Biol* 2007;36(1):53-60.

298. Tamai I, Ohashi R, Nezu JI, Sai Y, Kobayashi D, Oku A, et al. Molecular and functional characterization of organic cation/carnitine transporter family in mice. *J Biol Chem* 2000;275(51):40064-40072.

299. Moaddel R, Patel S, Jozwiak K, Yamaguchi R, Ho PC, Wainer IW. Enantioselective binding to the human organic cation transporter-1 (hOCT1) determined using an immobilized hOCT1 liquid chromatographic stationary phase. *Chirality* 2005;17(8):501-506.

300. Moaddel R, Ravichandran S, Bighi F, Yamaguchi R, Wainer IW. Pharmacophore modelling of stereoselective binding to the human organic cation transporter (hOCT1). *Br J Pharmacol* 2007;151(8):1305-1314.

301. Jonker JW, Wagenaar E, Mol CA, Buitelaar M, Koepsell H, Smit JW, et al. Reduced hepatic uptake and intestinal excretion of organic cations in mice with a targeted disruption of the organic cation transporter 1 (Oct1 [Slc22a1]) gene. *Mol Cell Biol* 2001;21(16):5471-5477.

302. Jonker JW, Wagenaar E, Van Eijl S, Schinkel AH. Deficiency in the organic cation transporters 1 and 2 (Oct1/Oct2 [Slc22a1/Slc22a2]) in mice abolishes renal secretion of organic cations. *Mol Cell Biol* 2003;23(21):7902-7908.

303. Kerb R, Brinkmann U, Chatskaia N, Gorbunov D, Gorboulev V, Mornhinweg E, et al. Identification of genetic variations of the human organic cation transporter hOCT1 and their functional consequences. *Pharmacogenetics* 2002;12(8):591-595.

304. Shu Y, Leabman MK, Feng B, Mangravite LM, Huang CC, Stryke D, et al. Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. *Proc Natl Acad Sci U S A* 2003;100(10):5902-5907.

305. Kolka CM, Rattigan S, Richards S, Clark MG. Metabolic and vascular actions of endothelin-1 are inhibited by insulin-mediated vasodilation in perfused rat hindlimb muscle. *Br J Pharmacol* 2005;145(7):992-1000.

306. Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coovert DD, et al. SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet* 2005;14(6):845-857.

307. Caccia S, Fong MH. Kinetics and distribution of the beta-adrenergic agonist salbutamol in rat brain. *J Pharm Pharmacol* 1984;36(3):200-202.

308. Hayer-Zillgen M, Bruss M, Bonisch H. Expression and pharmacological profile of the human organic cation transporters hOCT1, hOCT2 and hOCT3. *Br J Pharmacol* 2002;136(6):829-836.

309. Ruderman NB, Houghton CR, Hems R. Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem J* 1971;124(3):639-651.

310. Colquhoun EQ, Hettiarachchi M, Ye JM, Richter EA, Hniat AJ, Rattigan S, et al. Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sci* 1988;43(21):1747-1754.

311. Rattigan S, Clark MG, Barrett EJ. Hemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* 1997;46(9):1381-1388.

312. Hettiarachchi M, Parsons KM, Richards SM, Dora KA, Rattigan S, Colquhoun EQ, et al. Vasoconstrictor-mediated release of lactate from the perfused rat hindlimb. *J Appl Physiol* 1992;73(6):2544-2551.

313. Rattigan S, Dora KA, Colquhoun EQ, Clark MG. Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not in incubated muscle: a role for the vascular system. *Life Sci* 1993;53(20):1545-1555.

314. Lipworth BJ, McDevitt DG, Struthers AD. Systemic beta-adrenoceptor responses to salbutamol given by metered-dose inhaler alone and with pear shaped spacer attachment: comparison of electrocardiographic, hypokalaemic and haemodynamic effects. *Br J Clin Pharmacol* 1989;27(6):837-842.

315. Wu X, George RL, Huang W, Wang H, Conway SJ, Leibach FH, et al. Structural and functional characteristics and tissue distribution pattern of rat OCTN1, an organic cation transporter, cloned from placenta. *Biochim Biophys Acta* 2000;1466(1-2):315-327.

316. Valenzuela B, Lopez-Pintor E, Perez-Ruixo JJ, Nacher A, Martin-Villodre A, Casabo VG. Modelling intestinal absorption of salbutamol sulphate in rats. *Int J Pharm* 2006;314(1):21-30.

317. Quinque D, Kittler R, Kayser M, Stoneking M, Nasidze I. Evaluation of saliva as a source of human DNA for population and association studies. *Anal Biochem* 2006;353(2):272-277.

318. Ng DPK, Koh D, Choo S, Chia KS. Saliva as a viable alternative source of human genomic DNA in genetic epidemiology. *Clin Chim Acta* 2006;367(1-2):81-85.

319. Neuhaus T, Geisen G, Bolt HM, Janzen V, Kraemer A, Vetter H, et al. Reliability of non-invasively acquired human genomic DNA as a substrate for real-time PCR-assisted analysis of genetic polymorphisms. *Arch Toxicol* 2004;78(7):390-396.

320. Etter JF, Neidhart E, Bertrand S, Malafosse A, Bertrand D. Collecting saliva by mail for genetic and cotinine analyses in participants recruited through the Internet. *Eur J Epidemiol* 2005;20(10):833-838.

321. Golenberg EM, Bickel A, Weihs P. Effect of highly fragmented DNA on PCR. *Nucleic Acids Res* 1996;24(24):5026-5033.

322. Miller SA, Dykes DD, Polesky HF. A Simple Salting out Procedure for Extracting DNA from Human Nucleated Cells. *Nucleic Acids Research* 1988;16(3):1215-1215.

323. Nasiri H, Forouzandeh M, Rasaee MJ, Rahbarizadeh F. Modified salting-out method: high-yield, high-quality genomic DNA extraction from whole blood using laundry detergent. *J Clin Lab Anal* 2005;19(6):229-232.

324. Killeen AA. Quantification of nucleic acids. *Clin Lab Med* 1997;17(1):1-19.

325. Gallagher SR, Desjardins PR. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Curr Protoc Protein Sci* 2008;Appendix 3:Appendix 4K.

326. Garcia-Closas M, Egan KM, Abruzzo J, Newcomb PA, Titus-Ernstoff L, Franklin T, et al. Collection of genomic DNA from adults in epidemiological studies by buccal cytobrush and mouthwash. *Cancer Epidemiol Biomarkers Prev* 2001;10(6):687-696.

327. Ng DPK, Koh D, Choo SGL, Ng V, Fu QY. Effect of storage conditions on the extraction of PCR-quality genomic DNA from saliva. *Clin Chim Acta* 2004;343(1-2):191-194.

328. Walker SR, Evans ME, Richards AJ, Paterson JW. The clinical pharmacology of oral and inhaled salbutamol. *Clin Pharmacol Ther* 1972;13(6):861-867.

329. Kauffman FC. Sulfonation in pharmacology and toxicology. *Drug Metab Rev* 2004;36(3-4):823-843.

330. Coughtrie MW. Sulfation through the looking glass--recent advances in sulfotransferase research for the curious. *Pharmacogenomics J* 2002;2(5):297-308.

331. Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, et al. Human sulfotransferases and their role in chemical metabolism. *Toxicol Sci* 2006;90(1):5-22.

332. Dajani R, Cleasby A, Neu M, Wonacott AJ, Jhoti H, Hood AM, et al. X-ray crystal structure of human dopamine sulfotransferase, SULT1A3. Molecular modeling and quantitative structure-activity relationship analysis demonstrate a molecular basis for sulfotransferase substrate specificity. *J Biol Chem* 1999;274(53):37862-37868.

333. Kakuta Y, Pedersen LG, Carter CW, Negishi M, Pedersen LC. Crystal structure of estrogen sulphotransferase. *Nat Struct Biol* 1997;4(11):904-908.

334. Wang L, Yee VC, Weinshilboum RM. Aggresome formation and pharmacogenetics: sulfotransferase 1A3 as a model system. *Biochem Biophys Res Commun* 2004;325(2):426-433.

335. Hildebrandt MA, Carrington DP, Thomae BA, Eckloff BW, Schaid DJ, Yee VC, et al. Genetic diversity and function in the human cytosolic sulfotransferases. *Pharmacogenomics J* 2007;7(2):133-143.

336. Johnson AD, Wang D, Sadee W. Polymorphisms affecting gene regulation and mRNA processing: broad implications for pharmacogenetics. *Pharmacol Ther* 2005;106(1):19-38.

337. Bosch TM, Meijerman I, Beijnen JH, Schellens JH. Genetic polymorphisms of drug-metabolising enzymes and drug transporters in the chemotherapeutic treatment of cancer. *Clin Pharmacokinet* 2006;45(3):253-285.

338. Lipworth BJ, McDevitt DG. Inhaled beta 2-adrenoceptor agonists in asthma: help or hindrance? *Br J Clin Pharmacol* 1992;33(2):129-138.

339. Nathan RA. Beta 2 agonist therapy: oral versus inhaled delivery. *J Asthma* 1992;29(1):49-54.

340. Louridas G, Kakoura M, Galanis N, Patakas D, Kastritsi K. Bronchodilatory effect of inhaled versus oral salbutamol in bronchial asthma. *Respiration* 1983;44(6):439-443.

341. Neville A, Palmer JB, Gaddie J, May CS, Palmer KN, Murchison LE. Metabolic effects of salbutamol: comparison of aerosol and intravenous administration. *Br Med J* 1977;1(6058):413-414.

342. Kalow W. Pharmacogenomics: historical perspective and current status. *Methods Mol Biol* 2005;311:3-15.

343. Bader JS. Systems approaches for pharmacogenetics and pharmacogenomics. *Pharmacogenomics* 2008;9(3):257-262.

344. Gibbs JR, Singleton A. Application of genome-wide single nucleotide polymorphism typing: simple association and beyond. *PLoS Genet* 2006;2(10):e150.

345. Hawkins GA, Weiss ST, Bleecker ER. Clinical consequences of ADRbeta2 polymorphisms. *Pharmacogenomics* 2008;9(3):349-358.

346. Sterling R, Henderson GE, Corbie-Smith G. Public willingness to participate in and public opinions about genetic variation research: a review of the literature. *Am J Public Health* 2006;96(11):1971-1978.

347. Keogh LA, van Vliet CM, Studdert DM, Maskiell JA, Macrae FA, St John DJ, et al. Is uptake of genetic testing for colorectal cancer influenced by knowledge of insurance implications? *AMJ* 2009;191(5):255-259.

348. Ogawa Y, Calhoun WJ. The role of leukotrienes in airway inflammation. *J Allergy Clin Immunol* 2006;118(4):789-798.

349. Garcia-Marcos L, Schuster A, Perez-Yarza EG. Benefit-risk assessment of antileukotrienes in the management of asthma. *Drug Saf* 2003;26(7):483-518.

350. Klotsman M, York TP, Pillai SG, Vargas-Irwin C, Sharma SS, van den Oord EJ, et al. Pharmacogenetics of the 5-lipoxygenase biosynthetic pathway and variable clinical response to montelukast. *Pharmacogenet Genomics* 2007;17(3):189-196.

351. Lima JJ. Treatment heterogeneity in asthma: genetics of response to leukotriene modifiers. *Mol Diagn Ther* 2007;11(2):97-104.

352. Hoshiko S, Radmark O, Samuelsson B. Characterization of the human 5-lipoxygenase gene promoter. *Proc Natl Acad Sci U S A* 1990;87(23):9073-9077.

353. Silverman ES, Du J, De Sanctis GT, Radmark O, Samuelsson B, Drazen JM, et al. Egr-1 and Sp1 interact functionally with the 5-lipoxygenase promoter and its naturally occurring mutants. *Am J Respir Cell Mol Biol* 1998;19(2):316-323.

354. Fowler SJ, Hall IP, Wilson AM, Wheatley AP, Lipworth BJ. 5-Lipoxygenase polymorphism and in-vivo response to leukotriene receptor antagonists. *Eur J Clin Pharmacol* 2002;58(3):187-190.

355. Telleria JJ, Blanco-Quiros A, Varillas D, Armentia A, Fernandez-Carvajal I, Jesus Alonso M, et al. ALOX5 promoter genotype and response to montelukast in moderate persistent asthma. *Respir Med* 2008;102(6):857-861.

356. Sanak M, Simon HU, Szczeklik A. Leukotriene C4 synthase promoter polymorphism and risk of aspirin-induced asthma. *Lancet* 1997;350(9091):1599-1600.

357. Lee SY, Kim HB, Kim JH, Kim BS, Kang MJ, Jang SO, et al. Responsiveness to montelukast is associated with bronchial hyperresponsiveness and total immunoglobulin E but not polymorphisms in the leukotriene C4 synthase and cysteinyl leukotriene receptor 1 genes in Korean children with exercise-induced asthma (EIA). *Clin Exp Allergy* 2007;37(10):1487-1493.

358. Sayers I, Barton S, Rorke S, Beghe B, Hayward B, Van Eerdewegh P, et al. Allelic association and functional studies of promoter polymorphism in the leukotriene C4 synthase gene (LTC4S) in asthma. *Thorax* 2003;58(5):417-424.

359. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386, 2000.

360. Kim SH, Bae JS, Suh CH, Nahm DH, Holloway JW, Park HS. Polymorphism of tandem repeat in promoter of 5-lipoxygenase in ASA-intolerant asthma: a positive association with airway hyperresponsiveness. *Allergy* 2005;60(6):760-765.

361. Sayers I, Barton S, Rorke S, Sawyer J, Peng Q, Beghe B, et al. Promoter polymorphism in the 5-lipoxygenase (ALOX5) and 5-lipoxygenase-activating protein (ALOX5AP) genes and asthma susceptibility in a Caucasian population. *Clin Exp Allergy* 2003;33(8):1103-1110.

362. Nelson KA, Smith SR, Trinkaus K, Jaffe DM. Pilot study of oral montelukast added to standard therapy for acute asthma exacerbations in children aged 6 to 14 years. *Pediatr Emerg Care* 2008;24(1):21-27.

363. Pelaia G, Vatrella A, Cuda G, Maselli R, Marsico SA. Molecular mechanisms of corticosteroid actions in chronic inflammatory airway diseases. *Life Sci* 2003;72(14):1549-1561.

364. Wenzel SE. The role of leukotrienes in asthma. *Prostaglandins Leukot Essent Fatty Acids* 2003;69(2-3):145-155.

365. Kedda MA, Worsley P, Shi J, Phelps S, Duffy D, Thompson PJ. Polymorphisms in the 5-lipoxygenase activating protein (ALOX5AP) gene are not associated with asthma in an Australian population. *Clin Exp Allergy* 2005;35(3):332-338.

366. The International HapMap Project. *Nature* 2003;426(6968):789-796.

367. Wright KL, Ting JP. Epigenetic regulation of MHC-II and CIITA genes. *Trends Immunol* 2006; 27(9):405-412.

368. Adcock IM, Tsaprouni L, Bhavsar P, Ito K. Epigenetic regulation of airway inflammation. *Curr Opin Immunol* 2007;19(6):694-700.

369. Tantisira KG, Lima J, Sylvia J, Klanderman B, Weiss ST. 5-Lipoxygenase pharmacogenetics in asthma: overlap with Cys-leukotriene receptor antagonist loci. *Pharmacogenet Genomics* 2009;19(3):244-247.

370. Desmet C, Gosset P, Henry E, Garze V, Faisca P, Vos N, et al. Treatment of experimental asthma by decoy-mediated local inhibition of activator protein-1. *Am J Respir Crit Care Med* 2005;172(6):671-678.

371. Lane SJ, Adcock IM, Richards D, Hawrylowicz C, Barnes PJ, Lee TH. Corticosteroid-resistant bronchial asthma is associated with increased c-fos expression in monocytes and T lymphocytes. *J Clin Invest* 1998;102(12):2156-2164.

372. Sousa AR, Lane SJ, Soh C, Lee TH. In vivo resistance to corticosteroids in bronchial asthma is associated with enhanced phosphorylation of JUN N-terminal kinase and failure of prednisolone to inhibit JUN N-terminal kinase phosphorylation. *J Allergy Clin Immunol* 1999;104(3):565-574.

373. Adcock IM, Lane SJ, Brown CR, Lee TH, Barnes PJ. Abnormal Glucocorticoid Receptor Activator Protein-1 Interaction In Steroid-Resistant Asthma. *J Exp Med*1995;182(6):1951-1958.

374. Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. *Immunol Cell Biol* 2001;79(4):376-384.

375. Gonzalez MV, Jimenez B, Berciano MT, Gonzalez-Sancho JM, Caelles C, Lafarga M, et al. Glucocorticoids antagonize AP-1 by inhibiting the

activation/phosphorylation of JNK without affecting its subcellular distribution. *J Cell Biol* 2000;150(5):1199-1207.

376. Umino Y, Hohjoh H, Tokunaga K. Noval single nucleotide polymorphisms (SNPs) at positions 497 (T/G)Q and 829 (T/C) in the human c-fos hene and haplotype association. *Human Mutat* 2000;16:279.

377. SNP [database on the Internet]. *PubMed database*: National Center for Biotechnology Information. [cited Mar 2008] Available from: http://www.ncbi.nlm.nih.gov/sites/entrez

378. Explore the Homo sapiens genome [database on the Internet]. *Ensembl 49*: E-ensembl. [updated Jul 2008, cited Sep 2008] Available from: http://www.ensembl.org/Homo_sapiens/Info/Index

379. Reid DW, Johns DP, Feltis B, Ward C, Walters EH. Exhaled nitric oxide continues to reflect airway hyperresponsiveness and disease activity in inhaled corticosteroid-treated adult asthmatic patients. *Respirology* 2003;8(4):479-486.

380. Reid DW, Misso NL, Aggarwal S, Thompson PJ, Johns DP, Walters EH. Tolerance and rebound with zafirlukast in patients with persistent asthma. *J Negat Results Biomed* 2008;7:3. doi:10.1186/1477-5751-7-3

381. Fahy JV. A safe, simple, standardized method should be used for sputum induction for research purposes. *Clin Exp Allergy* 1998;28(9):1047-1049.

382. Louis R, Sele J, Henket M, Cataldo D, Bettiol J, Seiden L, et al. Sputum eosinophil count in a large population of patients with mild to moderate steroid-naive asthma: distribution and relationship with methacholine bronchial hyperresponsiveness. *Allergy* 2002;57(10):907-912.

383. Lex C, Ferreira F, Zacharasiewicz A, Nicholson AG, Haslam PL, Wilson NM, et al. Airway eosinophilia in children with severe asthma: predictive values of noninvasive tests. *Am J Respir Crit Care Med* 2006;174(12):1286-1291.

384. van Rensen EL, Straathof KC, Veselic-Charvat MA, Zwinderman AH, Bel EH, Sterk PJ. Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* 1999;54(5):403-408.

385. Zacharasiewicz A. Fractional exhaled nitric oxide and induced sputum. *Paediatr Respir Rev* 2007;8(1):94-96.

386. Koh GC, Shek LP, Goh DY, Van Bever H, Koh DS. Eosinophil cationic protein: Is it useful in asthma? A systematic review. *Respir Med* 2007;101(4):696-705.

387. Holz O, Buhl R, Hausen T, von Berg A, Weber M, Worth H, et al. [Measuring airway inflammation in clinical practise - application and interpretation]. *Pneumologie* 2007;61(3):194-201.

388. Jatakanon A, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. *Am J Respir Crit Care Med* 2000;161(1):64-72.

389. Pizzichini E, Pizzichini MM, Efthimiadis A, Dolovich J, Hargreave FE. Measuring airway inflammation in asthma: eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood. *J Allergy Clin Immunol* 1997;99(4):539-544.

390. Wilson N. Measurement of airway inflammation in asthma. *Curr Opin Pulm Med* 2002;8(1):25-32.

391. Fujimoto K, Yamaguchi S, Urushibata K, Hanaoka M, Koizumi T, Honda T, et al. Characteristics of asthma resistant to moderate dose inhaled corticosteroid treatment on bronchial hyperresponsiveness. *Intern Med* 2006;45(14):843-849.

392. Jayaram L, Parameswaran K, Sears MR, Hargreave FE. Induced sputum cell counts: their usefulness in clinical practice. *Eur Respir J* 2000;16(1):150-158.

393. Fortuna AM, Feixas T, Gonzalez M, Casan P. Diagnostic utility of inflammatory biomarkers in asthma: exhaled nitric oxide and induced sputum eosinophil count. *Respir Med* 2007;101(11):2416-2421.

394. Korzeniewska A, Soloniewicz A, Stelmach P, Sobocinska A, Stelmach I. [Serum and sputum eosinophil cationic protein levels and clinical status in cystic fibrosis patients]. *Pneumonol Alergol Pol* 2006;74(4):372-376.

Appendices

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Appendix XIII Letter addressed to local (Southern Tasmania) respiratory physicians: Investigation of relationship between genetic variation and steroid insensitive asthma (Chapter 8).

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Appendix I

Patient information sheet and consent form :Circulating salbutamol enantiomers levels in acute COPD (Chapter 3)

Patient information Sheet

Stereoselective metabolism of salbutamol as a determinant of the extrapulmonary effect in exacerbation of Chronic Obstructive Pulmonary Disease (COPD).

This study has received approval from the Human Research Ethics Committee (Tasmania) Network

Contacts:

Chief investigator: Dr. Glenn Jacobson, School of Pharmacy, University of Tasmania.
Telephone: 03-6226 2202; e-mail: glenn.jacobson@utas.edu.au

Primary clinical contact: Prof. E H Walters, Respiratory Specialist, Discipline of Medicine, Royal Hobart Hospital, University of Tasmania. Telephone: 03-6226 4870

Background

Salbutamol (Ventolin, Asmol, Respolin, Respax) is a widely used medication for respiratory diseases including COPD. Salbutamol is administered as a mixture of two forms (enantiomers or mirror images), the active R-salbutamol and inactive S-salbutamol. People exhibit differences in how long it takes to clear salbutamol (both forms) from the body and some may be at a greater risk of serious adverse effects from the medication, in particular the effects on the cardiac system (heart function).

This study will examine the relationship between plasma levels of (R) and (S)-salbutamol and adverse effects including the cardiac system, among adult patients with exacerbations of COPD.

Purpose of the study

This study aims to examine how the treatment of salbutamol in people with COPD effects the heart.

Why have you been contacted?

You are being contacted because you have presented to the Department of Emergency Medicine (DEM) at the Royal Hobart Hospital with an exacerbation of COPD.

Study procedures

This study is observational in design. You will not be required to perform any additional test or procedure if you choose to participate the study.

You will be asked to allow the remaining blood sample, taken during your DEM presentation, to be sent to the School of Pharmacy, University of Tasmania, for the analysis of salbutamol enantiomers. You will also be asked to allow your medical information, including your heart function (eg ECG reading) and blood test result (eg. serum potassium level) as well as medical history and demographic information, to be collected from the hospital medical record.

- If you are willing to participate in the study, you have to sign and date the consent form (attached) witnessed by a third party.

If you have any further questions about the study, please contact Mr Kwang Choon Yee on 03-6226 2232.

Benefits

Although your participation may not lead to any direct personal benefit, it may contribute to a better understanding of the response to treatment in the management of COPD. This may lead to an improvement in care of COPD sufferers in the future.

Possible risks or discomforts

There will be no potential risk or discomfort by taking part in the study.

Confidentiality

The blood sample and material derived from the sample will be given a study code. Information such as your age, sex, race and relevant health condition will be identified by the code. All identifying information will remain confidential and be securely stored at the Royal Hobart Hospital. Your personal information will not be used in reports or publications resulting from this study..

Freedom to refuse or withdraw
Participation is entirely voluntary. You can decide not to take part in this study or withdraw your participation from the study at any time, without any prejudice to your future care.

Concerns or complaints
The Southern Health and Medical Human Research Ethics Committee (Tasmania) have approved this study. If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Executive Officer of the Human Research Ethics Committee (Tasmania) Network on (03) 6226 7479 or Email: human.ethics@utas.edu.au.

This information sheet and a copy of your signed consent form will be for you to keep for any future reference

Consent Form

Stereoselective metabolism of salbutamol as a determinant of the extrapulmonary effect in exacerbation of Chronic Obstructive Pulmonary Disease (COPD).

1. I have read and understood the 'Information Sheet' for this study, and have had the opportunity to have any questions answered

2. I understand that in consenting to participate:

   - **I will allow the remaining blood sample, taken during my presentation to the Department of Emergency Medicine (DEM) at the Royal Hobart Hospital, to be sent to the School of Pharmacy at the University of Tasmania, for the analysis of salbutamol enantiomers.**
   - **I will allow the medical information regarding my medical condition and treatment received, including heart function, blood test result, as well as medical history and demographic information, to be collected from the hospital medical records.**

3. I have been informed that the results of the study may not be of any direct benefit to my medical management.

4 I agree to participate in this investigation and understand that I may withdraw at anytime without prejudice.

5. I agree that research data gathered for the study may be published provided that I cannot be identified.

Name of **subject** ..... Signature .....

Date .....

Name of **witness** ..... Signature .....

Address .....

Date .....

Appendix II

Subject information sheet and consent form: Collection, Quantification and Quality of Genetic Material (Chapter 5).

Method for DNA sample collection --

Evaluating the most suitable and cost effective method for DNA sample collection and extraction for polymorphisms analysis.

1 Investigators:

1. Chief investigator
Mr. Kwang Choon **Yee**, School of Pharmacy, University of Tasmania (PhD student)
Tel: 03-6226-2232
Email: kyee@utas.edu.au

2. Chief investigator and supervising investigator
Dr. Glenn A **Jacobson**, School of Pharmacy, University of Tasmania
Tel: 03-6626-2202
Email: glenn.jacobson@utas.edu.au

2 Ethical Issues:

This study involves DNA collection and extraction from volunteer subjects for an asthma project. The samples will be handled in accordance with relevant guidelines.

Due to the nature of this study, which involved only volunteer among the staff and Post-grad students at the school, this study does not require ethic approval from the HREC, as advised by the ethic committee.

3 Research Plan:

3.1 Background

DNA sample collection and extraction is a crucial step for researchers involved in genetic investigations. A good quantity and quality of DNA sample is required for genotyping, and the amount of DNA required varies between different types of genetic tests. A typical SNP test require about 3 ng of DNA; where a microsatellite genotyping method requires a much greater amount of DNA.

A convenient and non-intrusive way of collecting DNA samples from subjects is also crucial to increase recruitment rates from potential participants of the study.

There are a number of different methods and numerous products on the market to assist with DNA collection, ranging from simple cotton tip swabs to high performing extraction kits, with a matching range in prices.

3.2 Aim

Investigate the efficacy of different method of extracting DNA. The study will serve to identified the most suitable and cost effective method for DNA collection for the Montelukast DUE study and chiral salbutamol studies, as well as future research involving genetic polymorphisms studies.

3.3 Method

3.3.1 Study design

The study will evaluate some commonly used methods in DNA collection and extraction.

3.3.1.1 Method 1: Oral swap

A sterilised cotton tip swab will be rubbed against the inside of the cheek of each subject for 30 seconds (minimum 8 times), a second sterilised cotton tip swap will be rubbed against the other side of the cheek of the same subject for 60 seconds. The swaps will then be place in a plastic bag and sealed, before being stored at -20□C until the DNA was ready for extraction.

Subjects will be requires to rinse their mouth with water and to not eat or drink (except water) for at least 30 minutes prior to the sample collection, in order to minimise contamination and maximise DNA yield.

3.3.1.2 Method 2: Finger prick blood in Eppendorf tube

Blood sample will be collected from each subject's middle finger or ring finger by finger pricking. The blood samples from the finger prick will be transferred to Eppendorf tubes with disposable micropipettor tips. Each Eppendorf tube will be weighed prior to and after the blood collection to measure the amount of blood being collected. The blood samples will then be stored at -20□C until the DNA is ready for extraction.

In order to maximise the blood samples being collected, each subject will has their hand(s) run under warm water for 1 minute and be given a gentle massage at the base of the finger prior to the blood collection.

3.3.1.3 Method 3: Finger prick blood on filter paper

Blood sample will be collected from each subject's middle finger or ring finger by finger pricking. The blood sample will be placed on a piece of filter paper (low acid, cotton base WhatmanTM filter paper). Each filter paper will be weight prior and after the blood collection to measure the amount of blood being collected. The filter paper will then be placed in a plastic bag before being stored at -20□C until the DNA was ready for extraction.

In order to maximise the blood samples being collected, each subjects will have their hand(s) run under warm water for 1 minute and be given a gentle massage at the base of the finger prior to the blood collection.

3.3.1.4 Method 4: OrageneTM DNA Purification (saliva) kit

Subjects will be asked to spit their saliva into the OrageneTM container, up to the indicator on the container within 30 minutes. The saliva required is about 2 mL (not containing foam). The cap will then be screw onto the container to release DNA stabilising solution. The sample would then be mixed gently with the solution and be stored at room temperature (up to 30 months at 24□C or 180 days at 50□C) until the DNA is ready for extraction.

Subjects will be required to rinse their mouth thoroughly with water and to not eat or drink for at least 5 minutes prior to the sample collection, in order to minimise contamination and to maximise DNA yield. In order to increase saliva production, a small amount of plain white sugar can be given to subjects.

3.3.2 Participants

Participants of this study will be the volunteers among the staff and/or post-grad students at the School of Pharmacy, University of Tasmania. About five subjects are required for this study, and each subject was required to sign a consent form prior to the study.

3.3.3 Measurement

The DNA sample collected from the first three methods will be extracted by using the QIAampTM DNA blood mini kit, in the laboratory of the School of Pharmacy. DNA samples collected from OrageneTM DNA Purification kit will be extracted as per Oragene protocol.

The concentration of the DNA will be measured by spectrophotometry, at the 260 nm wavelength (A260). The sensitive range of the light absorbance at 260 nm (through 10mm cuvette) for measuring DNA concentration is within 0.1 to 2 units. The concentration of DNA will be calculated with the coefficient of 50, which 1 unit reading of absorbance at 260 nm represent the DNA concentration of 50 □g/mL. Amount of DNA been extracted from each method will then be calculated accordingly. (Dilution of the original samples may be required if the reading fall outside of the sensitive range)

The purity of the DNA being extracted will be calculated with the ratio of A260/A280, as DNA produces a maximum absorption at 260 nm wavelength and proteins or other contaminants produce maximum absorption at 280 nm wavelength. A highly purified DNA sample would produce the ratio of >1.7.

DNA extracted will then been sent to AGRF for SNPs determination, in order to demonstrate that the DNA extracted from individual method is suitable for SNPs tests as required for the pharmacogenomics studies.

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VOLUNTEER CONSENT FORM

Method for DNA sample collection trial II

1 I have read and understood the 'Information Sheet' for this study, and I was given every opportunity to have any of my questions answered.

2. I understand that the study involves the following procedures:

   I will be asked to provide two buccal cells samples by oral swab, and
   I will be asked to provide one saliva sample

3 I volunteer to participate in this investigation without any pressure from the school, my colleague or the research team, and remain the right to withdrew from the study at any time without any prejudice

4. I agree to allow my samples to be used for genetic determination as described to me and the samples will not be used for purposes other than those agreed to in this consent form

5. I understand that I remain the owner of my DNA samples and if I decide to withdraw from the study, my DNA samples may be destroyed under my request.

6. I have been informed that the results of the study are purely for research purpose.

7. My test result will not be revealed to any other person or organisation without my written consent except under court order.

8. The group result of the study may be used as reference for future study design, given that my personal information can not be identified

Name of **subject** .... Signature .....

Date ....

Name of **witness** ..... Signature .....

Date .....

Appendix III

Media release for subject recruitment: Pharmacogentic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients (Chapter 6).

MEDIA RELEASE

NEWS FROM THE UNIVERSITY OF TASMANIA
DATE: THURSDAY, 7 SEPTEMBER 2006
ATTENTION: Chiefs of Staff, News Directors

Clearing the airways

SALBUTAMOL SIDE-EFFECTS UNDER THE MICROSCOPE IN NEW STUDY

A common drug used to treat asthma is being investigated for possible side effects.

UTAS School of Pharmacy PhD student Kwang Choon Yee (Tasmanian Asthma Foundation Scholarship holder) is examining how people with asthma metabolise the drug salbutamol commonly found in asthma medications such as Ventolin.

Salbutamol has been used as first line treatment for acute asthma for decades.

The drug widens the air passages to the lungs to ease breathing by relaxing bronchial muscles.

Recent studies have shown that a person's genes may affect how well the drug works in the lungs as well as the activity of enzymes which metabolise, or clear the body, of salbutamol.

The investigating team are concerned that salbutamol may be ineffective, or even irritate the airways and worsening symptoms in some people, due to these genetic differences.

The investigating team are examining the effect of genetic differences in salbutamol treatment and are currently recruiting participants for their study.

Participants must be those individuals between the ages of 18 and 65 who have been diagnosed with asthma.

Participants will be excluded if there are problems relating to other diseases such as diabetes, heart disease and epilepsy.

The study will be conducted in the Centre for Clinical Research at the Royal Hobart Hospital.

For more information please contact:

Kwang Choon Yee, UTAS School of Pharmacy
6226 2232

Information Released by:
The Media Office, University of Tasmania
Phone: 6226 2124 Mobile: 0417 517 291
Email: Media.Office@utas.edu.au

Appendix IV

Subject recruitment flyer A: Pharmacogenetic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients (Chapter 6).

If you have asthma, you can help with our research.

Are you between 18 to 65 years of age?

The University of Tasmania and the Royal Hobart Hospital are conducting a study to find out if genetic differences affect how the body eliminates the anti-asthma drug salbutamol (also known as Airomir®, Asmol®, Epaq® and Ventolin®).

The study is seeking volunteers who are willing to provide a DNA sample and attend a clinic session at the Royal Hobart Hospital.

If you are willing to participate or would like more information, please contact **Mr Kwang Choon Yee on (03)-6226-2232 or email kyee@utas.edu.au**.

The study has been approved by the Tasmanian Human Research Ethics Committee.

Mr Kwang Choon Yee holds an Asthma Foundation of Tasmania PhD Scholarship.

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asthma foundation of tasmania

Appendix V

Subject recruitment flyer B: Pharmacogenetic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients (Chapter 6).

Do you suffer from asthma?

Are you between 18 to 65 years of age?

The University of Tasmania and the Royal Hobart Hospital are conducting a study to find out if genetic differences affect how the body eliminates the anti-asthma drug salbutamol (also known as Airomir®, Asmol®, Epaq® and Ventolin®).

The study is seeking volunteers who are willing to provide a DNA sample and attend a clinic session at the Royal Hobart Hospital.

If you are willing to participate or would like more information, please contact **Mr Kwang Choon Yee on (03)-6226-2232 or email kyee@utas.edu.au**.

The study has been approved by the Tasmanian Human Research Ethics Committee.

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Appendix VI

Subject recruitment radio advertisement (transcript): Pharmacogenetic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients (Chapter 6).

COUGHING UP FOR ASTHMA community service announcement)

MEDIUM: Radio

DURATION: 30 secs

CLIENT: ASTHMA FOUNDATION OF TASMANIA

VOICE OVER *(light, comical and obviously nervously hesitant about the indelicacy of what he is about to say)*:

(CLEARING THROAT)

Look ... it's a bit difficult to say this politely ...but if you have asthma – we'd like you to cough up.

No we don't want your money. We want something far more valuable than that.

We need your (CLEARING THROAT) ... spit.

You see if you're aged between 18 and 65 and you happen to have asthma, your spit will help us with some ground breaking research.

All you have do is ring The Asthma Foundation – under A for Asthma in the phone book - and we'll tell you how to ... cough up.

Please give generously.

Appendix VII

Invitation letter: Pharmacogenetic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients (Chapter 6).

Dear «First_Name»,

Thank you for your inquiries about our study.

I have attached a copy of information sheet, as discussed earlier, which explain the nature of our study. I have also attached a copy of consent form and a return paid envelope with this letter.

We are looking forward to hearing from you.

Regards,

Mr. Kwang Choon Yee
(PhD student, School of Pharmacy, UTAS)

Appendix VIII

Patient information sheet and consent form: Pharmacogenetic determinants of pharmacokinetics of inhaled salbutamol enantiomers in asthma patients (Chapter 6).

Enantioselective pharmacokinetics of salbutamol: Effects of genetic differences in the enzyme responsible for metabolism (SULT1A3)

This study has received approval from the Human Research Ethics Committee (Tasmania) Network

Contacts:
Chief investigator: Dr. Glenn Jacobson, School of Pharmacy, University of Tasmania.
Telephone: 03-6226 2202; e-mail: glenn.jacobson@utas.edu.au
Primary clinical contact: Prof. H Walters, Respiratory Specialist, Discipline of Medicine, Royal Hobart Hospital, University of Tasmania. Telephone: 03-6226 4870

Background
Salbutamol (Ventolin, Asmol, Respolin, Respax) is a widely used medication for asthma. Salbutamol is administered as a mixture of two forms (enantiomers or mirror images), the active R-salbutamol and inactive S-salbutamol. People exhibit differences in how long it takes to clear salbutamol (both forms) from the body and some may be at a greater risk of worsening asthma control, possibly through indirect effects of accumulation of the medication.

Salbutamol is cleared from the body (metabolism) by some special enzymes – SULT1A3. Recent advances in knowledge have shown that differences in an individual's genetic make up may affect the activity of these enzymes, and in turn affect the metabolism of salbutamol. These differences are known as single nucleotide polymorphisms or SNPs. Similarly, differences at the site of action of salbutamol (the receptors) in the lung can also affect individual's response to salbutamol.

Purpose of the study
This study is set up to examining how people with asthma clear the drug salbutamol, and how the genetic differences among individuals affect the metabolism and clearance of both forms of salbutamol.

Why have you been contacted?
You are being contacted because you have been visiting the Respiratory Clinic at the Royal Hobart Hospital for the treatment of asthma, or you have responded to advertising flyers through the Tasmanian Asthma Foundation.
However if you have uncontrolled problems due to any of the following diseases; hyperthyroidism, diabetes, high blood pressure, heart disease and epilepsy, you will not be eligible to take part in this study.

Study procedures
This study was divided into two parts. You may be asked to participate in only the first phase of the study or both the first and the second phase, based on the result obtained from the first part of the study.

In the first phase of the study, you will be asked to provide either a finger prick blood sample or a saliva sample which will be used to analyse the SNPs related to the enzyme that metabolises salbutamol. Subjects with a certain genetic make-up will then be asked to take part in the second phase of the study

In the second phase of the study, you will be invited to attend a four hour study session at the Centre for Clinical Research at the Royal Hobart Hospital. During the study session, an intravenous cannula will be inserted for blood sampling, which may cause some local discomfort or bruising. You will then be given a dose of salbutamol (400 μg or 4 puffs), from a metered dose inhaler (puffer) using a spacer. Blood samples (10 ml) will be collected just prior to the dose of salbutamol and at 15 minutes, 30 minutes, 1, 2 and 4 hours after the salbutamol dose. At all times during this period, you will be clinically observed and monitored by the research team. In addition, a non-invasive lung function (blowing test) will be performed. You will need to stay at the centre until the last blood sample has been taken (four hours after the salbutamol dose), and you will be free to leave after that. Lunch or refreshment will be provided during the study session. Should you feel unwell in any way after you leave the Centre, you should contact the research team using the emergency telephone number provided.

- If you are willing to participate in the study, you have to sign and date the consent form (attached) witnessed by a third party and return it in the reply paid envelope provided. A suitable time and method (either finger prick blood sample or saliva) will be arranged for your contribution to the first phase of the study, and later advise you on whether you are invited for the second phase of the study. If you have any questions about the study, please contact Mr Kwang Choon Yee on 03-6226 2232.

➢ If you do not wish to participate in the study, please tick the *"do not consent"* box on the accompanying consent form, with your signature and date, and return it in the reply paid envelope provided. This will ensure you are not contacted again regarding this study.

Genetic ethics concern

The blood, saliva and genetic material collected will be destroyed in the genetic analysis for SNPs and for salbutamol. All information gathered for this study (including genetic material) will only be used by the investigators for research into genetic differences relating to asthma treatments. Any remaining genetic material will be destroyed at the completion of the study. Genetic material will not be retained for any further unspecified genetic testing. There will be eight SNPs tested which account for less than 0.0000003% of the total genetic code contained in your DNA. You will continue to be the owner of your genetic material and if you decided you would like to withdraw from the study at any time, you have the right to have your sample destroyed by contacting the researcher.

Benefits

Although your participation may not lead to any personal benefit, it may contribute to a better understanding of the response to treatment in the management of severe asthma. This may lead to an improvement in care of asthma sufferers in the future.

Possible risks or discomforts

There may be a minor physical discomfort and possible bruising during cannula insertion and blood sample collection during the second phase of the study. There is also a minor risk of infection at the site of needle insertion.

Confidentiality

The blood sample and material derived from the blood sample will be given a study code. Information such as your age, sex, race and relevant health condition will be identified by the code. All identifying information will remain confidential and be securely stored in the Respiratory Unit of the Royal Hobart Hospital. Your personal information will not be used in reports or publications resulting from this study..

Freedom to refuse or withdraw

Participation is entirely voluntary. You can decide not to take part in this study or withdraw your participation from the study at any time, without any prejudice and prejudice to your future care.

Concerns or complaints

The Southern Health and Medical Human Research Ethics Committee (Tasmania) have approved this study. If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Executive Officer of the Human Research Ethics Committee (Tasmania) Network, Ms Amanda McAully on (03) 6226 2763.

This information sheet and a copy of your signed consent form will be for you to keep for any future reference

UTAS

Enantioselective pharmacokinetics of salbutamol: Effects of genetic differences in the enzyme responsible for metabolism (SULT1A3)

1. I have read and understood the 'Information Sheet' for this study, and have had the opportunity to have any questions answered

2. I understand that the study involves the following procedures:

   - **I will be asked to provide an initial blood or saliva sample. This sample will be used to analyse genetic material (an extremely small section of my DNA) relevant to the metabolism of the anti-asthma drug, salbutamol.**
   - **I may be asked to participate the second phase of the study if I meet the study criteria, based on the result of the SNPs tests, which will involve:**
     - ***Attendance at the Centre for Clinical Research at the Royal Hobart Hospital for four hours***
     - ***Inhalation of 400 µg (4 puffs) of salbutamol via metered dose inhaler and spacer***
     - ***Insertion of an intravenous cannula and the collection of 10 ml blood samples pre salbutamol inhalation as well as at 15 minutes, 30 minutes, 1, 2, and 4 hours after the dose.***
     - ***Disclosure of information about my asthma and medical history to the researcher***
     - ***Lung function testing***

3. I understand insertion of the intravenous cannula may involve some discomfort and may result in bruising or infection.

4. I have been informed that the results of the study may not be of any direct benefit to my medical management.

5. I agree to allow my blood sample to be used for genetic determination as described in the information sheet, and the DNA sample will not be used for purposes other than those agreed to in this consent form. I understand that the blood sample and genetic materials may be destroyed at my request.

6. The result of my genetic test, and the fact that I had a test, will not be revealed to any other person or organisation without my written consent except under court order.

7. I agree to participate in this investigation and understand that I may withdraw without prejudice.

8. I agree that research data gathered for the study may be published provided that I cannot be identified.

Name of **subject** ..... Signature .....
Date .....

Name of **witness** ..... Signature .....
Address .....
Date .....

Which of the following time(s) is suitable for you if you are invited for the second phase of the study?
☐ Weekday morning (9.00 am – 1.30 pm) ☐ Weekday afternoon (1.00 pm – 5.30 pm)
☐ Weekday evening (6.00 pm – 10.30 pm) ☐ Weekend morning (9.00 am – 1.30 pm)

☐ Others (please specified) .....

❑ I do NOT consent to the SNP analysis.

Appendix IX

Instruction for subjects: use of the Oragene™ DNA Self-Collection kit.

Dear <insert name>,

Thank you for participating in our study. Attached with this letter is a DNA collection kit (Oragene™ DNA Self-Collection kit), which is designed to collect a sample of your DNA from saliva.

In order to collect the best quality DNA, please follow the steps as described below:

1. Rinse your mouth with water (or brush your teeth if you just eaten some food) and wait for 10-15 minutes.
2. Do not eat or drink (except clear water) within this period.
3. Remove the Oragene™ container from the plastic container (tube) and spit your saliva into the Oragene™ container.
4. Keep spitting until the amount of liquid saliva (not containing foam) reaches the top of the white label.
5. Tighten (screw) the cap very firmly.
6. Gently mix your saliva by turning the container upside down several time – do not shake.
7. Place the Oragene™ container back into the original plastic container (tube).

Once you have deposited a small volume of saliva (approximately 2 mL) into the container following the instructions, please send it back to us with the pre-paid return envelope provided as soon as possible. This will allow us to extract and analyse the small section of your DNA related to the study as described to you previously.

I have also attached a copy of the information sheet of this study, the same copy as provided to you previously.

If you have any questions, please do not hesitate to contact us.

Phone: 03-6226 2232 or E-mail: kyee@utas.edu.au

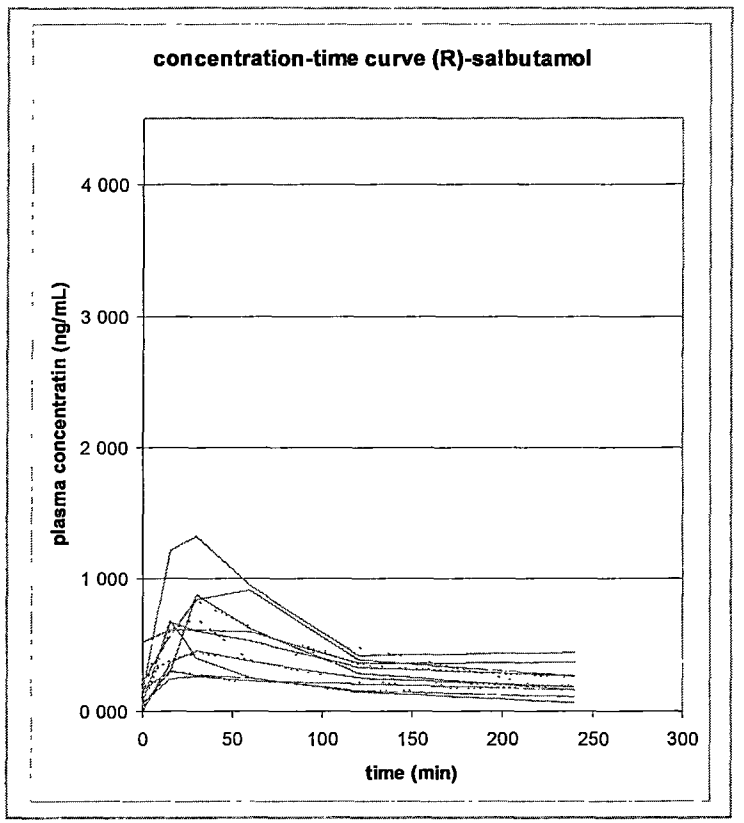
Regards,

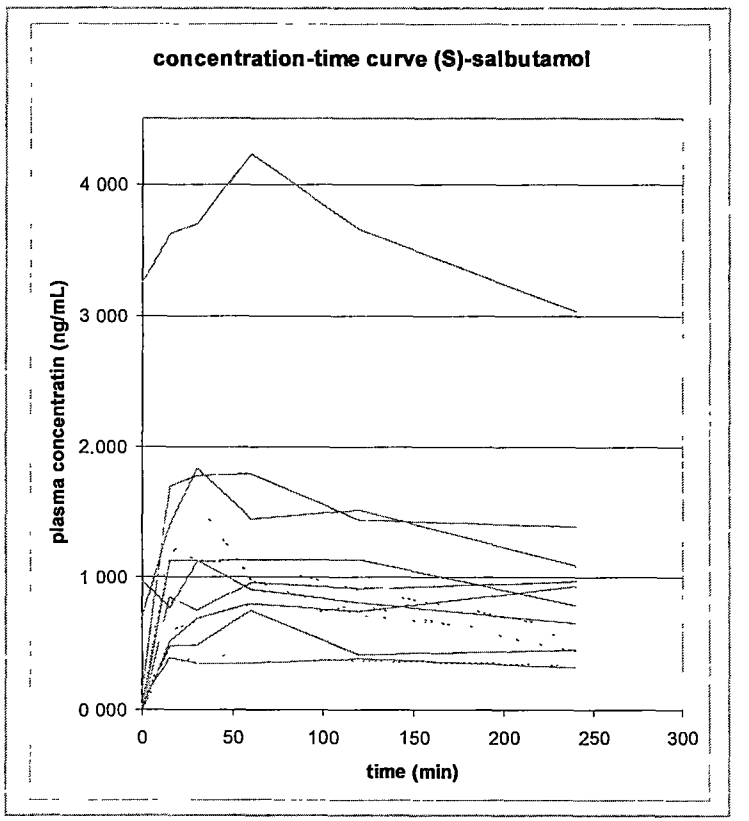
Kwang Choon Yee (BPharm)
PhD student,
School of Pharmacy, UTAS

Appendix X

The concentration-time curve of (R)- and (S)-salbutamol after 400 µg of inhaled *rac*-salbutamol, in all subjects who take part in the pharmacokinetic study (Chapter 6)

* The solid line represent subject woth W/T genotype (control); the broken line represent subjects with mutant genotype (case)


concentration-time curve (R)-salbutamol
plasma concentratin (ng/mL)
4 000
3 000
2 000
1 000
0 000
0
50
100
150
200
250
300
time (min)


concentration-time curve (S)-salbutamol
plasma concentratin (ng/mL)
4 000
3 000
2.000
1 000
0 000
0
50
100
150
200
250
300
time (min)

Appendix XI

Invitation letter *: Montelukast pharmacogenetics in clinical setting (Chapter 7)

* Letter was printed on standard letterhead of Respiratory Medicine. RHH and Respiratory Research Group, Menzies Research Institute, UTAS.

Dear <Insert patients name>,

The Respiratory Unit of the Royal Hobart Hospital (RHH) in conjunction with the School of Pharmacy at the University of Tasmania is undertaking a study to investigate the influence of genetics on response to the asthma therapy montelukast (Singulair®). We would like to invite you to participate in this study as you have been prescribed montelukast treatment from the RHH, which may dated back to 2001.

We have sent out a similar invitation letter asking for participants earlier this year, and the response we received is very encouraging. However, we found that there was a lack of response from patients who did not seem to benefit from the treatment (about five times less than those who does benefit from the treatment). It is important for our study, in order to achieve an accurate comparison of the genetic make up and to identified the key differences, that both patients who does and patients who does not response to the treatment are investigated. The treatment may not produce a significant improvement in your asthma control, however, your participation is crucial for the success of our investigation, and potentially improves the care of asthmatic sufferer in the future.

To participate in the study, all that is required is a sample of your DNA obtained from a non-invasive saliva sample which can be arranged by post. The section of DNA we are investigating is an extremely small portion (0.00000001%) of the overall genetic make-up in an individual.

An information sheet explaining this study and a consent form are attached with this letter. We would appreciate your consideration of participation in this research.

Your personal details will remain confidential to the Respiratory Unit of the Royal Hobart Hospital. This study is approved by the Human Research Ethics Committee (Tasmania) Network, and meets the ethical standards of genetic research in Australia.

Should you have any enquiries regarding to this study please contact Mr. Kwang Choon Yee (PhD student) Tel: 6226 2232 or Email: kyee@utas.edu.au

Yours sincerely,

A. Professor Richard Wood-Baker

Appendix XII

Patient information sheet and consent form: Montelukast pharmacogenetics in a clinical setting (Chapter 7).

Pharmacogenetics study of treatment response to montelukast (Singulair®) at the Royal Hobart Hospital (RHH) - A retrospective Drug Utilisation and Evaluation (DUE) study 2006

This study has received approval from the Human Research Ethics Committee (Tasmania) Network

Contacts:
Chief investigator: Dr G Jacobson, Senior Lecturer, School of Pharmacy, University of Tasmania. Telephone: 03-6226 2190
Primary clinical contact: Dr R Wood-Baker, Respiratory Specialist, Discipline of Medicine, University of Tasmania. Telephone: 03-6226 4870

Backgound
Montelukast (Singulair®) is a new asthma medication that works by blocking the effect of substances called leukotrienes in the body. Leukotrienes are a chemicals produced by cells in the body, especially during asthma exacerbations. These chemicals play a key role in asthma, causing narrowing of the breathing tubes. Blocking the effects of these chemicals using drugs such as montelukast), improves asthma control, although the degree of improvement varies widely between people.

The leukotriene substances are produced in the body by enzymes, such as 5-lipoxygenase and LTC_4 synthase. Differences between the genes controlling these enzymes may affect the amount of leukotriene produced in the body, which may in turn alter the effect of drugs which block leukotrienes, such as montelukast. The changes in the genes of people responsible for these differences are known as polymorphisms, and are contained on very small sections of DNA which make up the genes.

Purpose of the study
To investigate the differences in genetic make up of people with asthma to see if this alters how effective montelukast treatment is in asthma .

Why have you been contacted?
You have been contacted because you were given montelukast for the treatment of asthma through the Respiratory Clinic at the Royal Hobart Hospital, .

Study procedures
You will be asked to provide a saliva sample which can be organised by mail. DNA will be collected from this sample and then be analysed for your particular enzyme type.

Once we have received your consent:

1. A saliva collection kit designed for DNA collection and instructions can be posted to you with accompanying reply paid envelope. Saliva can be collected and returned to the investigators by mail.

2. Information related to your age, sex, race, asthma condition, drug treatment and other relevant clinical information such as response to drug therapy can be collected from the Royal Hobart Hospital records.

➢ **If you are willing to participate** in the study, please sign and date the consent form (attached) include the signature of a witness and return it in the reply paid envelope provided. If you have any questions about the study, please contact Mr Kwang Choon Yee on 03-6226 2232 or one of the study investigators listed above.

➢ **If you do not wish to participate** in the study, please tick the *"do not consent"* box on the accompanying consent form, with your signature and date, and return it in the reply paid envelope provided. This will ensure we do not contact you again.

Genetic ethics concerns
The saliva and genetic material we collect will be destroyed in the genetic analysis All information gathered for this study (including genetic material) will only be used by the investigators for research into the genetic differences relating to asthma treatments outlined in this information sheet. Genetic material will not be retained for any further unspecified genetic testing. There will be six genetic differences tested for, which accounts for less than 0.0000009% of the total genetic code contained in your DNA. You will continue to be the owner of your genetic material and have the right to have your sample destroyed at anytime up to the analysis by contacting the researchers.

Benefits
Your participation may contribute to a better understanding of the variable response to treatment of montelukast. This may lead to an improvement in care of asthma sufferers in the future.

Possible risks or discomforts
None.

Confidentiality
The sample derived from your saliva sample will be given a code for use in this study when the samples are sent for testing. Personal information such as your age, sex, race, asthma condition, drug treatment etc will be given the same code. Any identifying information and your hospital records will remain secure and confidential in the Respiratory Unit of the Royal Hobart Hospital. Your name and any other personal information will not be used in reports or publications resulting from this study.

Freedom to refuse or withdraw
Participation is entirely voluntary. You can refuse to consent to this study or withdraw your participation from the study at any time, without any prejudice and prejudice to your future care.

Concerns or complaints
The Southern Health and Medical Human Research Ethics Committee (Tasmania) have approved this study. If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Executive Officer of the Human Research Ethics Committee (Tasmania) Network, Ms Amanda McAully on (03) 6226 2763..

This information sheet will be for you to keep for any future reference

UTAS

Pharmacogenetics study of treatment response to montelukast (Singulair®) at the Royal Hobart Hospital (RHH) - A retrospective Drug Utilisation and Evaluation (DUE) study 2006

1. I have read and understood the 'Information Sheet' for this study.
2. I understand that the study involves the following procedures:
   - **I will be asked to provide a saliva sample, which will be used to analyse genetic (DNA) material relevant to the effect of anti-asthma drug montelukast.**
   - **Access to relevant clinical information at the Royal Hobart Hospital**
3. I have been informed that the results of the study may not be of any direct benefit to my medical management.
4. I agree to allow my sample to be used for genetic determination as described above, and the DNA sample will not be used for purposes other than those agreed to in this consent form. I understand that the saliva sample and genetic materials may be destroyed if requested.
5. The result of my genetic test, and the fact that I had a test, will not be revealed to any other person or organisation without my written consent except under court order.
6. I agree to participate in this investigation and understand that I may withdraw at anytime without prejudice.
7. I agree that research data gathered for the study may be published provided that I cannot be identified.
8. Any questions I have asked have been answered to my satisfaction.

Name of **subject** ..... Signature .....
Date .....

Name of **witness** ..... Signature .....
Address .....
Date .....

❑ I do NOT consent to the genetic analysis.

Appendix XIII

Letter addressed to local (Southern Tasmania) respiratory physicians *: Investigation of relationship between genetic variation and steroid insensitive asthma (Chapter 8).

* Letter was printed on standard letterhead of Respiratory Medicine. RHH and Respiratory Research Group, Menzies Research Institute, UTAS

Royal Hobart Hospital

Respiratory Medicine

PO Box 1061
HOBART TAS 7001
rhh.respiratory@dhhs.tas.gov.au

Director:
Dr Richard Wood-Baker

Consultants:
Dr David Reid
Dr Nick Harkness
Dr Rob Hewer
Dr Hugh Mestitz

Office:	6222 7353
	6222 8438
Fax:	6222 7579
TB Services:	6222 7293
Lung Function:	6222 7323
Respiratory RN:	6222 7485

Menzies Research Institute

Respiratory Research Group

Centre for Clinical Research

Researchers:
Prof E H Walters
A/Prof D Johns
A/Prof R Wood-Baker
Dr D Reid

Research Nurses:
Carol Phillips
Sue Davoren
Elizabeth Hammer

Office:	6222 8892
	6222 7068
	6222 7890
Fax:	6234 1660

Tasmania

Friday, August 3, 2007

Dear

I write to request your assistance with recruiting subjects for a study we are performing into the pharmacogenetic determinants of steroid dependent asthma. We are keen to investigate the relationship between steroid responsiveness and genetic abnormalities in the transcription factor activator protein-1 (AP-1) and the c-Jun NH2 terminal phase kinase (JNK), an enzyme responsible for regulating expression of the glucocorticoid receptor.

In order to assess the role of these factors in corticosteroid responsiveness in asthma we are looking to recruit cohorts of patients with steroid sensitive asthma (SSA) and steroid insensitive asthma (SIA). The definitions of these groups of patients are described below:

SSA patients will be defined as:

- Patients who show more than 15% improvement in FEV_1 or PEF after treatment with equal or less than maximum recommended doses of inhaled corticosteroid (1000 μg fluticasone daily or equivalent)
- Patients who do not require maintenance doses of oral corticosteroids
- Patients who do not experience significant deterioration of asthma more than once in every six months
- Patients who have not been prescribed anti-leukotriene agents, theophylline, mast cells stabilisers or immuno-modulating agents, for the treatment of asthma or other conditions.

SIA patients will be defined according to ATS criteria:

AMERICAN THORACIC SOCIETY CONSENSUS FOR TYPICAL CLINICAL FEATURES OF REFRACTORY ASTHMA *†

Major Characteristics

In order to achieve control to a level of mild–moderate persistent asthma:
1. Treatment with continuous or near continuous (50% of year) oral corticosteroids

2. Requirement for treatment with high-dose inhaled corticosteroids:

Drug	Dose (mcg/day)
Beclomethasone dipropionate	1,260
Budesonide	1,200
Flunisolide	2,000
Fluticasone propionate	880
Triamcinolone acetonide	2,000

Minor Characteristics

1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting beta-agonist, theophylline, or leukotriene antagonist

2. Asthma symptoms requiring short-acting beta-agonist use on a daily or near

daily basis

3. Persistent airway obstruction
   - FEV $_1$ <80% predicted
   - diurnal PEF variability >20%

4. One or more urgent care visits for asthma per year

5. Three or more oral steroid "bursts" per year

6. Prompt deterioration with 25% reduction in oral or inhaled corticosteroid dose

7. Near fatal asthma event in the past

* Requires that other conditions have been excluded, exacerbating factors treated, and patient felt to be generally adherent.
† Definition of refractory asthma requires one or both major criteria and two minor criteria.

To be included in the study patients should have no other respiratory diseases and fulfil the inclusion criteria of:

- Age over 12 years old
- Subjects diagnosed with asthma (according to NAC guidelines) for at least 12 months
- Subjects who have been prescribed corticosteroid for the management of asthma for at least 6 months, dose as described under the definition of SIA and SSA for case and control respectively.
- Subjects who are able to provide written informed consent (subject under the age of 18 years old will require written informed consent from their parent or legal guardian)
- Not taking oral xanthines or montelukast

We would be grateful for your assistance in recruiting participants to this study, and would be happy to assist with database searching to identify potential subjects if this is appropriate.

We will contact potential participants directly if you are able to give us their contact details, or alternatively provide an invitation letter for you to mail if this would be preferable.

I attach a copy of the patient information sheet and consent form for your information.

Many thanks in anticipation of your support for the research project.

Yours sincerely

Dr Richard Wood-Baker
RESPIRATORY PHYSICIAN

Appendix XIV

Patient information sheet and consent form: Investigation of relationship between genetic variation and steroid insensitive asthma (Chapter 8).

Pharmacogenetic determinants of steroid dependant asthma

This study has received approval from the Human Research Ethics Committee (Tasmania) Network

Investigators:

Chief investigator: Dr Glenn Jacobson, Senor Lecturer.
School of Pharmacy, University of Tasmania.
Telephone: **03-6226 2190**

Primary clinical investigator: Prof. E H Walters, Respiratory Specialist.
Discipline of Medicine, University of Tasmania, Head of Medicine RHH.
Telephone: **03-6226 4870**

Study coordinator: **Mr Kwang Choon Yee, PhD student,**
School of Pharmacy, University of Tasmania
Telephone: 03-6226 2232 or E-mail: kyee@utas.edu.au.

Backgound

Corticosteroids, also called steroids, are commonly used medications for preventing an asthma attack and reducing inflammation. Corticosteroids used for asthma treatment (including beclomethasone, budesonide and fluticasone) are delivered via devices called inhalers, including *Flixotide®*, *Pulmicort®* and *Qvar®*. Corticosteroids are also available in inhalers such as *Seretide®* and *Symbicort®*, as a mixture with other medications. Corticosteroids are very effective in treating asthma in most patients. However, some asthma patients do not respond well to normal doses of corticosteroids, and need much larger doses of corticosteroid to provide symptom relief.

Asthma is a chronic inflammatory disease, where inflammation affects the severity of the disease. Inflammation is a process involving a number of chemicals and enzymes interacting with each other in the body. Corticosteroids reduce inflammation by interfering with these chemicals and enzymes, therefore reduce the severity of asthma.

Recent advances in knowledge about these enzymes have shown differences in genetic make up among people. Some of these genetic differences are found to affect the enzymes' activities. The enzymes affected include AP-1 and JNK. These genetic differences (known as polymorphisms) may decide how well people respond to treatment with corticosteroids. These differences are contained on extremely small sections of the DNA.

Purpose of the study

To investigate if genetic differences in some enzymes will influence the outcome of corticosteroids treatment among patients with asthma.

Why have you been contacted?

You have been contacted because you (*or a patient less than 18 years old who is under your care*) has been visiting a participating Respiratory Physicians for the treatment of asthma. This treatment has involved corticosteroids.

Study procedures

This study involves the following procedures:

- The subject will be asked to provide a blood sample (5 mL), which will be used to extract the DNA. The DNA sample will be analysed for genetic variation (polymorphisms) of the AP-1 enzyme and JNK enzyme, which play an important role in corticosteroid treatment.
- The subject will be asked to attend a clinical session at the Centre for Clinical Research at the Royal Hobart Hospital, and to provide a sputum sample using inhaled nebulised hypertonic saline (salty water) method. During the clinical session, the subject will be asked to inhale some hypertonic saline (salty water) through a nebuliser for 30 seconds, which will loosen up secretions from the chest. The subject will then be asked to cough out sputum that the salty water has produced. The subject will

also be asked to perform a simple blowing test to ensure that chest tightness is not experienced during the procedure.

➢ If you are willing to participate in the study, you have to sign and date the consent form (attached) including a witness and return it in the reply paid envelope provided.

- ❖ *In the case where subject is less than 18 years old, the parent/legal guardian will also have to sign and date the consent form in conjunction with the subject,* ***provided both parties agree for the subject to participate the study.***

Genetic ethics concerns

The blood and genetic material derived from the blood sample will be destroyed in the genetic analysis. All information gathered for this study (including genetic material) will only be used by the investigators for this asthma study as described. Genetic material will not be retained for any further unspecified genetic testing. There will be six polymorphisms tested which account for less than 0.0000002% of the total genetic code contained in your DNA. The subject will continue to be the owner of their genetic material and has the right to have the sample material(s) destroyed at anytime during the investigation by contacting the researchers. (This study conforms to the NHMRC requirement for genetic studies)

- ❖ *In the case where subject is under 18 years old, both the subject and the parent/legal guardian have the right to have the sample material(s) destroyed at anytime during the investigation by contacting the researchers*

Benefits

Your participation may contribute to a better understanding of the differences in response to corticosteroid treatment in asthma. This may lead to an improvement in care of asthma sufferers in the future.

Possible risks or discomforts

There may be a mild physical discomfort during blood sample collection and sputum induction.

Confidentiality

The blood sample and material derived from the blood sample will be given a unique study code and only information including the subject's age, sex, race, health condition and other relevant clinical information (for example response to drug therapy) will be available from the study code. Your identifying information and hospital records are confidential and will remain secure with the Respiratory Unit of the Royal Hobart Hospital or the clinic of your Respiratory Physician. Your name, address and all other personal information will be de-identified, and will not be available with the study code, and will not be used in reports or publications resulting from this study. All information is presented in group form, and no one would be able to identify an individual person in these groups.

- ❖ *In the case where subject is less than 18 years old, the confidentiality as described will apply to both the subject and the parent/legal guardian.*

Freedom to refuse or withdraw

Participation is entirely voluntary. You can refuse to consent to this study or withdraw your participation from the study at any time, without any prejudice and prejudice to your future care.

- ❖ *In the case where subject is less than 18 years old, both the subject and the parent/legal guardian will have the freedom to refuse or withdraw as described.*

Concerns or complaints

The Southern Health and Medical Human Research Ethics Committee (Tasmania) have approved this study. If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Executive Officer of the Human Research Ethics Committee (Tasmania) Network, on (03) 6226 7479or Email: human.ethics@utas.edu.au

This information sheet will be for you to keep for any future reference

If you have any questions about the study, please contact Mr Kwang Choon Yee (PhD student) Telephone: 03-6226 2232 or E-mail: kyee@utas.edu.au.

Consent Form
Pharmacogenetic determinants of steroid dependant asthma

1. I have read and understood the 'Information Sheet' for this study.

2. I understand that the study involves the following procedures:

- **The subject will be asked to provide a blood sample, which will be used to analyse DNA material to determine common genetic differences (SNPs), in enzymes called AP-1 and JNK. The SNPs analysed are an extremely small section DNA relevant to the pharmacological effect of anti-asthma drug corticosteroids.**
- **I will be asked to attend a clinical session at the Centre of Clinical Research, Royal Hobart Hospital for sputum induction, using an inhaled nebulised hypertonic saline method.**

3. I understand, as a subject, there will be minor physical discomfort during blood sampling.

4. I have been informed that the results of the study may not be of any direct benefit to my (*or the subject less than 18 years old who is under my care*) medical management.

5. I agree to allow my blood sample to be used for genetic determination as described to me, and the DNA sample will not be used for purposes other than those agreed to in this consent form. I understand that the blood sample and genetic materials may be destroyed if under my request.
   - ❖ *In the case where subject is under 18 years old, both myself (parent/legal guardian) and the subject agree to the condition as described in consent form point number 5, and both the subject and myself (parent/legal guardian) may request for the blood sample and genetic material to be destroyed.*

6. The result of my genetic test, and the fact that I had a test, will not be revealed to any other person or organisation without my written consent except under court order.
   - ❖ *In the case where subject is under 18 years old, the result of the subject's genetic test and the fact that the subject have a test (under the condition that both the subject and myself consent to the test) will not be revealed to any other person or organisation without both the subject's and my written consent except under court order.*

7. I agree to participate in this investigation and understand that I may withdraw without prejudice.
   - ❖ *In the case where subject is under 18 years old, both myself (parent/legal guardian) and the subject agree for the subject to participate the study, and both the subject and myself (parent/legal guardian) may decide for the subject to withdraw from the study without prejudice to either the subject or myself(parent/legal guardian).*

8. I agree that research data gathered for the study may be published provided that I (*or the subject less than 18 years old who is under my care*) cannot be identified.

9. Any questions I have asked have been answered to my satisfaction.

Name of **subject**: ..... Signature: .....

Name of **parent/ legal guardian**: ..... Signature: .....
(if subjects is under 18 years old)
Date: .....

Name of **witness**: ..... Signature: .....
Address: .....
Date: .....