The role of magnesium in the treatment of acute asthma in adults and its effects on beta-2 adrenergic receptor function

Thesis submitted for the degree of Doctor of Philosophy

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Chapter 1 provides the relevant background information about the pathology, aetiology and current pharmacological treatments of asthma. It also explores the potential mechanisms of the action of magnesium and the evidence for its efficacy and introduces possible explanations for the heterogeneity of its effect.

Chapter 2

This laboratory study was designed to illustrate the potential mechanism of the effect of magnesium in acute asthma, by assessing the effects of magnesium on β-2 agonist-induced cAMP in human peripheral lymphocytes. This study demonstrated a reduction in β-2 agonist responses in acute asthmatics relative to non-asthmatics and suggests that there are intrinsic sex differences in susceptibility to down-regulating influences on β-2 responses. These differences corresponded with apparent sex differences in the efficacy of magnesium and support an effect of magnesium on agonist responses.

Chapter 3

This chapter is a clinical trial designed to optimize the selection of acute asthmatics presenting with exacerbations, who would benefit the most from magnesium treatment as an adjunct to standard treatment. The sample size was insufficient to draw any conclusions regarding the efficacy of magnesium. This study showed that the Asthma Control Questionnaire (ACQ) score was a significant predictor of an outcome of admission to hospital.

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This chapter is a re-analysis of the 3M clinical trial of magnesium in asthma. A sub-set of Caucasian asthmatics aged between 18 and 50 years old, who received intravenous magnesium vs. placebo, was analysed. The findings in this sub-set suggest a sub-group benefit limited to males and those with severe exacerbations.

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This chapter reviews the findings and provides a rationale for guidelines on the use of magnesium in adult asthma and explores the possibilities for future research in this area. It also discusses the further exploration of the sex/atopic differences observed in this study.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Key to abbreviations

AA	Arachidonic acid
AC	Adenylate cyclase
ACQ	Asthma Control Questionnaire
ADP	Adenosine di-phosphate
cADPR	Cyclic ADP ribose
AHR	Airway hyper-responsiveness
Arg	Arginine
ASM	Airway smooth muscle
ATP	Adenosine tri-phosphate
β-2AR	Beta-2-adrenergic receptor
BM	Basement membrane
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
DAG	Di-acyl glycerol
EB	Eosinophilic bronchitis
ECF	Extracellular fluid
ERK	Extra-cellular signal related kinase
FA	Focal Adhesion
FAK	Focal adhesion kinase
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
Gs	G protein stimulatory sub-unit
Gi	G protein inhibitory sub-unit
GDP	Guanine di-phosphate
GTP	Guanine tri-phosphate
GPCR	G protein coupled receptor
Gly	Glycine
Glu	Glutamic acid

Gln	Glutamine
GRE	Glucocorticoid response element
GRK	G protein receptor kinase
GR	Glucocorticoid receptor
GSDMB	Gasmerdin B
HASM	Human airway smooth muscle
IBMX	Iso-butyl-methyl xanthine
ICS	Inhaled corticosteroid
Ig	Immunoglobulin
IL	Interleukin
IF	Interferon
IP3	Inositol tri-phosphate
kD	Kilo Dalton
K_D	Dissociation constant
LABA	Long acting beta agonist
LAMA	Long acting muscarinic antagonists
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
Mg	Magnesium
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NAD	Nicotinamide adenine dinucleotide
NANC	Non-adrenergic non-cholinergic
NHANES	National health and nutritional examination survey
OCS	Oral corticosteroid
PDE	Phosphodiesterase
PEF	Peak expiratory flow
PBMC	Peripheral Blood Monocyte
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLP	Phospholipase
PLC	Phospholipase C
PKA	Protein Kinase A

RADS	Reactive airways dysfunction syndrome
Rho-A	Small GTP-ase
ROCK	Rho-A associated kinase
RSV	Respiratory syncytial virus
SABA	Short acting beta agonist
SERCA	Sarcoplasmic reticulum/endoplasmic reticulum ATPase
SR	Sarcoplasmic reticulum
SOCC	Store operated calcium channel
TAS2R	Bitter Taste-2 receptor
TNF-α	Tissue Necrosis Factor alpha
TGF	Tissue Growth factor
TH	Thymic Helper lymphocyte
TMD	Trans-membrane domain
VAS	Visual analogue scale
WEA	Work exacerbated asthma

Abstract

Asthma is a common respiratory condition that affects adults and children. Treatment options for acute exacerbations are limited. Magnesium could provide additional benefit when used in conjunction with β-2 agonists however, the evidence for its efficacy is lacking. Meta-analysis suggests that severity is an important factor in response to magnesium. The results of published clinical trials are heterogeneous, suggesting that individual factors may influence response to treatment with magnesium. The literature also shows that there are sex differences in aetiology of asthma which could influence treatment response. Phenotypes also vary with ethnicity and atopy and these factors could also influence treatment response.

The aims of the thesis were; to examine the effects of magnesium on β-2 agonist stimulation of the β-2-adrenergic receptor (β-2AR) on lymphocytes collected from asthmatics and compare these effects with non-asthmatics *in vitro,* and to determine if cellular responsiveness to magnesium is altered by asthma severity and patient sex or other factors such as atopy; to examine effects of the addition of intravenous magnesium to optimal standardised treatment in acutely exacerbating adult asthmatics in the emergency department; and to re-analyse 3M trial data to examine the effects of magnesium between severity subgroups and in relation to patient sex.

The interpretation of the results of the *in vitro* studies is limited by small sample sizes. The *in vitro* studies suggested that there are sex differences in β-2AR function which could influence treatment response. The *in vitro* studies found that β-2ARs in lymphocytes of healthy females were more responsive to agonist stimulation relative to healthy males however, lymphocytes of asthmatic females were less responsive to agonist stimulation relative to asthmatic males. Magnesium was more effective in male asthmatics relative to female asthmatics, in augmenting β-2 agonist stimulation of the receptors, as indicated by the difference in cAMP elevations. This suggests that magnesium may be more effective in improving β-2AR responsiveness in males. The sample size was too small to assess the effects of atopy on β-2AR responses and the effects of magnesium on those responses.

I was unable to determine a benefit from the use of magnesium for the treatment of acute asthma relative to standard care alone, due to the small numbers recruited to the trial. The small sample prevented me from determining any subgroup benefits.

In my re-analysis of the 3M trial data, I found that there was a benefit from magnesium in those with severe asthma, as defined by PEF 33-50% predicted. The data also showed that there were sex differences in response to magnesium. Magnesium's effect on reducing admission rates compared to placebo was greater for males relative to females. When the sexes were assessed separately, the sample size was too small to show a statistically significant benefit in males.

Individual differences in β-2AR responses may account for differences in efficacy of magnesium. These may relate to differences in sex, and possibly atopic status and ethnicity, with potential interactions between sex and atopy. Further study may be warranted to confirm the initial findings and to further explore the effects of sex-atopy and ethnicity in a larger sample of asthmatics. It is likely that magnesium is a safe and inexpensive optional addition to the treatment of acute asthma in those patients failing to respond to standard treatment however, its utility and effectiveness may be limited in Caucasian populations, to males with severe asthma.

Chapter 1: Introduction: background and rationale for the research

1.1 Asthma Overview

1.1.1 Definition and diagnosis

There is no gold standard or universally agreed or accepted definition of asthma. The term asthma, describes a group of disorders that share the common clinical manifestations of recurrent episodes of wheezing, often accompanied by cough, chest tightness and breathlessness. These symptoms are due to bronchial or airway hyper-responsiveness (AHR) which causes episodic attacks of airways obstruction. The episodes either resolve spontaneously or require treatment [1]. This type of wheezing is distinct from chronic obstructive pulmonary disease, where the symptoms and the obstruction are persistent and less responsive to treatment. International and national asthma guideline definitions incorporate such descriptions [2-4].

The universal clinical characteristic used in all definitions, is that of airway hyper-responsiveness (AHR) with reversible airflow obstruction [1] and the exclusion of other causes of wheezing, such as cystic fibrosis and alpha-1-antitrypsin deficiency. These conditions have distinct pathophysiological and aetiological causes. There are no universally agreed gold standard criteria for asthma diagnosis. National and international respiratory disease authorities, including the International Classification of Diseases Registry (ICD-10), The Global Initiative for Asthma (GINA), British Thoracic Society (BTS) and the National Asthma Council Australia (NACA), list various criteria for diagnosis. Recommendations for diagnosis are a combination of history, clinical features and lung function measurement, with or without provocation tests. Central to the diagnosis, is the history of episodic symptoms and demonstration of reversible airways obstruction.

Airways obstruction can be demonstrated during the measurement of a forced expiratory manoeuvre, (after a full inspiration to total lung capacity), such as a peak expiratory flow (PEF). The use of PEF measurements for asthma diagnosis, are no longer recommended [2]. Alternatively, the volume of air expelled in one second during forced expiration (FEV1), can be measured against the total volume that is expelled during forced expiration (FVC). This produces an FEV1/FVC ratio that can be used to demonstrate obstruction. A fixed ratio of less than 0.7 in adults, suggests airflow limitation [4]. More recently, the use of this ratio has been discouraged and the use of an age-related ratio is recommended, as this ratio varies with age [5]. A ratio below the lower limit of normal for age, ethnicity and sex, is recommended to diagnose the presence of lung disease [5]. A bronchodilator induced improvement in FEV1 $\geq$12% and $\geq$ 200mL from the pre-bronchodilator value, is generally

accepted as a prerequisite for a diagnosis of asthma [2-4, 6]. Demonstration of reversibility may distinguish asthma from other forms of obstructive airway disease, such as emphysema and chronic bronchitis, but there can be overlap of these diagnoses [7].

For patients where a clinical diagnosis is made but spirometry is normal, measurement of airway hyper-responsiveness (AHR) may assist with diagnosis. AHR is measured by the concentration of inhaled agonist (methacholine or histamine) that induces a decrease in FEV1. It is defined as the provocative concentration to cause a 20% fall in FEV1 (PC 20). The sensitivity and specificity of the PC 20 result depends on the concentration of agonist [8]. Patients breathe incrementally increasing concentrations of the agonist until a fall in FEV1 of 20% is reached. If a patient tolerates a concentration of 16mg/mL, this excludes asthma with reasonable certainty. If a patient has a PC 20 of 1mg/mL, the specificity and positive predictive value for the presence of AHR is close to 100%. A positive result does not confirm the diagnosis, as patients with other forms of obstructive lung disease may demonstrate AHR. Alternatively, a provocative dose of the inhaled agonist to cause a 20% fall in FEV1 (PD 20) may be used to measure AHR. A PD 20 of 100 μg is equivalent to a PC 20 of 1mg/mL.

The presence of AHR is not limited to asthmatics or those with lung disease, as AHR has been found to occur in healthy children, in the absence of other features of asthma [9]. The lack of a gold standard diagnosis and an absolute definition of the disease asthma can create difficulties when attempting to assess asthma prevalence and the results of clinical studies.

1.1.2 Prevalence, distribution and burden of disease

Much of the information on worldwide trends in the prevalence of asthma has been derived from surveys of atopic wheeze in children, therefore, is not applicable to all asthmatics. These large multi-centre studies have used self-reporting/parental reporting of symptoms of wheeze, as well as physician diagnosed asthma [10, 11]. Although this method of assessment is valid [12-16], under-reporting [15, 17, 18] and disparities between the methods of surveys [19] have been shown to occur. National medical databases have also been used to determine prevalence [20]. These databases are arguably a more accurate method of assessment however, they are reliant on clinician diagnosis of a condition without gold standard diagnostic criteria.

Given these limitations in data collection, asthma is estimated to be responsible for 1% of the total global disease burden and responsible for 250,000 deaths annually worldwide [3]. It is also the 24th most common cause of lost productivity due to disability worldwide [21]. Its prevalence has increased since the second world war, particularly during the 1970s and 1980s [22].

The prevalence of asthma tends to vary depending on geographic location and socio-economic status. Asthma prevalence in children is highest in the developed world, with up to 15-fold differences in prevalence rates between countries [23]. English-speaking countries and Latin America have the highest prevalence. Under-developed regions of South East Asia and the Indian subcontinent have the lowest asthma prevalence however, this varies between regions and is highest within their affluent populations. Prevalence studies in adults show a similar trend [10]. Within geographic regions, further variation in asthma prevalence is associated with the degree of urbanization [24, 25], with a lower prevalence of asthma in children raised on farms compared with urban centres [26, 27]. Meta-analysis of epidemiological studies suggests that the prevalence of asthma symptoms in adults and children is increasing [28] and is reflective of increases in urbanization around the world.

Asthma in Australia

The statistics from Asthma Australia, as obtained from the Australian Bureau of Statistics, show that 2.7 million Australians (1 in 9) have a diagnosis of asthma [29]. The prevalence of asthma is twice as high for indigenous Australians relative to non-indigenous Australians. In 2015, an estimated $1.2 billion was spent on asthma treatment, with the largest portion on prescription medicines (50%) and hospital admissions accounting for 20% of the cost [30]. Deaths due to asthma are relatively few, with less than 500 per year in 2017 or less than 1 per 5000 asthmatics. Asthma deaths are more likely to occur in those living in remote areas, in those of low socio-economic status and in indigenous Australians [31].

Self-monitoring of symptoms, appropriate use of disease controlling medications and early management of exacerbations are important in managing asthma [32]. Formal written action plans are recommended for all those with asthma [32] however, only 21% of adults and 57% of children with asthma have formal action plans [31]. Increasing the proportion of asthma sufferers with formalized action plans and appropriate prescriptions of preventer medications, through better awareness and education, could reduce exacerbations and hence, reduce hospital presentations/admissions [33]. Improved disease control, through novel treatments for chronic symptoms and acute exacerbations, could also contribute to improved outcomes and reduced financial costs [34].

1.1.3 Aetiology and pathogenesis

There is no single aetiologic factor that has been found to be solely responsible in the development of asthma within an individual. Many different environmental factors interact with an individual's genetics to influence development of the disease. An International Epidemiological Survey (ISAAC) found significant positive associations between asthma and the gross national product per capita (GNP), high dietary trans fatty acids, paracetamol usage and maternal smoking [35]. Twin studies

have implied a genetic influence [36, 37] however, not all those who suffer from asthma have a positive family history. Genetics can also affect the severity of illness in childhood asthma [38].

Multiple minor mutations in a variety of genes, affecting differing aspects of the asthma phenotype, act synergistically to influence the expression of the phenotype. These genes are influenced by environmental factors [39]. The risk of developing asthma may be modified via genetic polymorphisms [40, 41], as was illustrated in the Childhood Study of Asthma (COAST) study of genetics of asthma. This study found that the same genes could confer an increased or decreased risk of asthma, depending on the environmental exposure [42]. It was also found that none of these genes alone, nor environment alone, had a significant effect on the phenotypes.

Although there are more than 170 genes on 15 chromosomes thought to be associated with asthma [43], these associations are not consistent. Variation in allele frequencies between different populations [44], sex differences [45] and ethnicity [46], have been shown to influence phenotypic expression. For example, the GPR154 gene located on chromosome 7p, linked to the G-protein coupled receptor present in smooth muscle and epithelium, was associated with AHR in an Asian population [47]. The ORMDL3 gene located on chromosome 17q21, was specifically associated with childhood asthma and asthma across three non-Caucasian populations [48]. Thus, in addition to environmental influences, genetic and ethnic influences on asthma expression appear to be population-specific.

Theories of asthma pathogenesis

There are three main theories of asthma pathogenesis. The first two pertain to early immune exposures, while the third postulates an intrinsic dysfunction of the β-2AR within the airway [49]. None alone explains the disease, as there are numerous different phenotypes with varying associated inflammatory patterns.

The role of inflammation in the pathogenesis of asthma is debated, as there is evidence from human biopsy specimens to show that certain types of inflammation can be dissociated from AHR [50-52]. Eosinophilic bronchitis is a condition that is associated with eosinophilic inflammation of the airways however, there is no associated AHR [50, 51]. A study of asthmatic patients showed that the degree of inflammation, as determined by sputum, bronchial lavage or lung biopsy, was not correlated with the degree of AHR, as determined by methacholine inhalation [52]. At the same time, inflammatory mediators have been demonstrated to alter airway smooth muscle (ASM) properties [53], induce AHR in normal ASM [54], and exacerbate AHR in mild asthma [55].

Inflammation has a role in promoting exacerbations [56], contributes to the severity of symptoms[57] and influences long-term lung function [58, 59]. Variation in the pattern of inflammation and predominant inflammatory cell type, also contribute to variations in phenotypic expression [60] and

structural alterations associated with asthma [61]. Thus, the pattern of inflammation influences the clinical features.

The Thymic lymphocyte is the primary immune cell associated with asthma, and when activated, induces clonal expansion of T-cell sub-sets. These sub-sets are classified as helper (TH) cells and regulate the acquired immune response. The two main pathways of inflammation differ in their cytokine profiles and cell types recruited and activated. The TH_1 pathway, is associated with neutrophilic inflammation and the TH_2 pathway of eosinophilic inflammation, is associated with both atopic and non-atopic variants of asthma [62, 63].

The atopic theory, proposes that early sensitisation to inhaled antigens stimulates eosinophilic/TH_2 inflammatory pathways, resulting in eosinophilic inflammation in the lungs and consequently, AHR. Although allergen inhalation induces eosinophilic inflammation and bronchoconstriction in asthmatics [64], the presence of eosinophilic airways inflammation in humans, has been shown to exist in the absence of AHR [50, 51, 65]; thus, atopic sensitisation as a mechanism for atopic asthma in humans remains controversial. Atopy has been shown to modulate the disease process [66]. Reduced sensitization to antigens improves symptoms [67] and influences the frequency/severity of exacerbations. Atopic status may be an important factor in influencing or modulating asthma phenotype.

The 'hygiene hypothesis', postulates that 'un-hygienic' contact with infection, or early exposure to microbes, may stimulate innate (TH_1) pathways and down-regulate acquired or TH_2 pathways, and that this contact may be protective against atopic sensitisation and asthma [68]. Prenatal exposure, specifically to endotoxin, has been associated with lower asthma rates [26, 27]. Childcare attendance in the first six months, or households with multiple siblings, are also protective against developing asthma in childhood [69]. These outcomes suggest microbial exposure may be protective against atopic disease.

1.2 The Pathology of Asthma

1.2.1 Overview

As with the aetiology, asthma pathology is diverse. Although all forms of asthma share a similar pattern of functional and structural alterations within the airways, there is variation in the severity of these changes, as well as the underlying or driving inflammatory processes. Human lung is composed of parenchyma and tubular air conducting passages or bronchi. It is within the conducting segments of

the lung, that histopathological abnormalities are found. In addition to these visible changes in lung tissue of asthmatics, there are dynamic changes in the properties of ASM.

There are several inflammatory cell types involved, and the predominating infiltrate varies between individuals. These inflammatory cells are derived from the pool of lymphocytes known as T-helper cells (TH), which are derived from Thymus tissue. Within the airways, are increased cellular infiltrates with T lymphocytes and varying numbers of eosinophils, neutrophils and macrophages [70]. Eosinophils predominate in allergic/atopic asthma [70, 71], while non-atopic asthmatics have a more mixed population of inflammatory cells [62]. Inflammation drives exacerbations [56] and contributes to severity of symptoms [57], and in the long term, reduces lung function [58, 59]. Variations in these inflammatory patterns are responsible for the phenotypic variations previously described [60].

The pathology of asthma also involves structural changes in the lung tissues. Structural changes that can be seen histologically, involve the sub-epithelial or basement membrane (BM), the extracellular matrix (ECM) and the smooth muscle. These changes are referred to as remodelling. Thickening of the BM is only observed in the presence of eosinophilic inflammation [71, 72]. Mechanical factors are also thought to be significant contributors to these changes, due to the observation *in vivo*, that thickening of the BM was stimulated by methacholine challenge in asthmatic patients, independent of any additional recruitment of eosinophils [64]. Remodelling also involves an increase in both the size and number of smooth muscle cells, with hypertrophy predominating in the small airways and hyperplasia in the larger airways [73]. The ASM mass is increased proportionately to eosinophilia and mast cell numbers [74, 75]. In cultures of human airway smooth muscle (HASM), mechanical strain has been observed to induce the proliferation of HASM cells [76]. Increased ASM mass has been associated with asthma severity [77, 78], treatment resistance in paediatric asthma [79], and fatal adult asthma [80].

1.2.2 Airway smooth muscle and asthma

Regulation of airway smooth muscle tone

To maintain airway patency and adapt to the constantly changing loads imposed during the cycle of breathing, HASM tone is regulated by the balance of excitatory and inhibitory influences. This is largely regulated by input from the parasympathetic nerves of the autonomic nervous system, onto muscarinic receptors on the muscle membrane [81, 82]. The major neurotransmitter is acetylcholine (ACH), which causes bronchial smooth muscle contraction via activation of muscarinic receptors (M3) [83]. Noxious stimuli mediate the release of various neuropeptides, including Neurokinins, which can directly initiate ASM contraction [81]. Relaxation is mediated through activation of β-

adrenergic receptors. These receptors are of the beta-2(β2) subtype [84] and are activated via endogenous β-adrenergic agonists in the blood [85]. The β-2 adrenergic receptor (β-2AR), is the only β-adrenergic receptor sub-type in airway smooth muscle, and the density of these receptors increases, as the diameter of the airway lumen decreases [84]. In contrast, the density of parasympathetic ganglia is greatest at the proximal bronchi [86].

Molecular mechanisms of ASM contraction

Activation of the M3 receptor in ASM and activation of its associated G-protein by ACH, causes ASM contraction via the production of second messengers. These second messengers regulate many down-stream events. Contraction/relaxation of ASM involves the formation and activation of the actin-myosin cross-bridge, like that of skeletal muscle, however, there is an additional process that involves polymerisation of cytoskeletal actin and myosin and re-arrangement of specialised filaments. This is dependent on and initiated by increasing intracellular calcium levels, which are regulated via release of intracellular calcium stores [87]. Unlike other smooth muscle types, membrane depolarisation and voltage gated calcium channels are not generally considered to be involved in ASM contraction [88].

The release of intracellular calcium is achieved via activation of muscarinic receptors of the M3 sub-type [82, 83], which stimulates its associated G-protein α sub-unit to activate the enzyme phospholipase C [89]. Phospholipase C (PLC) catalyses the production of the second messenger inositol-1-4-5 triphosphate (IP3) [90], via the IP3 receptors that are associated with calcium channels on the membrane of the sarcoplasmic reticulum (SR) [91]. Phospholipase C also effects the release of calcium from the SR that surrounds the myo-filaments [92-93]. Calcium is returned to its stores at the end of the contraction via sarcoplasmic reticulum/endoplasmic reticulum ATPase (SERCA), which is a calcium pump located on the surface of the SR [94].

The intracellular increase of calcium occurs as a series of oscillatory waves [92]. The frequency of these oscillations are modulated in response to agonist concentrations and highest at the initiation of contraction [95]. The amplitude and frequency of these oscillations, influences ASM behaviour. An increase in oscillation frequency is associated with an increase in contraction [96].

The contractile apparatus

In all muscle, the basic unit of contraction is formed by a thick filament composed of myosin and a thin filament composed of actin [97]. These filaments are arranged to overlap and form cross-bridges between the thin filament and the ‘head’ of the thick myosin filament [98]. The head contains the binding sites for ATP [99] and actin [100] and a regulatory light chain (MLC20), which contains the

enzyme acto-myosin-ATPase [99]. The thin filament is primarily formed by a double stranded helix of filamentous actin (F-actin) [101] and the associated regulatory actin-binding proteins [102].

A contraction is produced by the shortening of the muscle fibre that occurs when myosin activation creates a sliding of actin on the myosin [98]. This 'sliding filament' theory was proposed by H. E. Huxley in 1969 [103] and is based on early studies in the skeletal muscle of animals [97, 98, 104]. Myosin is activated by an increase in intracellular calcium [105]. In the presence of calcium, actin binds to the myosin head, along with ATP, and the enzyme acto-myosin-ATPase is activated. This results in hydrolysis of ATP [106]. The energy released by ATP hydrolysis causes the tilting of the actin heads which are attached to myosin via cross-bridges [103] and results in shortening and stiffening of the muscle. A cross-bridge cycle of attachment-detachment occurs, as each ATP molecule is hydrolysed [107]. Muscle tension can be maintained for a time after the initial stimulus. This is thought to be due to the 'latch state' in which the cross-bridges remain attached but are not cycling [108].

Acto-myosin ATP-ase activity is dependent on the phosphorylation status of the MLC20 [109-111], which is regulated by intracellular calcium, in a process known as calcium sensitization [112-114]. This process is regulated by the two opposing enzymes, myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) [115]. MLCK is activated through its binding of calcium and increases MLC20 phosphorylation [116]. MLCP decreases MLC20 phosphorylation, independently of calcium concentration [109]. MLCP activity is inhibited by M3 receptor activation through activation of Rho-GEF [117] which in turn activates of Rho-A [118].

The role of the cytoskeleton in contraction of ASM

In ASM, 'stiffness' or isometric tension can be uncoupled from active contractile force [129]. In sheep trachea, acetylcholine-induced stiffness was shown to be modulated by Rho-kinase, independently of calcium and myosin light chain phosphorylation [131]. This stiffness was found to be calcium dependent, and increasing intracellular calcium was found to augment the degree of stiffness [132]. Thus, the acto-myosin cross-bridge activation and cytoskeletal modifications regulate ASM tone and contractility, and the cytoskeleton can be regulated independently of acto-myosin cross-bridges.

ASM contraction requires polymerisation of cytoskeletal actin [119], together with the re-arrangement of other cytoskeletal proteins and filaments, such as soluble of G-actin, for force development [120]. Cholinergic M3 stimulation of ASM leads to polymerisation of G-actin, which is also regulated by Rho-A [121].

F-actin is anchored at dense bodies or focal adhesion complexes (FAs), which are located throughout the cytoplasm and at the cell membrane. The dense bodies at the membrane are associated with

integrin proteins [122] which are anchored to the surrounding ECM [123]. These structures provide a structural framework for the transmission of force between cells and throughout the smooth muscle tissue [124]. Contractile activation stimulates recruitment of dense bodies and association with integrins at the cell membrane [122]. This is regulated by Rho-A [125].

The cytoskeleton is also involved in the maintenance phase of a contraction or 'stiffness'. Ex *vivo* studies in animal airway muscle, demonstrate cytoskeletal modulation of 'stiffness' of the muscle at different lengths [126-128]. Transfer of the load to the cytoskeleton by re-arrangement of the filaments, is thought to be responsible for the maintenance of force, while allowing the actin-myosin unit to re-initiate the contraction [129, 130].

Relaxation of human airway smooth muscle

The β-2 adrenergic receptor

The β-2AR is a prototypical member of the family of G protein coupled receptors. The mechanism of β-2AR activation is well defined. Activation of its stimulatory subunit (Gsα) results in the activation of adenylate cyclase (AC), a membrane bound enzyme which catalyses the production of cAMP [133]. It is initiated via β-2 agonist binding which then promotes association of the receptor with the Gsα sub-unit of the G-protein [134]. This coupling activates GTPase of the Gsα and dissociation of the sub-unit from the G-protein [134]. Adenylate cyclase (AC) is activated by hydrolysis of GTP on the Gsα subunit [135]. The Gsα sub-unit has spontaneous AC activating abilities however, β-2 agonist binding to the receptor is catalytic in the activation of AC by several fold [136], as it can couple with multiple G-proteins [137].

To explain receptor activation by agonist, a computer-generated model of ligand efficacy, the "Ternary complex model" of activation, was derived [138]. This model predicted that agonist ability to bind receptor and stabilize the receptor -G-protein complex, determined the degree of response. Full and partial agonists are defined in this model, by the relative abilities of the ligands to stabilize this complex and maintain the "active" state of the receptor. The use of NMR spectroscopy permitted the observation of different conformational states and provided further information regarding β-2AR dynamics [139-142]. When un-liganded or inverse agonist bound, two inactive conformations of the receptor exist in dynamic equilibrium [139]. The agonist alone is insufficient to stabilize a fully active state of the receptor and requires the presence of the Gsα protein [141, 142] or an intracellular G protein mimetic [139, 140]. Agonists shift the equilibrium toward a conformation capable of coupling to Gsα, although there is heterogeneity in the conformational changes, resulting in the coexistence of inactive, intermediate and active states [140]. When bound by agonist, an intermediate state is the most populated state of the receptor [139]. Efficacy is related to the population of active conformations of receptor relative to intermediate or inactive conformations [140].

Agonist stimulated production of cyclic AMP results in phosphorylation of the receptor and uncoupling from its Gsα sub-unit. This process is rapid and reversible and is part of the normal process of regulation and maintenance of receptor function [143]. This functional uncoupling is followed by removal of receptor from the surface, otherwise known as sequestration. Receptor sequestration transiently reduces the population of surface receptors, but recycling occurs rapidly within minutes [144] and is an important step in re-sensitisation of the receptor's responses [145, 146]. Long-term exposure to β-2 agonist stimulation however, results in a down regulation of β-2AR numbers, as determined by radio-ligand binding studies [147-150].

Molecular mechanisms of relaxation

Beta-2 adrenergic receptor activation of animal and HASM causes a reduction in active tension *in vitro* [151]. Relaxation of ASM via β-2 agonist stimulation is generally accepted as an important mechanism of relaxation in human and animal tissues [85, 152].

In HASM, the model of β-2AR mediated relaxation involves the second messengers cAMP and PKA, which reduce intracellular calcium via several mechanisms [85]. In HASM, cAMP is rapidly degraded by the enzyme phosphodiesterase 4D (PDE4D) [153] therefore, the effect of β-2 stimulation on cAMP production is short.

The suggested mechanisms by which cAMP may affect relaxation have been demonstrated in animal tissues via reduction in intracellular calcium and calcium sensitivity [154]. This β-2 agonist cAMP/PKA pathway has been shown to inhibit calcium entry via SOCCs [155], to inhibit calcium release via IP3 inhibition [156], reduce frequency of calcium oscillations [157] and increase re-uptake of calcium via stimulation of the internal calcium pump (SERCA) [151]. Studies in animals suggest that β-2 agonist mediated increases in cAMP, also induce relaxation due to alterations in calcium sensitisation, calcium sensitivity via MLCK inhibition [151], enhanced activity of MLCP [151, 158] and de-phosphorylation of MLC20 [159]. It is possible that other mechanisms independent of MLCK phosphorylation may regulate long-term calcium sensitization via direct G protein stimulation [160]. Animal studies have also shown that in the absence of de-phosphorylation of MLC20, an increase in cAMP is insufficient to relax animals' ASM [159], emphasising the importance of de-phosphorylation of MLC20.

The relevance of these mechanisms in human tissue is not clear. In HASM, β-2 agonist mediated cAMP/PKA mechanisms have been shown to be involved in relaxation of pre-contracted tissue via the inhibition of receptor-activated calcium influx [161]. The involvement of PKA was challenged due to the discovery of an alternative cAMP activated exchange protein (Epac), which was found to increase MLCP phosphorylation and ASM relaxation [162]. The inhibition of Epac had no effect

while PKA inhibition abolished β-2 agonist induced relaxation [161]. These data suggest that PKA rather than Epac activation, is involved in β-2 agonist mediated relaxation.

Animal studies in intact bronchial segments, demonstrated that β-2 agonists act synergistically with oscillatory strain to induce relaxation [163]. This suggests that the cAMP/PKA pathway may also be involved in regulating ASM cytoskeletal dynamics. Previous studies in cultured HASM cells found heat shock protein 20 (HSP 20) was phosphorylated via β-2 agonist induced PKA, and in animal ASM, this phosphorylation of HSP 20 resulted in relaxation via depolymerisation of F-actin [164, 165]. Evidence from cultured HASM, suggests that both PKA and PKA independent pathways are involved in β-2 agonist regulation of cytoskeletal dynamics [166, 167].

The details of the mechanisms of a β-2 agonist effect on calcium sensitisation in HASM remains to be defined. It may involve additional pathways independent of PKA, such as the inhibition of Rho-kinase as a means of reducing calcium sensitisation [168] or via Myosin light chain kinase (MLCK) phosphorylation [169].

Stretch induced relaxation and the behaviour of ASM in vivo

The mechanisms of ASM contraction and relaxation are largely derived from experimental observations in static models and isometric contractile states of ASM. Historically, the property of length adaptation has been attributed to ASM, based on *in vitro* studies on isolated ASM strips in animal tissues [130, 170] and comparative studies in humans and animals [171]. Contraction in response to stretch has been observed in cultured HASM [172], mediated via a stretch receptive calcium channel that is usually stimulated by changes in osmolality [173]. A contractile response to stretch has also been observed in an *in vitro* study in an intact animal lung [174]. In contrast to these findings, the property of length adaptation is not evident when physiological conditions are imitated in animal ASM [175] or in HASM *in vitro*[176], nor in *in vivo* human systems [177]. The response of HASM *in vivo*, is relaxation. Despite this, some argue that stretch does not induce relaxation of HASM [178].

The more recent models of ASM behaviour, recognise the property of stretch-induced relaxation or force-fluctuation-induced re-lengthening (FFIR), actin-myosin cross-bridge detachment cycling and cytoskeletal re-organisation, as important events in regulating the contractility of ASM [179-183]. Recently, experimentally derived mathematical equations have been constructed which describe the relationship between force and length [127, 179]. These equations predict force-length relationships that cannot be accounted for by rates of cross-bridge cyclic alone, further supporting the role of the cytoskeleton in the dynamic regulation of ASM tone.

The properties of ASM have been likened to that of soft glassy materials. Although the explanation for the behaviour of these materials in not well understood, soft glassy materials are observably 'scale free', in that there is no absolute alteration in force per unit change in length or direct relationship between stiffness and rate of any internal process [180]. These observations are consistent with the predictions of the afore described mathematical models of ASM dynamics. As ASM displays these same properties, it is suggested that analogous to glass, where plasticity is modulated by internal temperature, ASM plasticity is modulated via remodelling of its cytoskeletal structure [180].

Integrating the roles of the cross-bridge and the cytoskeleton in the mechano-sensitivity of ASM

Early studies in animal ASM provided some preliminary data on the mechanisms of mechano-sensitivity. Stretch-induced changes were shown to involve actin-myosin cross-bridge interactions [184, 185]. Stretch was also shown to induce changes in actin-cytoskeletal-integrin interactions, whereby stretch down-regulated the cross-linking of these filaments [127]. Focal adhesion kinase (FAK) was found to be involved in both the regulation of intracellular calcium and MLC20 phosphorylation [186], suggesting that the two processes are inter-dependent. Using a cultured human non-smooth muscle cell line transfected with contractile proteins, a model was developed which demonstrated a mechanism of interaction of contractile elements and the regulation of the assembly of cytoskeletal components, in response to mechanical factors [187]. The study showed that focal adhesion complexes (FAs) are regulated by acto-myosin generated forces, and inhibition of acto-myosin generated tension leads to FA disassembly. Different filaments of the FA (paxillin, vinculin and zyxin) showed different response times.

Subsequently, and most recently, further details consistent with these findings were obtained via *in vitro* study of animal fibroblast cells [188]. This study observed heterogeneous responses of zyxin, vinculin, paxillin and FAK, to Rho-associated kinase (ROCK)-mediated perturbations of actin-myosin contractility. The responses of the FAs were determined by their location, size, filament composition and length history. The effects of these ROCK-imposed mechanical perturbations in different focal adhesions, had distinct paths. Within different focal adhesions, the protein exhibiting the strongest response to ROCK perturbations also varied.

These findings explain the ability of ASM to behave in a 'glassy' fashion in response to mechanical stress. Disruption to these pathways may underlie the dynamic abnormalities observed in asthmatic ASM.

The effects of asthma on the behaviour of ASM

Airway hyper-reactivity is manifested as an increased but reversible bronchoconstriction response to pharmacological broncho-constricting agonists [1]. Evidence presented below, suggests that this is

also accompanied by an alteration in the ASM dynamic response to mechanical stimuli, wherein the property of the ASM itself is altered.

Dynamic abnormalities

The human lung *in vivo* is subject to dynamic alterations in load/tension, such that there is no static equilibrium between contractility/relaxation. More relevant to the function of ASM in health and disease, is how these properties of the muscle are dynamically related and regulated. Studies of intact healthy human systems *in vivo* have shown that a deep inspiration induces relaxation [177]. Furthermore, increasing length, as in a deep inspiration (DI), reverses bronchoconstriction [189, 190] and protects against contractile stimuli [191, 192]. Thus, the normal response of ASM to dynamic changes in length and tension that occur with the respiratory cycle, allows the muscle to adapt to these changes and maintain airway patency over a wide range of lung volumes, as occurs during tidal breathing and deep inspiration. This mechanism is impaired in asthmatic ASM.

It was noted as early as 1859, that there are dynamic abnormalities in the function of asthmatic ASM [193]. Since then, few studies have examined the dynamic behaviour of asthmatic ASM *in vivo*. In a recent *ex vivo* comparative study of ASM strips in healthy controls and asthmatics, asthmatic ASM was observed to have greater passive stiffness at longer lengths relative to non-asthmatic ASM. The ability of the ASM to relax after stretch was reduced by almost 50% in asthmatic ASM relative to non-asthmatic ASM [194].

Studies of broncho-motor responses to DI, as measured by plethysmography [195-197] and later direct imaging of the airways [177], have shown that asthmatics have a bronchoconstriction response to deep inspiration relative to healthy non-asthmatics. Studies of the effects of a DI on pre-constricted lungs as measured by PEF or FEV1, showed that the ability of a DI to reverse constriction is impaired in asthma relative to non-asthmatics [189, 190, 192, 197]. Spirometry evaluations and direct imaging of the airways of asthmatics, showed impairment of DI-induced bronchodilation after constriction with methacholine occurred in those whose FEV1 was <75% predicted [198]. The ability of a DI to reverse bronchoconstriction was positively correlated with airway distensibility in the large and medium-sized airways. Thus, an increase in ‘stiffness’ was associated with impaired ability to relax in response to stretch. The ratio of residual lung volume to total lung capacity (RV/TLC) was negatively correlated with airway distensibility and the ability of a DI to reverse bronchoconstriction. Distensibility was dependent on RV/TLC, and not an independent predictor of the ability of DI to reverse bronchoconstriction. These relationships were lost after bronchodilation with a β-2 agonist.

These data show that this impaired ‘stretch-relaxation’ response and increased ‘stiffness’, contribute to the obstructive defect in asthma and suggest a functional abnormality in asthmatic ASM. This concept is further supported by a recent comparative electron microscopic study of *ex vivo* human

tracheal muscle, in which no significant qualitative or quantitative differences were found in the sub-cellular structure between asthmatics and non-asthmatics [199].

A comparative study of asthmatics and non-asthmatics, using forced oscillations to measure minimal airway resistance following maximal inspiration R (min), showed that R (min) is greater in asthmatics relative to non-asthmatics, and performed better than PEF or FEV1 in detecting AHR as determined by methacholine challenge [200]. This study supports a reduction in airway distensibility as a contributing factor in AHR and demonstrates a non-invasive method of predicting AHR, that may be very useful in assessing and diagnosing asthma.

Changes in distribution of ventilation

Studies of asthmatic lungs show that bronchoconstriction is associated with, and results in, ventilation heterogeneity [201-203]. Using MRI, it was demonstrated that at baseline, ventilation heterogeneity is proportionate to the reduction in FEV1, and that methacholine and exercise challenges increase, while β-2 agonists decrease heterogeneous ventilation. Non-asthmatics did not develop any regions of heterogeneous ventilation in response to these challenges [201]. Another study, using a histamine challenge and multiple-breath-washout (MBW) to measure heterogeneity of ventilation, showed that reduction in FEV1 followed the degree of in-homogeneity of ventilation that occurred in the conducting zones of the lung. The degree of in-homogeneity in the non-conducting/gaseous exchange zones or acinar zone, corresponded to the degree of AHR, i.e. those with a decrease of FEV1>20% in response to histamine had a higher baseline acinar in-homogeneity of ventilation relative to those with <20% reduction in FEV1 [202]. In another study of asthmatics, using MBW, baseline heterogeneity was found to predict AHR, independent of airway inflammation [203]. These data illustrate that ventilation distribution and inequalities thereof, are important determinants of AHR and may determine the severity of the disease. In a comparative study of larger areas of lung with normal and poor ventilation, using Positron Emission Tomography (PET), heterogeneity of ventilation was also demonstrated. Within segments of poorly ventilated lung, there were greater numbers of smaller 'units' of poorly ventilated lung, while within normally ventilated segments, there were few poorly ventilated 'units'[204]. This suggests that small clusters of poorly ventilated areas, resulting from constriction of the small airways, can impact on larger areas of the surrounding lung. The images obtained in this study are also consistent with airway closure from bronchoconstriction of small airways, as the mechanism of loss or reduction in ventilation [201].

The cause of this abnormal bronchoconstriction and the factors responsible for these alterations in dynamic ASM properties, are not known. Abnormalities in contractility via alterations in calcium handling and calcium sensitivity, have been postulated as mechanisms of increased response to otherwise non-constricting stimuli, as a potential cause of AHR. Changes in the dynamic properties of ASM may be caused by abnormalities in cytoskeletal dynamics. Additionally, alterations in β-2

receptor function may impair relaxation and contribute to AHR. There may be an interaction between the presence of inflammation and alterations in intracellular calcium.

Potential mechanisms of abnormal smooth muscle behaviour

Mechanical/structural abnormalities

In non-asthmatics, AHR as measured by sensitivity to methacholine, was able to be induced by inhibiting DIs [205]. Another study of similar methodology failed to demonstrate this [197], suggesting that more than just mechanical factors are required for the development of AHR. Structural alterations associated with remodelling have been proposed as a cause of altered dynamics [206] and are considered to have a significant impact on clinical disease expression [207, 208]. These changes may be indicative of functional disorder, rather than the cause.

A comparative study of the ultra-structural features in post mortem ASM tissue of asthmatic and non-asthmatics, failed to detect any large or statistically significant differences in ASM cell cross-sectional area, thick or thin filament density or aggregation of dense bodies [199]. In asthmatics, there was evidence of remodelling, as demonstrated by an increase in the ASM area, a near significant difference in BM thickness ($p=0.09$), together with active eosinophilic inflammation relative to non-asthmatics [199]. This data suggests that functional changes may be more important than structural ones.

Abnormal calcium handling

Some associations between calcium handling and cytokines associated with airway inflammation in asthma, have been demonstrated. These cytokines may be responsible for the AHR observed in asthma. The most commonly implicated cytokines, IL13 and TNF-α, as well as being associated with asthma exacerbations, have been observed to increase intracellular calcium by a variety of mechanisms. In *ex vivo* non-asthmatic HASM, IL-13 and TNF-α were shown to increase SERCA expression and decrease the rate of fall of [Ca2+]I [209], increase calcium responses to agonist [210] and increase Ca2+ influx and the expression of the regulatory Na+-Ca2+ exchanger [211]. In cultured HASM cells, IL-13 was shown to increase agonist stimulated calcium responses and maximal force generation [212]. These data suggest a mechanism whereby, altered calcium handling may lead to AHR and potentially increased contractility and 'stiffness'. In *ex vivo* human tracheal smooth muscle cells, TNF-α was found to produce increased intracellular calcium responses to contractile agonist [213] and to induce AHR, in non-asthmatic ASM [214]. Levels of TNF-α are also increased via increases in intracellular calcium [215] hence, there is a positive feedback effect interaction between calcium and inflammation.

Altered calcium sensitivity has been observed in asthmatic HASM. Biopsies of lung tissue showed maximal shortening velocity and capacity were increased in asthmatic ASM relative to non-asthmatic ASM and coincided with an increased expression of MLCK [216]. This may explain the increase in airway contractile responses observed in asthma.

Beta-2 receptor abnormalities

In 1962, Andor Szentivanyi proposed the 'β-adrenergic theory of the atopic abnormality in bronchial asthma', on the basis that atopy alone cannot account for the disease process, and he proposed that the disease was due to an abnormality in the β-2AR itself [49]. There is evidence that suggests dysregulation of β-2AR function occurs in asthmatics, however, it is difficult to distinguish between intrinsic differences and the effect of regular β-2 agonist use.

Genetic polymorphisms could account for observed differences in responsiveness to β-2 agonist, among asthmatics. A mutation in the catalytic domain of Adenylate Cyclase (AC) type 9 was shown to confer reduced basal and β-2 agonist stimulated cAMP activity [217]. A polymorphism in the peptide encoded by the 5LC region of the β-2AR gene, has been shown to influence receptor synthesis [218]. This peptide has a polymorphism at position 19 Arginine (Arg)/ Cysteine (Cys). In HASM cells natively expressing β-2AR, the Cys19 polymorphism was associated with a two-fold greater expression of receptors relative to the Arg 19 genotype. These data show that innate β-2AR responsiveness is influenced by genetic factors, which may contribute to the asthma phenotype. These data also show that genetic factors could contribute to β-2 agonist responses independently of the asthma phenotype.

There is evidence that inflammation can contribute to impaired relaxation responses to β-2 agonist. Interleukin-13 has been shown to reduce β-2 agonist ability to relax HASM cells [219]. Interleukin-1β has also been shown to alter β-2AR function in HASM via uncoupling of the receptors from Gsα [220, 221]. Tumor necrosis factor-α has been shown impair β-2 agonist induced relaxation in cultured HASM [222] and to be synergistic with IL-1β in reducing β-2 agonist induced relaxation [223]. The Cysteine-leukotriene LTD4, has been shown to reduce maximal isoprenaline stimulated cAMP in cultured HASM and inhibit β-2 agonist induced relaxation in *ex vivo* HASM [224], suggesting desensitisation of receptor responses. A study of cultured HASM, found that asthmatics expressed twice the level of PDE4D relative to non-asthmatics [225]. Inhibition of PDE4D in both asthmatics and controls, gave a similar response in terms of β-2 agonist induced cAMP production. In theory however, increased PDE4D levels could lead to a more rapid decline in β-2 agonist stimulated cAMP and hence, impair ASM relaxation.

Altered regulation of cytoskeletal dynamics

Cytoskeletal dysfunction as a mechanism of AHR, has recently been supported [226]. In this study, the model for the mechanism was first developed in mice. Subsequently, post-mortem airway smooth muscle tissue from those with fatal asthma was examined. Zyxin has been identified and characterised in human smooth muscle [227]. It is a zinc binding protein that is widely expressed in human tissues and forms part of focal adhesions (FA), via binding to actinin, in association with mature complexes, and is important for microfilament organisation [228]. It is the primary mechanosensitive filament of the FA [229]. Asthmatics that died from asthma, had increased accumulation of zyxin relative to those who died from other causes and non-asthmatics. These data strongly implicate altered cytoskeletal function mediated by zyxin in the pathogenesis of asthma.

Summary of the asthmatic ASM

In summary, ASM function may be altered by factors that influence calcium sensitivity and cytoskeletal dynamics. Calcium sensitivity, regulating actin-myosin cross-bridge cycling, may be altered in asthma via increase in MLCK expression therefore, magnifying intracellular calcium increases.

Rho-A/ROCK activation potentiates MLCK driven increase in contractility via inhibition of MLCP, the counter-regulator of MLC20 phosphorylation. Cytoskeletal fragility in response to mechanical stress is the normal response, however, this response may be altered in asthma via increased zyxin expression. Rho-A/ROCK activation promotes cytoskeletal reinforcement via actin, actinin and paxillin accumulation however, this process is also regulated by cross-bridge cycling which is mechanosensitive. Both processes are influenced by Rho-A/ROCK pathways hence, they are a common denominator in the regulation of ASM function. The most commonly implicated cytokines in asthma pathogenesis, TNF-α and IL-13, have been demonstrated in animal and HASM, to up regulate Rho-A/ROCK pathways [230-232]. Oral corticosteroids inhibit IL-13 induced up regulation of Rho-A [233].

Pro-contractile influences mediated by Rho-A/ROCK, are also opposed via cAMP/PKA pathways. These pathways involve inhibition of MLCK, de-phosphorylation of MLC20 and depolymerisation of F-actin via phosphorylation of HSP 20. Increase in expression of PDE sometimes associated with asthma, could limit the effect of cAMP elevation on relaxation. Beta-2 agonists mediate cAMP/PKA pathways but can also inhibit actin polymerization, Rho-A/ROCK pathways and MLCK, via PKA independent pathways.

1.2.3 Role of inflammation in asthma

Leukocyte pathways and inflammatory mediators

Inflammatory mediators are involved in acute exacerbations of asthma, and more than one of the immune pathways may be involved [234]. The main cytokines produced by TH cells are; interleukin-13 (IL-13) [235, 236], which activates mast cells and stimulates allergic inflammation [237], interleukin-5 (IL-5), which activates eosinophils [238], tumour necrosis factor (TNF-α), which is associated with both allergic and non-allergic asthma [239, 240] and interleukin-8 (IL-8), which is associated with non-allergic asthma [241]. Both TNF-α and IL-8, activate and recruit neutrophils [242, 243], while TNF-α is also a common denominator in both T-helper pathways described. It is also increased in asthmatic airways when compared to healthy controls [239, 240], and TNF-α gene expression is increased in severe asthmatics [240]. Monocytes and macrophages from asthmatics have also been shown to have increased IL-8 and TNF-α production *in vitro* [244]. The production of nuclear transcription factors that promote inflammation, such as NF-kappa-B (nuclear factor-kappa-B), are also increased by TNF-α [215]. In non-asthmatics, TNF-α has been shown to induce AHR and induce sputum neutrophilia [54] and increase severity of AHR mild asthmatics [55].

More recently, another T-lymphocyte pathway, TH_{17} and its associated cytokine IL-17A, have been identified in allergic asthma [245]. The cytokine was first identified in T-lymphocytes infected with herpes virus [246] and was subsequently identified in high concentrations in sputum and broncho-alveolar lavage fluid in asthmatics relative to controls [247]. Interleukin-17A was associated with IL-8 and neutrophilic inflammation and asthma severity [248] and was demonstrated *in vitro* in both animal and HASM, to increase contractile force in response to methacholine [249]. It was shown to reduce the efficacy of budesonide on TNF-α induced IL-8 inhibition, through induction of changes in gene expression, thereby blocking potential pathways for corticosteroid inhibition of cytokine production [250]. The cytokine IL-17A is now recognised as an important mediator of airway inflammation and its resistance to treatment with corticosteroids.

Recently discovered is another class of immune cell, a non-B/non-T lymphocyte, which is designated as an innate lymphoid cell. It has similar properties to T-helper cells, in that it also influences the behaviour of other inflammatory cells. These cells, found in the sputum of asthmatic patients but not healthy controls, produced IL-13 and IL-5 [251]. These immune cells, therefore, can stimulate allergic inflammation and recruit eosinophils.

Cystyl-leukotrienes (LTs) are a group of inflammatory mediators associated with allergy and anaphylaxis. They are produced in all leukocytes by the metabolism of arachidonic acid (AA) within the cell membrane. The AA pathway is implicated in asthma, as inhibition of the receptors for LTs can improve asthma symptoms [252], particularly in aspirin-intolerant asthma [253].

The mast cell

The mast cell was one of the first cell types to be associated with asthma. Mast cell degranulation was first implicated in asthma pathogenesis in the 1960s [254] and became the target of asthma treatment with mast cell stabilisers [255]. Subsequently, inhaled corticosteroids replaced these treatments as first line for prevention of asthma symptoms [256]. More recently, the mast cell has been recognised as a key mediator of several aspects of asthma pathology. Studies of bronchial washings in asthmatics showed mast cells to be increased in asthma relative to non-asthmatics [257]. In both eosinophilic and non-eosinophilic asthma, mast cells are seen to infiltrate ASM [71, 75]. Mast cells are a source of the cytokines IL-4 [239, 258] and IL-5 [239] which are involved in the recruitment of eosinophils. Interleukin-13 [258] and TNF-α [239], which are important inflammatory mediators in asthma, are also produced by mast cells.

Mast cell numbers in ASM correlate with the severity of the disease [259]. Recently, a specific mast cell sub-type was identified as a major source of IL-17A [260]. This mast cell sub-type was associated with increased AHR [261], eosinophilic inflammation [261, 262] and resistance to corticosteroid treatment [262].

1.2.4 Clinical features

General features

Asthma symptoms are caused by the physiological abnormality of hyper-responsiveness of ASM. In response to a variety of contractile stimuli, there is an abnormal contractile response, which results in a state of prolonged/excessive contraction or "bronchospasm", resulting in airflow obstruction. Inflammation within the airways is often present to varying degrees and can precipitate and modify the hyper-excitability of the airways. Inflammation also causes swelling to the airways, which further contributes to the obstruction. These abnormalities are experienced as episodic bouts of wheezing/coughing and/or chest tightness and associated breathlessness. The bouts are usually brief and self-resolving but may be persistent or recurring. When persistent, these episodes are often relieved with an inhaled bronchodilator.

Frequency and severity of exacerbations varies between individuals and may be influenced by the type and amount of inflammation present. In many cases, the degree of inflammation correlates with the severity and frequency of exacerbations [56]. The obstruction caused by bronchospasm is reversible, but with time it may become fixed due to alteration of the muscle structure and properties. This may occur when inflammation is difficult to control and symptoms persist, leading to a decline in lung function and fixed obstructive defect [263]. In some asthmatics, despite control of the

inflammation, there is persistent AHR which leads to the development of rapidly progressive fixed obstruction [264].

Asthma symptoms can progress at any time in to a much more acute condition, in which the bronchospasm is persistent and there is a rapid worsening of airways obstruction and a decline in lung function. The exacerbation is usually due to increased inflammation as the result of a respiratory tract infection or an environmental trigger but can occur without an identifiable trigger [265]. Severity and frequency of exacerbations tends to reflect the activity of the disease. Exacerbations may be unavoidable and difficult to manage and may occur abruptly and without warning.

The severity of an exacerbation varies greatly in terms of degree of obstruction and respiratory distress. Response to treatment can also be variable, with some responding well to emergency treatment and being discharged home, while others requiring hospital admission and intensive treatment. Exacerbations can be life-threatening and even fatal [266]. Death results from closure of the small airways and complete obstruction to airflow. This leads to respiratory arrest, followed by eventual cardiac arrest from hypoxia. Exacerbations of such severity are often termed 'status asthmaticus' [267] and are difficult to manage.

Classification of severity varies between guidelines [2-4] but generally indicates severity on a scale of mild to near-fatal. Mild exacerbations are generally classified as those without hypoxia and increased work of breathing, while increased work of breathing, increased respiratory effort and reduction in oxygen saturation, indicate more severe exacerbations. The degree of distress or respiratory effort determines the severity of the exacerbation which, if life-threatening, is usually accompanied by significant reduction in oxygen saturation. A saturation of <92%, with or without supplemental oxygen, is a feature of a life-threatening exacerbation [4]. Exhaustion may intervene and is a sign of impending respiratory arrest and death. Guidelines include the use of PEF to assess severity in acute asthma exacerbations. Both BTS and GINA use a PEF <50% predicted to classify an exacerbation as severe [4, 268]. BTS classifies a PEF <33% as life-threatening [4].

Diversity within the asthma phenotype

Those with a diagnosis of asthma share the common features of AHR and airways inflammation, together with a clinical pattern of acute paroxysms of bronchospasm in isolation and/or acute exacerbations, superimposed on chronic airflow limitation. Despite these general similarities, there are individual differences in clinical patterns of disease. Patients with similar disease patterns can be "grouped" based on phenotypes and endotypes however, there are other individual factors that can influence clinical expression of disease, such as sex, atopy and possibly ethnicity.

Intrinsic vs. extrinsic asthma

Traditionally, asthma has been broadly classified into two sub-types, as determined by the presence or absence of specific IgE antibodies to common allergens or triggers [269]. Patients with specific IgE antibodies against aeroallergens or positive skin prick tests (SPTs), were classified as having extrinsic or allergic/atopic asthma, whereas those without IgE markers or positive SPTs, were classed as intrinsic or non-allergic/non-atopic.

Clustering of asthma phenotypes

Classification of asthma sub-types is useful, as there are differences in inflammatory phenotypes and underlying aetiology which influence the clinical expression of the disease, therefore, also the treatment response. The optimal method of classification would predict best treatment and treatment responses, which no classification predicts perfectly. The 'clustering' approach of sub-grouping asthma expands on the traditional intrinsic/extrinsic classification. It is based on common inflammation patterns, clinical features and treatment response. It distinguishes inflammatory types by the presence or absence of one inflammatory cell type, the eosinophil, which is often associated with allergy.

Atopic asthmatics have early onset disease, eosinophilic inflammation and are more responsive to inhaled steroids. This 'cluster' represents the 'extrinsic' asthma type and is described by several authors [270-272]. A separate cluster of late onset asthma is like the 'extrinsic' phenotype in being atopic, eosinophilic and corticosteroid sensitive, but symptoms appear in adulthood [270, 271]. The other phenotypes are characterised by non-eosinophilic inflammation or a mixed inflammatory pattern. These include one cluster characterised by late onset of symptoms, obesity, predominantly female sex and a neutrophilic inflammatory profile [270, 273, 274]. Patients in this group have more severe symptoms, poor response to an inhaled steroid and a greater degree of fixed obstruction.

Endotypes

The recently developed concept of endotypes, recognises asthma sub-types with a demonstrable aetiology and distinct pathological mechanisms, such as occupational, aspirin-sensitive asthma and exercise-induced asthma [271]. Such classification allows identification of those with specific treatment requirements and allows avoidance of triggers for improved symptom control.

Occupational asthma refers to asthma that occurs in the absence of pre-existing airways disease and is a direct result of occupational exposure. It can be irritant-induced, otherwise known as reactive airways dysfunction syndrome (RADS) [275-277]. This occurs immediately upon exposure to irritant and without immunological sensitization. Alternately, it can be immunologically mediated due to sensitisation to work-place allergens that evoke an IgE mediated response [275-277]. Pre-existing

asthma may be exacerbated by environmental factors at work and is considered as a separate entity known as labelled Work Exacerbated Asthma (WEA) [275].

Aspirin-sensitive asthma refers to a triad of asthma, nasal polyposis and exacerbations that are precipitated by exposure to non-steroidal anti-inflammatory medications or aspirin. In susceptible asthmatics, these medications enhance the synthesis of cystyl-leukotrienes (Cl-T) which induce mucus production and bronchospasm [278]. It is estimated, that 10% of asthmatics without prior use of aspirin/ NSAID may be sensitive to these medications, and it is generally advised that all asthmatics avoid these medications [279].

Asthma in elite athletes occurs in the absence of pre-existing disease. The condition has been observed in competitive swimmers and skiers and responds to a reduction in the intensity, or the cessation of training [280]. Release of inflammatory mediators triggered by drying of the airways is believed to be the mechanism, and the mast cell is believed to be the source of these mediators. Hyper-responsiveness of the airways is accompanied by neutrophilic and sometimes eosinophilic inflammation. Sodium cromoglycate, a mast cell stabiliser, is very effective in this group of asthmatics if taken immediately prior to exercise [281] however, it is less effective when taken alone than in combination with an inhaled β-2 agonist [282].

Recently, blood biomarkers and gene clusters have been correlated with specific endotypes in a group of 146 children [283]. These genes were involved with immune cell function and inflammatory patterns. The clinical markers included obesity and atopy, identifying profiles like that previously described in the endotype models [271]. There was no correlation between the type of medication or amount of medication used and the endotype. Larger studies may be able to correlate these endotypes with treatment requirements, otherwise the utility of such biomarkers is largely academic.

Sex differences

There are significant sex differences in several aspects of asthma. For example, the prevalence of asthma in childhood is greater in males relative to females. This is reversed in adult asthma [284]. The change in the sex ratios in asthma prevalence from childhood to adulthood, is not correlated with the stage of puberty [284]. This, and other data, suggest more than just an effect of hormones.

Some of these sex differences have implications for treatment, including differences in the phenotypic expression of asthma. Severity of the disease follows opposite sex trends in childhood asthma relative to adult asthma. In childhood asthma, males have greater severity and admission rates relative to females [285-288]. In adult asthma, women have more severe symptoms and frequency of exacerbations relative to men [285, 289-294], are more likely to have difficult to treat asthma [295], use oral corticosteroids [291] and are more likely to be admitted to hospital with an exacerbation relative to men [292, 296]. Differences in outcomes were also reported in the larger of these studies.

Women had longer hospital stays relative to men, yet men were more likely to die during admission [292]. In this study, females were more likely to be Caucasian (37.9%) relative to men (34.2%). This data suggests that asthma in women is more persistent and chronic relative to asthma in men, while in men, asthma may be a more acute life-threatening event.

In studies of the immunological cell lines in asthmatics, sex differences in the immune responses of TH_1 and TH_2 pathways were found. The differences in immune response patterns may explain the sex differences in the severity patterns that have been described. Interleukin 2 (IL-2) is a cytokine that induces clonal expansion of T-cells [297]. In an *ex vivo* study, it was used to stimulate proliferation of TH_1 (IFN-γ+) and TH_2 (IL-13+) cells. Stimulation of a population of peripheral lymphocytes, resulted in a greater increase in IL-13 and IFN-γ producing cells in asthmatics relative to non-asthmatics [298]. The increase in IL-13 (TH_2) producing lymphocytes was dependent on sex, with a significant increase in the number of IL-13 producing cells occurring in female asthmatics only. There were no sex differences in the number of IFN-γ (TH_1) producing cells. The increase in T-lymphocytes was found to be due to an increased lymphocyte survival and not an increase in proliferation. This suggests that asthmatic females have a greater TH_2 response relative to asthmatic males. Inhaled corticosteroid (ICS) had no effect on the production of cytokine producing lymphocytes in either sex.

Sex differences in immune responses have also been found in relation to the β-2AR. In a study of healthy non-asthmatics, the effect of β-2 agonist on the function of neutrophils was assessed [299]. There were almost three times more β-2 binding sites on neutrophils of females relative to males. The β-2 agonist isoprenaline, increased non-directional locomotion (chemokinesis), stimulated release of a chemotactic factor from neutrophils of females, but had no effect on neutrophils from males. Isoprenaline also inhibited IL-8 induced chemotaxis in neutrophils of females only.

The β-2AR is also important in asthma as the target of β-2 agonist bronchodilator treatment. Stimulation of the β-2AR on ASM cells results in an increase in cAMP production and consequently, relaxation of the muscle and relief of bronchospasm. Beta-2 receptor-stimulated cAMP in lymphocytes of healthy females, during the luteal phase of the menstrual cycle (when oestrogen and progesterone levels are highest), has been shown to be greater relative to males [300, 301]. This variation in cAMP was related to hormonal influences on receptor density [300]. Paradoxically, female asthmatics have been shown to down-regulate β-2 responses in response to cyclic changes in hormones relative to non-asthmatic females [302]. This may account for the increase in symptoms that some asthmatic women experience prior to menses [303, 304].

Atopy

Atopy is another factor influencing asthma phenotype. The effect of atopy on phenotype was discussed earlier in the section on “endotypes”. Broader differences in clinical features between atopic

and non-atopic asthmatics have also been described in the literature. Non-atopic asthma was associated with more frequent requirement for ICS preventers, greater use of intermittent oral corticosteroid (OCS) [305] and more severe and persistent symptoms [305, 306] relative to atopic-asthmatics. These differences may relate to the differences in inflammation patterns as previously described, thus, atopy may be an important determinant of disease expression and treatment response.

Sex vs Atopy

The effects of sex on asthma outcomes may be in part related to the effects of atopy. The incidence of non-atopic asthma is higher in females relative to males in all age groups [307]. Non-atopic asthmatics are more likely to be female, have lower FEV1 values, require ICS and OCS for control of symptoms and have higher severity scores relative to atopic asthmatics [305]. These differences may be due to an interaction between atopic status and sex, that influences the development of asthma in adults.

Female sex was associated with an increased risk of asthma in adulthood relative to males (OR 1.99; CI 1.54-2.57) $p<0.05$ [294]. There was also an increased risk of asthma in atopic relative to non-atopic individuals (OR 3.04; CI 2.40-3.85); however, the effect of atopy on asthma incidence was greater in males (OR 4.9; CI 3.29-7.30) relative to females (OR 2.28; CI 1.68-3.08), and the effect of sex on asthma incidence was greater in non-atopic individuals (OR 3.2; CI 2.12-4.85) relative to atopic individuals (OR 1.4; CI 1.02-2.02). In the same study, the risk of asthma in atopic females decreased with age, but the risk did not decrease in men. Other studies have found a decrease in the incidence of asthma [308] and a decrease in atopy [309], in post-menopausal relative to pre-menopausal women. The effect of menopause on asthma was lost when oestrogen replacement was given [308]. Thus, atopy is a greater risk factor for adult onset asthma in males relative to females, and the decrease in risk of asthma with age in females (unless hormone replacement is given) is due to a decline in the prevalence of atopy. In the absence of atopy, the female sex is a greater risk for adult onset asthma relative to the male sex.

Ethnicity

Differences in asthma phenotypes between African Americans and “white” populations have been described. One USA study found that among asthmatic adults presenting to an emergency department, African-Americans had a greater degree of airways obstruction, as measured by PEF values and were more likely to have PEF <50% and PEF <35% predicted, relative to Caucasians [310]. Atopic disease and asthma among relatives were higher in African Americans relative to “whites” and Hispanics [311]. Differences in endotypes between African American and “white” children with asthma have recently been identified. Eleven cytokines, including IL-4, IL-5, IL-13 and IL-17A, were found to influence asthma severity and symptom control in African American children, while only IL-5 was a

significant factor in symptom control and severity in non-African American children [312]. In acute care facilities, where a high proportion of those presenting with acute asthma exacerbations are African-Americans, there are disparities in asthma outcomes, with African-Americans having the highest frequency of presentations to emergency, admissions and asthma-related deaths compared with 'whites' [313, 314]. These differences are independent of socio-economic status [315]. Genetic variation in the β-2AR gene and higher rates of atopic sensitization relative to "whites", are thought to contribute to these disparities in outcomes [316].

1.3 Management of Asthma

1.3.1 Overview of asthma management

The principles of asthma management are to control the underlying inflammation and relieve the airway obstruction caused by the hyper-responsiveness of the airways. Long-term treatment aims to minimise the frequency and severity of acute exacerbations, while the aim of treatment of acute exacerbations is to prevent deterioration in respiratory function and death. National and international guidelines have similar recommendations for the management of asthma symptoms and acute exacerbations [2-4]. Management of asthma involves pharmacological treatment, directed towards reducing the frequency of symptoms and preservation of lung function, by relieving bronchoconstriction and controlling inflammation. A variety of types of medication are available to manage the underlying pathological processes.

A short acting inhaled β-2 agonist (SABA), either via a metered dose inhaler (MDI) or via wet nebulization, is used to treat the symptoms of bronchoconstriction such as wheezing or cough. The main pharmacological treatment used, to control inflammation and maintain symptom control, is an inhaled corticosteroid (ICS), either alone, or in combination with a long-acting β-2 agonist (LABA). Oral corticosteroids (OCS) are sometimes required to treat an acute exacerbation and may also be required to assist in long-term control of symptoms. Other add-on treatments are also available for long-term management of symptoms.

Good asthma management also includes self-monitoring, avoidance of triggers, abstinence from cigarette smoking and early recognition of and management of acute exacerbations. It also includes regular review by the general practitioner to ensure the prescription of appropriate medications and to facilitate consultation with a specialist, when and if this is required

1.3.2 Management of asthma symptoms

Beta-2 agonists

The β-2 agonists are proven to dilate human airways [317]. They are, and have remained, the mainstay of bronchodilator treatments since their introduction in the 1960s and are used primarily to relieve the symptoms of wheeze, cough or breathlessness. They are the only agents to relax ASM regardless of the contractile stimulus [318] and are therefore, an effective treatment of broncho-constriction in asthma regardless of the trigger. They stimulate the production of the second messenger cyclic adenosine monophosphate (cAMP), which is believed to effect smooth muscle relaxation. Beta-2 agonists are classified as short acting (SABA) or long acting (LABA), dependent on their respective durations of action. Beta-2 agonists also reduce airways hyper-responsiveness (AHR) as demonstrated by an increase in the PD 20 for histamine [319]. Long acting β-2 agonists are also effective in reducing AHR as defined by provocation challenges [320-322]. The β-2 agonists also display intrinsic anti-inflammatory effects [323] and have synergistic effects on inflammation when combined with corticosteroids [324]. In clinical trials in both adults and children, the addition of LABA to ICS therapy provides a modest improvement in symptom control relative to ICS alone [325].

Short acting β-2 agonists such as salbutamol and terbutaline, are the primary pharmacological agents to relieve episodes of bronchoconstriction and wheeze. They are available in a metered dose inhaler (MDI), and the dose can be titrated to effect. They are also available in a solution that can be delivered via an air or oxygen driver nebulizer. The long acting β-2 agonists, formoterol and salmeterol can be used as relievers. Due to concerns about increased mortality and life-threatening events, particularly in African-Americans [326, 327], they are only used in combination with ICS, when ICS alone is insufficient.

Beta-2 agonists can also have adverse effects. Regular SABA use has been shown to increase AHR in atopic asthmatics [328]. With chronic excessive administration, β-2 agonist can interfere with the anti-inflammatory actions of corticosteroids and contribute to worsening of asthma symptoms [329, 330].

Other bronchodilators

Ipratropium and tiotropium are muscarinic antagonists that induce bronchodilation by inhibiting the effect of acetylcholine at the muscarinic receptors (M3) in the airways. Tiotropium is a long acting muscarinic antagonist that was first used in chronic bronchitis. It has been shown to be effective as add-on therapy in those whose symptoms are not well controlled on the combination of LABA/ICS and in reducing the need for oral corticosteroid (OCS) [331].

Although still considered an optional add-on treatment in chronic asthma management [4], theophylline and others in its class are rarely used. Theophylline and its soluble salt aminophylline are inhibitors of phosphodiesterase, the enzyme responsible for the breakdown of cAMP. They are presumed to relax airway smooth muscle through increases in the intracellular content of cAMP via inhibition cAMP breakdown and are also believed to have some mild anti- inflammatory properties [332].

Anti-inflammatory treatment

Corticosteroids with glucocorticoid activity are the mainstay of anti-inflammatory treatment. By reducing inflammation, they reduce AHR and airways reactivity, thereby reducing the number and frequency of episodes of bronchoconstriction and obstruction. Due to their efficacy, they remain the first line anti-inflammatory treatment.

Corticosteroids cross the plasma membrane and bind to the glucocorticoid receptor (GR) in the cell cytoplasm. The hormone receptor complex is then transported to the nucleus where it binds to DNA on glucocorticoid response elements (GRE) [330, 333]. The anti-inflammatory action is thought to be dual. GRE-GR complexes can increase the transcription of anti-inflammatory mediators and directly inhibit inflammatory gene expression via inhibition of nuclear transcription factors such as nuclear factor-κβ [330].

The inhaled corticosteroids (ICS) are effective in controlling symptoms in adults and children, when taken regularly [334, 335]. An inhaled corticosteroid is more effective when combined with a long acting β-2 agonist than when taken alone [325]. This may be due to the additional properties of β-2 agonists as anti-inflammatory agents [336, 337] and modulators of remodelling [338], and synergism with corticosteroids in inhibiting remodelling [337]. Prednisolone is an oral corticosteroid that can be used in short bursts to stabilise chronic asthma symptoms [4].

Additional agents for maintenance therapy

Leukotriene receptor antagonists (LTRA) are antagonists of leukotrienes, another class of inflammatory mediator. They are less effective than ICS [339] and are used as second line medication in controlling asthma symptoms. They can improve control as ‘add-on’ therapy to ICS [340] and are effective in aspirin intolerant asthma [253].

Sodium cromoglycate and nedocromil sodium are both structurally different but have similar mechanisms of action. They are administered as an inhalation. They inhibit cough, mast cell and eosinophil activation and other pathways in allergic responses via chloride channel inhibition [341]. They are less effective in controlling asthma symptoms relative to ICS, but useful in allergic/atopic asthma, cold air induced asthma and some forms of occupational asthma [342, 343].

Newer treatments

Monoclonal antibody therapy is a more recently available option for those with difficult to control symptoms, in place of ongoing oral corticosteroids. They have very specific indications for use in those with difficult to treat allergic asthma, and prescription is limited to those over the age of 12 who are under respiratory specialist care. Monoclonal antibodies block interleukin signalling, so can be effective in treating the persistent eosinophilic inflammation by reducing the production and recruitment of eosinophils.

Omalizumab is an anti-IgE antibody and mepolizumab, benralizumab and reslizumab are anti-IL5 antibodies. They are given as a subcutaneous injection monthly. These agents have been shown to reduce the frequency of exacerbations, improve lung function and reduce asthma symptoms as measured by ACQ scores [344-346]. Anti-IL13 antibodies have been developed but are not yet available for use [347].

1.3.3 Management of acute exacerbations

The treatment of an acute asthma exacerbation is directed at maintaining adequate oxygenation of the blood and reducing work of breathing by relieving airway obstruction. Mechanical support of respiratory function may be necessary in extreme situations, to prevent death from respiratory failure due to severe obstruction and the resultant fatigue. Treatment is tailored to the severity of the exacerbation.

Pharmacological treatments

Despite the variety of agents available and the recent pharmacological developments in the management of chronic symptoms, no new pharmacological agents have been developed for management of acute asthma exacerbations. There are also few agents with proven clinical efficacy and hence, treatment options for the management of acute exacerbations are limited.

Supplemental oxygen

Supplemental oxygen is indicated where oxygen saturation is <94% and is titrated to maintain an oxygen saturation between 94-98% [4]. Oxygen can be delivered via nasal prongs to a flow rate of up to 4 litres per minute. Higher flow rates may be delivered via a mask, a non-invasive ventilator or invasive mechanical ventilation.

Bronchodilators

Short acting β-2 agonists such salbutamol and terbutaline are used to alleviate bronchospasm and are the mainstay bronchodilators used in the treatment of acute exacerbations of asthma. They can be given as an inhalation via a metered dose inhaler (MDI) or via wet nebulisations. They are very effective in relieving airway obstruction via either route [348]. Inhaled β-2 agonist is given initially as 'rescue treatment' in bursts of three nebulisations during the first hour but can be given continuously for as long as is required [2, 4, 268]. Once breathing has improved, the frequency of inhalations can be titrated according to need, with the aim of reducing the frequency of inhalations and eventually weaning β-2 agonist treatment over two to three days, depending on the severity of the attack.

Inhalation via MDI is suitable for initial treatment of mild to moderate exacerbations. Guidelines recommend 400-1200mcg salbutamol inhalation via a holding chamber or spacer, every 20-30 minutes, until there is an effect [2]. Absorption of β-2 agonists via the inhaled route can be variable, dependent on airway calibre. Greater degrees of airway obstruction are associated with reduced systemic absorption and reduced bronchodilating effect, necessitating higher doses [349].

The nebulised route is preferred for the more severe exacerbations and should be used in clinical situations where supplemental oxygen is required [2, 4, 268]. Salbutamol nebulisations of 5mg can be given intermittently at 20-minute intervals [2]. Continuous nebulisations are more effective than intermittent nebulisations, in reducing admission to hospital and improving obstruction and are recommended for severe and life-threatening exacerbations [350]. Although the intravenous route has been utilised for severe/acute exacerbations, it is now discouraged, as it has been shown to have an adverse side-effect profile without any clinical benefit over nebulised treatment [351]. Its use is reserved for patients where inhalation/nebulisation is unreliable [4].

Excessive or high dose β-2 agonist use in acute cases, can lead to biochemical disturbances such as lactic acidosis [352, 353] and hypokalaemia [354]. Lactic acidosis may contribute to increased respiratory effort [355] and complicate assessment of severity, leading to inappropriate further high dose use of β-2 agonist [353]. Although hypokalaemia may predispose to adverse cardiac events, a direct link between hypokalaemia and asthma morbidity/mortality is difficult to establish [354].

Ipratropium has been shown to be an effective adjunct in the treatment of acute asthma in children and adults. When administered with initial treatment of inhaled or nebulised β-2 agonist, as a single inhalation or nebulisation, ipratropium was found to improve airways obstruction and reduce admission rates relative to β-2 agonist alone [356-358]. The greatest improvements in airways obstruction were found in those with the lowest initial FEV1 or PEF values [357]. Ipratropium can be administered via inhalation or wet nebulisation, concurrently with β-2 agonist. Guidelines vary in recommendations. The British guidelines suggest 500mcg via nebulisation to be given as either as a

single initial dose with rescue treatment, followed by repeated dosing 6 hourly until resolution of symptoms [4]. The Australian guideline advises 500mcg nebulised concurrently with salbutamol, for three doses at 20-minute intervals for the first hour [2].

Corticosteroids

Oral corticosteroids, such as prednisolone, are effective in treating acute exacerbations [359], reducing hospital admissions [360] and preventing relapse [361]. Hydrocortisone is a corticosteroid that can be given intravenously where oral administration is not practical, i.e. life-threatening exacerbations and mechanically ventilated patients. Guidelines recommend 50mg of oral prednisolone within the first hour of treatment, regardless of asthma severity [2, 4, 268]. If the intravenous route is preferable, hydrocortisone can be given intravenously at 100mg 6 hourly [2, 4, 268]. Corticosteroid treatment should be continued daily for at least 5 days, or until recovery, for up to 10 days [2, 4, 268].

Optional add-on treatments

If there is no response or insufficient response to the above treatments, then there are no clinically proven and few guideline-recommended additional treatment options. The use of magnesium in acute asthma is discussed in detail in the following section.

Aminophylline

In those not already taking oral theophylline, aminophylline is given as a 5mg/kg loading dose infusion over 20 minutes, followed by a continuous infusion of 0.5-0.7 mg/kg/hour [4]. When compared with intravenous salbutamol [362] or added to high dose inhaled β-2 agonist [363, 364], aminophylline provided no clinical advantage relative to salbutamol alone and resulted in an increase in adverse effects, such as vomiting and abnormal cardiac rhythms [364]. Although aminophylline is included in the current BTS guideline, as an optional add-on treatment for those non-responsive to initial treatment, its use is discouraged due to the adverse side effect profile and lack of proven benefit in acute asthma [2, 4, 268].

Ketamine

Ketamine is listed as an option in the BTS/SIGN guideline, as there is some evidence that it is an effective bronchodilator [365]. An intravenous bolus followed by an infusion may reverse bronchoconstriction, improve symptoms and prevent the need for mechanical ventilation in status asthmaticus and assist in the weaning of mechanical ventilation [365]. It does not appear to have any benefits in those with less severe exacerbations, in terms of reduced hospital admission rates or improved airways obstruction [366, 367].

Heliox

Heliox is a gas that is a mixture of oxygen and helium. The principle behind its use is to reduce the work of breathing by delivering oxygen in a vehicle with a lower airflow resistance relative to the nitrogen present in air. Its use alone in asthma is not recommended [4, 268, 368] however, a recent meta-analysis suggests that it is effective as a vehicle for delivering β-2 agonist nebulisations and produces greater improvements in airways obstruction and reductions in admission to hospital, in adults and children, relative to the standard oxygen driven methods [369]. The reviewer suggests that its use in routine care of acute asthma is appropriate, but this practice has not been recommended in the more recent guidelines [2, 4, 268]. This may relate to the necessity of specialised breathing circuits for the delivery of heliox.

Treatment of respiratory failure

Fatigue or severe obstruction, with collapse and occlusion of the small airways, results in severe hypoxia and ultimately death if respiratory function is not supported. Retention of carbon dioxide as measured on arterial blood gas, or indicated clinically by drowsiness, heralds an impending respiratory arrest. In this circumstance, support of respiratory function is required to prevent death [4].

Assisted ventilation

Intubation and mechanical ventilation are a last resort, but necessary to prevent death from hypoxia. This procedure must be undertaken cautiously by those clinicians with experience in managing mechanically ventilated asthmatics, as there are potential adverse consequences of overexpansion of the lungs, raised intra-thoracic pressure and tension pneumothorax [370]. Indications for intubation and mechanical ventilation are drowsiness, hypoxia despite supplemental oxygen and respiratory arrest [4]. Once ventilated, bronchodilators may be administered via the endotracheal tube or intravenously.

Non-invasive ventilation (NIV) has been used to avoid the need for mechanical ventilation. The efficacy of non-invasive ventilation in severe exacerbations has been reviewed in a meta-analysis [371]. The reviewers were unable to draw conclusions because the data was insufficient. Although listed in guidelines as an option for respiratory failure, it is acknowledged that there is a lack of evidence in support of its use [2, 4, 268].

Pulmonary bypass

Extracorporeal membrane oxygenation (ECMO) is an invasive method for supporting cardio-respiratory function and was originally used during cardiac bypass surgery [372]. It can provide oxygenation of the blood through an external apparatus bypassing the lungs [373]. This procedure can only be performed in an intensive care setting. It has recently been reviewed as a method for

supporting respiratory function in mechanically ventilated asthmatics and was effective in patients where mechanical ventilation was unable to provide satisfactory oxygenation [374]. No patient suffered adverse neurological sequelae however, the sample size in this review was only16 patients. This treatment is not listed in current asthma guidelines [2, 4, 268].

1.4 Magnesium and asthma

1.4.1 Overview

Magnesium is listed as an optional add-on therapy in severe asthma and in those non-responsive to treatment [2-4]. The evidence is limited therefore, its use is not strongly advocated. The clinical trials of magnesium in asthma varied greatly in methodology, inclusion criteria and discharge criteria. This may be a reason for the inconsistent results however, individual factors have yet to be considered when assessing magnesium's efficacy. There are several factors that may contribute to this individual variation in response to magnesium. These factors should be taken in to consideration when assessing the utility of magnesium in the treatment of acute asthma.

There is much variation within the asthmatic population with respect to clinical phenotype, as well as underlying pathogenic processes and response to treatment. It would be reasonable to hypothesise that these differences could translate to a difference in response to magnesium and hence, there may be subgroups that benefit from magnesium more than others. This is the situation for treatment of asthma with monoclonal antibodies which are effective in those with high sputum eosinophil counts. Expert opinion advocates for targeted therapy based on asthma endotypes [375]. This principle could be relevant to the use of intravenous magnesium in acute asthma. Asthma endotypes may be important to the efficacy of magnesium, given the wide variations in efficacy between studies.

1.4.2 Efficacy of magnesium in the treatment of acute asthma

Overall effects of intravenous magnesium

Although there has been anecdotal evidence from case reports, the clinical trials assessing its efficacy along-side standard treatments have had varied results, therefore, there is a lack of conclusive evidence to support the use of intravenous magnesium in acute asthma. There have been few published clinical trials, most of which have been small and with varied results (Table 1). Comparisons between studies is made difficult by the inconsistency in methodology between studies and a lack of well-defined admission/discharge criteria. In addition, some studies were underpowered

to detect the differences in outcomes reported [376-378]. Meta-analysis has attempted to assess magnesium's efficacy [379, 380], but is limited by the factors previously mentioned. The most recent meta-analysis suggests that magnesium is an effective adjunct to treatment in adults presenting to emergency departments with acute asthma exacerbations however, the overall effect on admission rates is small (7%) [380]. Given the effect size, larger numbers would be required to confirm the utility of routine use of magnesium to treat acute asthma exacerbations.

Severe asthma subgroups.

Some studies have reported large significant benefits in small studies of severe asthmatics [381-383] and within the severe subgroups of larger studies [384]. Magnesium use was associated with a reduction is admission rate and greater improvements in spirometry relative to placebo; thus, the Cochrane group concluded that asthma severity and non-response to treatment in terms of improvements in airways obstruction, were factors in determining treatment response to magnesium [379].

Skobeloff found that acutely exacerbating asthmatics, whose initial PEF was <200L/min and failed to double after 2 salbutamol nebulisations, showed a 32% improvement in absolute PEF values within 2 hours of treatment with intravenous magnesium, while those receiving placebo showed no improvement in PEF [382]. In another study, intravenous magnesium was also found to reduce admission rates by 24% in those with a presenting FEV1 <30% predicted, accompanied by a modest (6%) but significantly greater improvement in airways obstruction relative to the placebo group [383]. Bijani et al found that for those with an initial PEF <200L/min, who failed to increase their PEF >200L/min after 6 hours of treatment with bronchodilators and steroids, intravenous magnesium produced a greater improvement in airways obstruction relative to standard care alone [385]. PEF data were reported as the percentage predicted, and the mean initial PEF was similar for both groups at <30% predicted. Those in placebo failed to increase their PEF to <50% predicted, while those treated with magnesium had a mean PEF >80% predicted at the end of the study period.

Porter failed to find any benefit from magnesium in a group of severe asthmatics with an initial FEV1 of <25% predicted [386]. Green also failed to find a benefit from magnesium in a group with an absolute initial PEF <150 L/min [377]. In both studies, those in placebo and treatment groups doubled their initial PEF values with nebulisations of β-2 agonist. These patients may have been excluded from the Skobeloff and Bijani studies as they responded well to initial β-2 agonist.

Tiffany replicated Skobeloff's study protocol, with the addition of a third group that received a magnesium infusion of 2g(8mmol)/hour for 4 hours. This study failed to demonstrate any difference in effect on improvements in airways obstruction between magnesium and placebo [387]. Both

magnesium and placebo groups had similar initial PEF values (120 vs. 130L/ min), and both groups had limited response to further β-2 agonist treatment, with PEF values remaining below 200L/min.

Studies where patients of all severities were included, also had variable results. Silverman found that the use of magnesium in a subgroup of those with FEV1<25% predicted, resulted in a greater improvement in FEV1 predicted relative to standard care alone [384]. The effect was greatest in those with an initial FEV1<20% predicted. The mean baseline FEV1 was 16% predicted, and the administration of magnesium increased FEV1 to 41% predicted relative to 34% predicted in placebo. There was a smaller benefit in those with FEV1 25-29% predicted which was not statistically significant. The admission rate overall was not reduced however, had admission criteria of a final FEV1 >50% predicted been strictly applied, the use of magnesium would have resulted in a 10% reduction in admission rate.

Bloch found also found that in the subgroup of those with an FEV1 < 25% predicted, magnesium produced a 20% greater improvement in FEV1 predicted relative to placebo [381]. Those receiving a placebo failed to improve their FEV1 with further β-2 agonist, despite a small initial improvement. There was also a significant 45% reduction in admission rate in the magnesium group relative to placebo.

Table 1. Randomised control trials of intravenous magnesium in asthma. Comparison of severity and patient demographics (over page).

Author (year)	N	% female	Age range (yrs)	Predominant ethnicity	Severity	Subgroup	Dose of Mg	Improved lung function relative to placebo	Reduction in admission rate
Skobelloff (1989)	38	73.5	18-70	African american	PEF <200L/min post beta-2		1.2g (4mmol)	32%	42%
Green (1992)	120	76.6	18-65	White	symptomatic after single beta-2 agonist treatment		2g (8mmol)	no difference	no difference
Tiffany (1993)	48	47	18-60	not stated	PEF <200L/min on arrival		2g (8mmol)	no difference	N/A
Bloch (1995)	135	72	18-65	not stated	FEV1 <75% predicted after single beta-2 agonist treatment	FEV1<25% predicted	2g (8mmol)	15% **	45%**
Boonyavourakul (2000)	34	88	15-65	Thai	FISCHL index ≥ 6		2g (8mmol)	favours Mg	no difference
Bijani (2001)	81	53	12-85	Iranian	PEF <200L/min on arrival		0.025g/kg (0.01mmol/kg)	favours Mg	N/A
Porter (2001)	42	64	18-55	not stated	PEF<100L/min or <25% predicted		2g (8mmol)	no difference	no difference
Silverman (2002)	248	52	18-60	Hispanic/African american	FEV1 < 30% predicted	FEV1 <25% predicted	2g (8mmol)	10%**	N/A
Bradshaw (2008)	129	57.4	>16	not stated	PEF <75% predicted		1.2g (4mmol)	no difference	no difference
Singh (2008)	60	52	18-60	Indian	FEV1 <30% predicted		2g (8mmol)	6%	23%
Goodacre (2013)	752	70	> 16	White	PEF <50% predicted		2g (8mmol)	no difference	ns

** severe subgroup only

In two other studies in acute asthma of mixed severities, magnesium was not shown to have any significant effect on admission rates or improvements in PEF relative to placebo, nor in the sub-group of severe/life-threatening asthma [378, 388]. Bradshaw failed to find any beneficial effect from magnesium on PEF or a significant reduction in admission rates in those with PEF <33% [378]. The Goodacre (3M) study (which included an inhaled magnesium group) also failed to find any beneficial effect on PEF or a significant reduction in admission rates in any sub-group analyses of severity [388]. The overall improvement in PEF was similar in both studies. These results contrast the Silverman and Bloch studies.

Magnesium's efficacy may be influenced by individual factors, in addition to severity and response to initial bronchodilator treatment, and individual variation in response is likely to be a contributing factor to the small overall effect. It is, therefore, important to examine these studies in detail to determine which factors may contribute to efficacy and to identify those more likely to benefit.

Factors that may limit or influence magnesium's efficacy

Magnesium dose

It is possible that there is a dose-response relationship between magnesium and efficacy, as magnesium dosing varied between the studies. However, both 1.2g(5mmol) [382] and 2g(8mmol) [384] were effective in small studies, while 2g was ineffective in the larger Goodacre study [388]. This suggests that factors other than dose may be involved.

Ethnicity

There may be an interaction between ethnicity and severity, in terms of bronchodilator responsiveness, that may also influence magnesium's efficacy. The ethnicity of populations studied varied greatly, however, the results appeared to favour magnesium in populations of mostly non-whites with "severe" asthma. Skobeloff's study, which demonstrated the greatest benefits from magnesium, had a large proportion (n=34, 89%) of African-Americans. These 'non-responders' were true non-responders and failed to increase PEF in response to β-2 agonist without the addition of magnesium to their treatment. This study demonstrated the greatest reduction in hospital admission rates for magnesium over placebo (42%) of all studies to date. Studies in mixed populations of African Americans and non-white Hispanics [384] have had similar success from magnesium, although, not with the same magnitude of effect as Skobeloff. Racial demographics for the Tiffany and Bloch studies were not reported, limiting an assessment of the effect of race on the β-2 responsiveness and efficacy of magnesium in these studies. Bijani, in an Iranian population [385] and Singh, in an Indian population [383], found benefit from the use of magnesium. Boonyavorakul's study in Thailand favoured magnesium, but the sample size was too small to conclude any benefit [376].

Green [377], Bradshaw [378] and Goodacre [388] failed to find benefit from the use of magnesium in acute asthmatics of mixed severities nor in the severe subgroups. The Green study in the US, consisted of >60% (n=74) white, 24% (n=29) Hispanics and 7.5% (n=9) African-Americans. In this study, there was a doubling of PEF values overall irrespective of treatment and the overall admission rate was low (20%), thus the potential for magnesium to show benefit would have been limited by the responsiveness of the cohort to bronchodilator. The major ethnicity in the Goodacre study was white (90 %, n=974), with less than 1% (n=11) classified as 'black' or 'black British'. The demographic of ethnicity was not reported in the Bradshaw study.

Ethnic differences in response to various asthma treatments is well documented. Asthmatic African-Americans were found to be less responsive to β-2 agonist relative to Caucasians asthmatics at maximal doses of inhaled β-2 agonist, but baseline lung function parameters were not significantly different [389]. Asthmatic African-Americans, Mexican Americans and Puerto-Ricans, were found to have below expected responses to β-2 agonist relative to Caucasians [390]. The same author found that in African American asthmatics, ICS does not increase bronchodilator response to β-2 agonist [391]. Clinical studies have also found that long-term LABA use resulted in increased adverse outcomes, including mortality, in African-Americans relative to other races [392]. As mentioned previously, the reason for the poorer outcomes for African Americans is thought to relate to racial differences in asthma phenotype and to be independent of socio-economic status [315, 316].

These data suggest that ethnicity is an important variable that may affect treatment response. Magnesium, therefore, may be more beneficial in certain ethnic groups. Further studies to investigate the effect of ethnicity on response to magnesium could prove African Americans to be one of those groups. The reason for a difference in efficacy may relate to a difference in β-2AR responses. Further studies in to the effect of ethnicity on outcomes related to the use of intravenous magnesium in asthma, may be worthwhile.

Other potential outcome benefits from the use of intravenous magnesium in acute asthma

Despite a lack of proven efficacy in Caucasian asthmatics overall, magnesium may have some benefits in terms of reduction in relapse rates and/or improvement in symptoms. Although the Green study failed to show any reduction in admission rate with the use of magnesium, there was an 8% relapse rate (n=5), requiring a return visit to the emergency department within 72 hours in the placebo group, while the relapse rate in the magnesium group was only 1.7% (n=1) [377]. Silverman also reported a difference in relapse rates, with 3% (n=4) of those in the placebo group and less than 1% (n=1) in the magnesium group re-admitted at 7 days [384]. These studies are small, and larger numbers of participants would be required to confirm such an effect on readmission to hospital. Relapse rates in the Goodacre study were less than 1% and therefore, too small for comparison [388].

Although Bradshaw reported no benefits from the use of magnesium, there was a non-significant trend towards a reduction in the number of β-2 agonist nebulisations administered to the magnesium group (n=20) relative to the placebo group (n=27) [378]. The study was small (n=129) therefore, it is not possible to draw conclusions regarding magnesium's effect on β-2 agonist use. Larger studies would be needed to further assess this.

Intravenous magnesium and status asthmaticus

There are no published clinical trials in which the utility of magnesium in status asthmaticus has been assessed. This is likely due to the difficulties in obtaining informed consent, or ethical dilemmas faced when withholding a potentially beneficial treatment in those patients whose life is under immediate threat. The only data available in the literature, is in the form of case reports and case series. Some studies reported that 1-2g of intravenous magnesium given as a rapid bolus, produced marked improvements in clinical symptoms in adults with impending respiratory failure and obviated the need for intubation [393, 394]. The use of 1.2g [395] and much larger doses, in the range of 10-20g (40-80mmol), have also been reported to be successful in reducing significantly increased airway pressures in mechanically ventilated asthmatics, where high airway pressures had persisted despite the use of maximal doses of intravenous β-2 agonist [396-398].

These data support the use of magnesium in status asthmatics, however, this data may be biased towards positive outcomes, as negative outcomes are less likely to be reported or published. Meaningful evaluation of magnesium's efficacy in these circumstances is limited.

Inhaled magnesium

There is little evidence in support of the use of inhaled magnesium in as a bronchodilator in acute or stable asthma in adults. Inhaled magnesium was not shown to be effective in stable asthmatics with chronic obstructive symptoms [399]. Nebulised magnesium was not shown to be an effective bronchodilator or influence airways resistance (Raw) in stable asthmatics [400]. The 3M study found no difference in admission rate between inhaled magnesium and placebo (79% vs. 78%) [388]. A Cochrane meta-analysis in 2005 supported the use of inhaled magnesium in acute asthma [401] however, more recent reviews are in conflict. One review found there were benefits related to reduced admission rates and lung function [402]. Others found no overall benefit in outcomes [403] and recommended intravenous over inhaled magnesium [404].

Inhaled magnesium may be of benefit in stabilization of AHR. Inhaled magnesium has been shown to reduce bronchoconstriction responses and prevent bronchoconstrictor responses in stable asthmatics, as defined by FEV1 >80% predicted [405-407]. When bronchial provocation response in asthmatics were measured by PD 20 or PC 20 to histamine and/or methacholine, inhaled magnesium increased

PD 20/PC 20 to histamine and methacholine relative to the placebo [405-407]. The effect of inhaled magnesium on PD 20 to histamine was also found to be dose-dependent [406]. Inhaled magnesium was also shown to reverse methacholine and histamine-induced bronchoconstriction [405, 407] and irritant induced bronchoconstriction [408].

The combination of inhaled magnesium and salbutamol has been effective in improving lung function in asthmatics [409]. A small trial showed inhaled magnesium in combination with inhaled salbutamol and intravenous corticosteroid, to improve airways obstruction and reduce admissions relative to standard bronchodilator therapy [410]. These data suggest that the addition of magnesium to standard bronchodilator therapy is more effective than inhaled magnesium alone. While Australian and British guidelines do not recommend inhaled magnesium in adults [2, 4], international guidelines (GINA) include inhaled magnesium as an option for dilution of salbutamol for nebulisation [268].

Magnesium in managing acute asthma in children

The data in clinical trials of children are more supportive of intravenous magnesium's clinical efficacy [411-416], and some recommend the use of intravenous magnesium in children to improve lung function and reduce admissions [402, 417]. The most recent Cochrane review was less supportive due to the small sample sizes in the studies reviewed [418]. Another recent review suggests that intravenous magnesium prevents one admission in every five children treated [419].

Inhaled magnesium has been shown to induce bronchodilation in children [420] however, it was less efficacious relative to salbutamol. The most recent review of magnesium use in children does not recommend the inhaled route [421].

The differences in the apparent efficacy of intravenous magnesium between adults and children, may be explained by the heterogeneity of adult asthma and associated co-morbidity, relative to the often 'otherwise healthy' childhood disease. Childhood onset asthma is often of the 'extrinsic' type which is associated with the presence of eosinophilic inflammation and a good response to steroids [270-272]. Adult asthma, though sometimes the persistence of a childhood onset disease, is often its own entity. The adult phenotypes sometimes share features in common with childhood disease [270, 271] however, they are more often persistent and severe [270, 273, 274]. Adult asthma may also be complicated by a greater degree of fixed obstruction due to the persistence of symptoms from childhood [422-424], co-morbid chronic airways disease [425] and/or the effects of cigarette smoking [426-428], which are generally absent in children. These differences may account for differences in response to magnesium between adults and children.

Current practice/usage in acute exacerbations

Intravenous magnesium sulphate has been recently included in guidelines for acute asthma treatment. It is suggested as a second line agent for those who fail to respond to standard bronchodilator treatment and severe asthma as determined by PEF or FEV1 [2, 3]. Guideline definitions of 'severe' asthma suggest PEF <50% predicted signifies a severe exacerbation [4, 268], while PEF or FEV1 <30% is classified as an indicator of a 'life-threatening' exacerbation [4, 268]. In contrast, early literature defined severe as PEF or FEV1 <30% [381-383]. Recommendations for its use in life-threatening asthma are weak [2, 4] or absent [268]. There is also a perception of potential for toxicity from the use of intravenous magnesium [4] however, this does not appear to have impacted on its usage [429].

The current practice of use of intravenous magnesium in adult acute asthma, has been assessed in two large surveys of emergency departments in Britain [429] and Canada/United States [430]. In Britain, magnesium was used to treat acute asthma in 93% of emergency departments. Reported reasons for its use, included non-response to treatment and severe or life-threatening asthma. Lack of evidence of efficacy was the most commonly stated reason for not using it. In the US, although 93% of respondents reported magnesium was available, magnesium was only used in 2.5% of patients with acute exacerbations. Reported reasons for its use were older age, previous need for intubation, high initial respiratory rate, low PEF and failure to respond to initial β-2 agonists. A recent small survey of 456 physicians in Turkey, found that only 48% were aware of its inclusion in the BTS, GINA asthma guidelines [431]. Usage was again restricted to severe and life-threatening exacerbations.

These data show consistency between guideline recommendations and usage however, perception of its efficacy and awareness of magnesium as an option for treatment of acute asthma exacerbations, may influence its use across the world.

1.4.3 Magnesium status and asthma

Magnesium status may also be important in asthma exacerbations and lung function however, the data are not consistent. The role of magnesium status in asthma is not well defined and results of studies vary, depending on the methods of assessment used.

Studies have not shown any differences in serum magnesium levels between asthmatics and non-asthmatics. No difference in serum magnesium was found between exacerbating asthmatics and controls [432], stable asthmatics and controls [433] or between children with asthma, exacerbating or stable, and non-asthmatics [434]. One study reported no differences in multiple measures of magnesium status between mild, well-controlled asthmatics and controls [435]. Although serum

levels were the same, there were some non-statistically significant differences, with asthmatics having slightly greater magnesium retention post IV loading and a slightly lower dietary magnesium intake relative to non-asthmatics. Low serum magnesium levels in asthmatics were associated with increased frequency of exacerbations and the need for preventative therapy [436]. This has also been found among patients with chronic airways limitation [437]. These data show that serum magnesium is not a surrogate marker of asthma, but magnesium deficiency may impact on disease severity in those with asthma.

This is supported by studies where total and free intracellular magnesium levels were measured. In exacerbating asthmatics, levels of erythrocyte magnesium but not serum levels, were reduced relative to both non-exacerbating asthmatics and healthy controls [438]. Exacerbating asthmatics also showed a return to normal intracellular levels when exacerbations were controlled [438]. Significantly lower erythrocyte magnesium levels in stable asthmatics relative to healthy controls have also been found [433]. These data suggest that low intracellular magnesium concentrations may be associated with acute exacerbations.

Histamine challenge in mild well-controlled asthmatics and non-asthmatics, was shown to reduce free intracellular erythrocyte magnesium levels without altering serum levels. The decrease in intracellular magnesium was not correlated with AHR [439]. Another study in asthmatics found that exacerbations were associated with elevated neutrophil counts, blood histamine, and inversely correlated with serum and red cell magnesium levels [440]. This effect was most pronounced in atopic relative to non-topic asthmatics. Low intracellular magnesium concentrations in neutrophils [441] and red blood cells [442, 443] was inversely correlated with AHR as measured by methacholine challenge but not severity of asthma as determined by FEV1 or PEF [442]. These studies suggest that reductions in serum/intracellular magnesium occur during an exacerbation and may be associated with AHR.

In adults with mild to moderate asthma, long-term (6.5 months) magnesium supplementation was shown to improve lung function, reduce AHR and improve quality of life, although, without significant changes in any of the multiple measures of magnesium status used [444]. It is also notable that only 2 of the 52 asthmatic patients were found to be deficient in magnesium thus, the improvements were independent of magnesium status. These data suggest that magnesium supplementation may be beneficial in terms of control of AHR, independent of magnesium status.

Sex may influence the effect of magnesium status. Poor dietary magnesium intake in children between 11 and 19 years was correlated with reduced lung function as measured by FEV1, in both asthmatics and healthy controls [445], however, only in females was there a difference in effect between healthy and asthmatic patients.

1.5 Gaps in Knowledge

1.5.1 Clinical benefits

The efficacy of magnesium in asthma has not been established however, future clinical trials may determine whether magnesium is efficacious in the treatment of acute exacerbations. It is clear from the heterogeneity in the results of published trials, that large numbers of patients may be required to demonstrate this.

The 3M trial is the largest clinical trial to date, yet it failed to demonstrate a significant reduction in admission rates or improvement lung function with intravenous magnesium (a 7% difference in favour of magnesium was not statistically significant) [388]. This suggests larger numbers of patients are needed to demonstrate an overall benefit. Additionally, there may have been other factors that limited magnesium's efficacy in this study. The trial included older patients, therefore, could have included those with co-morbid illnesses that predispose an admission to hospital, irrespective of treatment responses. The inclusion of asthmatics of all severities could also potentially mask any benefit from magnesium. It is, therefore, important for future studies to assess the effect of severity on treatment response and perhaps exclude those with significant co-morbid illness that could influence disposition. There were no pre-defined admission or discharge criteria in this multicentre study, which could also have influenced outcomes. Future studies should have well-defined admission and discharge criteria to reduce the influence of local admission policies on outcomes.

The heterogeneity in the results of clinical trials suggests that there is individual variation in response to treatment, therefore, there may be subgroups of patients more likely to benefit from the use of magnesium. A trial targeted towards those most likely to benefit, such as non-responders to initial β-2 agonist, may be more successful without the need for large numbers of patients. Ethnicity and other individual variations could also influence outcome therefore, larger numbers may still be required to determine any subgroup benefits. Future studies should collect demographic data such as race/ethnicity and atopic status, in addition to sex and other variables, to adequately assess the effect of individual variation on response to magnesium.

Whether the use of magnesium in acute asthma exacerbation may have other benefits, for example, in reducing relapse rates and β-2 agonist use, is yet to be determined. Outcome data should include relapse/readmission to hospital and frequency of nebulisations.

1.5.2 Potential mechanism of action

Bronchodilation

Magnesium has been shown to have bronchodilating properties. Magnesium was used as early as 1936, for the treatment of the bronchospasm associated with an acute asthma exacerbation, prior to the advent of β-2 agonists [446]. A small study of exacerbating asthmatics showed that 2g(8mmol) of intravenous magnesium had a direct bronchodilating effect, as shown by an improvement in FEV1 relative to placebo, but the effect of magnesium on FEV1 was less than that of inhaled salbutamol [447]. Thus, magnesium has a direct effect on airway smooth muscle relaxation. This could occur via several possible mechanisms

Calcium antagonism

In arterial smooth muscle, magnesium competitively inhibits L-type voltage gated calcium channels. Pharmacological blockade of these channels causes vasodilation in human vascular smooth muscle [448].When infused intravenously, magnesium has been shown to cause vasodilation in healthy volunteers [449], and the action of magnesium was blocked by simultaneous infusion of calcium gluconate [449]. These data show that magnesium causes vascular smooth muscle relaxation via competitive inhibition of L-type voltage gated calcium channels.

Inhibition of voltage gated calcium channels is unlikely to be the mechanism of HASM relaxation, as pharmacological blockers of L-type channels have very minimal beneficial effect on lung function in asthma [450], and when inhaled, they have been shown to induce bronchoconstriction in asthmatics [451]. The lack of effect is likely due to the voltages at which these calcium channels operate in HASM. These channels are not active at potentials of less than -20mV, which is outside the range at that occurs during HASM activity (-25mV and -55mV) [87]. The negative potential is maintained by potassium channels that maintain membrane polarity, despite changes in the flow of calcium [452, 453]. These data suggest L-type channel blockade is not the primary mechanism of action of magnesium in HASM relaxation, and that magnesium may compete with calcium via an alternative mechanism.

Lowering of intracellular calcium

Magnesium was shown to relax porcine ASM pre-contracted with potassium chloride (KCL) and a muscarinic agonist carbachol in a dose dependent manner, over a concentration range of 1.2 to 9.2 mM [454]. This was associated with a fall in intracellular calcium. Magnesium also inhibited KCL-induced but not carbachol-induced contractions, as shown by a rightward shift of the dose response curves, suggesting an inhibitory effect on voltage gated calcium channels [454]. Magnesium was

shown to relax rabbit ASM strips pre-contracted with acetylcholine in a concentration dependent manner, over a concentration range of 0.1mM to 2M [455]. The steepest decrease in contraction was observed at ≥10mM. Magnesium was also shown to relax resting, histamine, muscarinic agonist and electrically stimulated ASM in rabbits in a dose-dependent manner [456]. The lowest dose used (1mmol) was effective at relaxing resting ASM but not for contractile agonist or electrically stimulated ASM. Higher doses (≥ 5mmol) were also used and were able to relax contracted ASM. This relaxation was not antagonised by calcium, suggesting a different mechanism to L-type calcium channel antagonism.

In canine nerve membrane preparations, an inhibitory effect on the intracellular release of stored calcium via IP3 gated calcium channels has been shown to occur by competitive inhibition of IP3 binding [457]. The Na^{+}/Ca^{2+} exchanger (NCX) regulates calcium influx and is present in many cells including ASM [458]. Magnesium acts as a competitive antagonist of calcium influx through these channels [459]. Magnesium competitively inhibits calcium binding to the cytoplasmic binding sites and alters the conformation of NCX. This prevents calcium influx and occurs at intracellular magnesium concentrations of 0.5 to 1 mM. Magnesium may affect relaxation of HASM via decreased intracellular calcium via one or both mechanisms.

Effects on β-2 adrenergic receptor function and adenylate cyclase activation

Increased levels of intracellular magnesium may relax ASM through enhanced 'basal' AC/cAMP production via numerous mechanisms. In isolated membranes from yeast cells transfected with Sf9 AC, Magnesium was shown to catalyse AC in a concentration dependent manner [460]. Catalytic activation was observed at free magnesium concentrations of 0.1-1.0mM, which was up to 10-fold the normal free ionised levels. This range corresponded to the steepest part of the concentration/rate curve.

In intact mouse lymphoma cells, magnesium increased basal AC and GTP stimulated AC activity, but this effect was abolished in the presence of β-2 agonist [461]. Beta-2 agonists have been shown to affect magnesium transport across intact mouse lymphoma cell membranes via the β-2AR [462] but independent of cAMP [463]. This process was shown to undergo rapid desensitisation which was reversed after 30-60 minutes [464]. Magnesium uptake without influence of a β-2 agonist was shown to be dependent on extracellular concentrations, with a maximal rate of uptake at 50μM to 3mM [465]. The effect of the administration of intravenous magnesium on intracellular levels may be affected by concurrent β-2 agonist use however, this effect would be short-lived.

Inhibition of AC activity, as mediated through the Gβγ sub-unit, involves inhibition of GDP release and GTP binding [466]. Physiological concentrations of magnesium (1mM) caused this sub-unit to dissociate, and higher concentrations of magnesium promoted GDP dissociation and increased affinity

of GTP binding to the Gsα sub-unit [466]. These data suggest that greater than physiological levels may increase AC activity by antagonising the inhibitory effect of the Gβγ sub-unit.

Magnesium has also been demonstrated to influence β-2AR affinity. In intact mouse lymphoma cells [461] and rat lung membrane preparations [467], concentrations of 2-3mM magnesium were able to induce a high affinity state of receptor for agonist. Magnesium's effect on agonist affinity was limited to those receptors in the low activation state, i.e. not agonist/GTP bound. Thus, increasing intracellular magnesium may increase AC activity in systems not already stimulated by GTP or β-2 agonist.

Augmentation of β-2 agonist stimulated relaxation of ASM via enhanced production of cAMP.

Intravenous magnesium may augment β-2 agonist induced bronchodilation. In adults hospitalised for asthma, a bolus of 20mg/kg (0.08mmol/kg) of magnesium followed by infusion of 10mmol/kg/hour, when administered simultaneously during salbutamol inhalations, caused an increase in FEV1 responses to salbutamol relative to salbutamol alone [468]. Maximal bronchodilating response as measured by FEV1 was not altered however, the dose response curves were shifted to the left, which could reflect an increase in β-2 agonist binding to the receptor. These effects suggest that magnesium may augment β-2 agonist induced HASM relaxation. This may be due to an increase in agonist affinity for the receptor and consequently, an augmentation of cAMP production.

In isolated frog erythrocyte membrane preparations, magnesium was shown to increase β-2 agonist binding. Beta-2 agonist stimulated AC activation was increased and was proportionate to the intrinsic activity of the agonist [469]. This effect was also demonstrated in isolated S49 (lymphocyte) membranes [461]. Magnesium's effect on β-2AR affinity for the agonist was specific and not seen for other cations, and the effect was blocked by high levels of GTP, suggesting that this effect is limited to 'inactive' receptors. Receptor bound GTP reduces receptor affinity as a means of negative feedback regulation of receptor activity [470] however, in the absence of bound GTP, AC activity is low. Increased affinity for β-2 agonist increases nucleotide binding and exchange hence, AC activation and cAMP production. The relevance of this mechanism to intact human systems is not known however, this may be an important mechanism in maximising relaxation response of ASM to β-2 agonist bronchodilators.

Reduction of AHR and symptoms via anti-inflammatory actions

When administered intravenously, magnesium has also been shown to reduce AHR, as measured by bronchial provocation responses PC 20 to methacholine [471]. Intravenous magnesium resulted in 30% of asthmatics attaining a normal PC 20 relative to 10% in the placebo group. This suggests that

intravenous magnesium can reduce AHR in asthmatics. The mechanism may involve inhibition of the release of pro-inflammatory cytokines.

In cultured lymphocytes of asthmatic patients, magnesium has been shown to inhibit secretion of IL-5 and IL-13 at concentrations ≥ 5mM [472]. Magnesium has also been shown to attenuate the activation of neutrophils in asthmatic patients at physiological (1mM) and high concentrations (5mM) [473]. TNF-α, which is associated with AHR [54, 55] and IL-1β, which is associated with impaired β-2 agonist responses [223], are inhibited by magnesium. Magnesium was shown to suppress IL-1β, TNF-α and IL-8 in neonatal cord mononuclear cells [474] and IL-6 and TNF-α from maternal mononuclear cells [475], at clinically achievable serum levels (2.5mM). These studies show that magnesium has broad anti-inflammatory properties over a wide range of concentrations and has anti-TNF-α properties at clinically relevant concentrations thus, anti-inflammatory activity may be an important mechanism of action. An anti-inflammatory effect has not been demonstrated directly *in vivo* and the optimal serum concentration required to achieve this in asthma *in vivo* is yet to be established. Maximal non-toxic levels such as 2.5-3mM may be the most appropriate.

Restoration of intracellular magnesium depletion

The effects of an intravenous infusion of magnesium were assessed in one study of patients undergoing cardiac ablation. The 4g (20mmol) infusion produced an immediate increase in intracellular magnesium content in buccal mucosal cells, and serum concentrations remained elevated at 6 hours [476]. This demonstrates that an intravenous infusion of 4g magnesium is effectively distributed to both the intra and extracellular compartments. A definitive association between magnesium deficiency and acute asthma exacerbations has not been proven however, intracellular depletion of magnesium may impair β-2AR function and consequently, relaxation of HASM. Intracellular depletion may also increase inflammation. Intracellular deficiency may be restored with the administration of magnesium.

Magnesium and normal β-2 adrenergic receptor function

Magnesium has an important function in the regulation of the β-2AR. The β-2AR is the prototype of the family of G-protein coupled receptors (GPCRs), and receptor activation, AC stimulation and cAMP production, require coupling with a G-protein [133]. G-proteins are membrane bound transducers of receptor-activated signals, so named for the property and requirement of guanine-nucleotide binding. Magnesium is intimately involved in the G-protein regulation of β-2AR function.

Activation of the β-2AR is initiated via β-2 agonist binding which then promotes association of the receptor with a Gsα sub-unit of the G-protein [134]. This coupling activates GTP-ase of the Gsα and dissociation of the sub-unit [134]. Adenylate cyclase (AC) is activated by hydrolysis of GTP on the

Gsα sub-unit of the G-protein [135]. The binding of β-2 agonist to the receptor is catalytic in the activation of AC by several fold [136].

Magnesium is essential to normal β-2AR function. Normal physiological intracellular magnesium concentrations are required to produce cAMP from ATP, as Mg-ATP is the substrate for agonist induced activation of Gs/GTP-ase [477] and GTP binding/GDP dissociation [134, 466]. It is also necessary for agonist induced dissociation of Gsα from the Gβγ sub-unit [478] and detection of a high affinity state of receptor [479], i.e. that which promotes coupling with Gsα sub-unit. Magnesium is also required for basal, GTP and β-2 agonist stimulated AC activity, as demonstrated by competitive inhibition with Sc3+ [480].

Magnesium and immune function

Metabolic syndrome is characterised by insulin resistance, obesity, increased inflammation and oxidative stress and is associated with magnesium deficiency as determined by lower serum magnesium and low dietary intakes [481]. The most recent review reports that magnesium deficiency is a contributor to low grade inflammation, as indicated by TNF-α levels, and contributes to the pathogenesis of many chronic inflammatory diseases such as metabolic syndrome and coronary atherosclerosis [482]. Low serum magnesium levels have been associated with elevated TNF-α levels in obesity [483] and elevated serum levels of the inflammatory marker CRP (complement reactive protein), in association with lower glucose tolerance in otherwise healthy children [484]. Thus, magnesium has an important role in regulating inflammation and deficiency can have adverse consequences in human health.

Lymphocytes and the study of β-2 adrenergic receptor function

The β-2AR is the primary target of pharmacological HASM relaxation. In addition to a variation in response to magnesium in clinical studies, literature supports that there is individual variation in β-2AR function, as already discussed. It is, therefore, important to assess if there is any effect of magnesium on the β-2AR and/or β-2 agonist induced bronchodilation.

Human lung tissue may be difficult to obtain and therefore, impractical for research purposes. Human peripheral blood lymphocytes have abundant cell surface β-2 receptors [485] and are easily obtained, which makes them ideal, and is why they have been extensively used to study the function of β-2AR in asthma [486-496]. Studies have found the β-2AR on peripheral lymphocytes to have similar properties to those of HASM. Studies of the function of the β-2AR, in lung tissue and blood samples from patients undergoing lung resection for cancer and healthy cultured HASM tissues, have shown that the agonist responses of lymphocyte receptors are comparable to those of receptors in lung and HASM tissue.

Cyclic AMP responses to isoprenaline were shown to be of similar values in lymphocytes and homogenised lung parenchyma [497, 498]. In both lymphocytes and homogenised lung tissue [497,498] and intact segments of bronchial smooth muscle [497], salbutamol displayed partial agonist activity relative to isoprenaline, as determined by cAMP production. These data show that the lymphocyte β-2AR has similar agonist affinities thus, making lymphocytes a suitable vehicle to study the effects of magnesium on the β-2AR.

1.5.3 Safety and appropriate dosing of intravenous magnesium in acute asthma

Magnesium exists in the body as a metal salt. It is the fourth most abundant cation and the most common intracellular cation after calcium [499]. It is largely found in the intracellular compartment bound to organelles or complexed with anions, with only 2-3% 'free' or ionised [500]. It is this ionised form that is biologically active [501]. The normal reference range for total serum magnesium is between 0.75-0.9mM [502]. Toxicity due to intravenous infusions may occur however, not until levels are above 3mM. The earliest sign of toxicity is loss of the deep tendon reflexes which precedes more serious events [503]. More serious toxicity includes paralysis at levels above 5mM, cardiac conduction abnormalities at levels above 7mM and cardiac arrest at levels above 12.5 mM [504]. Magnesium is almost exclusively excreted in urine, with 90% of an intravenous dose being excreted within 24 hours [504]. Severe fluctuations in serum magnesium levels are only seen with intravenous administration, and accumulation is unlikely unless there is a co-existing renal impairment. Thus, dosing in someone with normal renal function would be safe, as long as serum levels were kept below 3mM.

A systematic review of pharmacokinetic studies in women with pre-eclampsia, found that the usual dosing regimen (4-6g [16-24mmol] as an intravenous bolus followed by an infusion of 1-2 g [4-8mmol] per hour) raised serum levels to approximately double the baseline [504]. In all women, levels were well below levels that induce any life-threatening toxicity (i.e. less than 2.5mM). In addition, magnesium clearance is reduced, the half-life is longer and steady state concentrations are higher in pre-eclamptic women relative to healthy pregnant women [505]. It could be concluded that if this dosing regimen is safe in pre-eclamptic women, that it is even more likely to be safe in males and non-pregnant females.

A dose of 5g (20mmol) given as a bolus, has been used to treat status asthmaticus in an un-intubated patient without producing any clinical signs of toxicity [506]. As previously noted, much larger doses have been used in ventilated asthmatics. A single bolus dose of up to 20g (80mmol) given over an hour, increased serum magnesium levels to 3.2mM without any signs of significant toxicity [398]. An

intravenous bolus dose of up to 5g (20mmol) is therefore, likely to be safe in an emergency department setting.

Asthma is not a listed indication for use of intravenous magnesium (2017 NPS MedicineWise, https://www.nps.org.au/), and there are no formal or established guidelines on the use of intravenous magnesium in acute asthma exacerbations. Any guideline for intravenous magnesium dose is limited to the treatment of pregnant women with pre-eclampsia and eclampsia. The recommended dose of intravenous magnesium, in guidelines for treating acute asthma exacerbations in adults, ranges from 2g (8mmol) [4, 268] to 2.5g (10mmol) [2]. Studies in conscious and spontaneously ventilating asthmatics, have reported successful outcomes from the use of magnesium in doses ranging from 1.2g (5mmol) [382] to 5g (20mmol) [506]. Doses of up to 20g (80mmol) have been used successfully in mechanically ventilated asthma patients [398]. Given magnesium's mechanism of action is not defined, the serum levels at which a therapeutic response may occur is not clear. There may also be a dose-response effect thus, appropriate dose may vary depending on individual sensitivity to magnesium's effects. Asthma guidelines may underestimate the required dose in some individuals.

Intravenous magnesium dosing in paediatric asthma is weight-based, with early clinical trials using 25-40mg/kg (0.1 -0.16 mmol/kg) as a single dose bolus infusion [411-416]. This would allow a dose of up to 2g (8mmol) to be administered to a 50kg child. A dose of 50-75mg/kg (0.2-0.3 mmol/kg) as a single bolus dose over an hour has also been used, with serum levels remaining below 1.8mM and no reports of toxicity [507]. More recently, even higher doses have been shown to be more effective. A small study of children with status asthmatics, showed that an infusion of magnesium at 50mg/kg/hour for 4 hours to a total of 200mg/kg (up to10g in a 50kg child) compared with a single dose of 50mg/kg, was safe and more effective in reducing length of stay to less than 24 hours [508]. There were no reports of toxicity.

These data suggest that higher intravenous doses than are currently recommended in the guidelines can be given safely to adults with an acute asthma exacerbation, and that larger dosing may be appropriate and safe for treating severe exacerbations within the emergency department. A dose of 5g would be safe for future clinical studies and optimizes the dose for any potential dose-response effects.

1.6 Hypothesis and Aims

Magnesium can improve asthma outcomes through several mechanisms including an improved response to β-2 agonist or directly via relaxation of airway smooth muscle. This study aims:

1. To demonstrate the mechanism of the action of magnesium, by assessing the effect of magnesium on β-2 agonist stimulated cAMP, in human peripheral blood lymphocytes.
2. To assess the effect of magnesium on hospital admission rates and bronchodilator response to β-2 agonist during an acute asthma attack, in patients who respond poorly to treatment, by comparing the addition of intravenous magnesium solution to the standard treatment with the standard treatment alone.
3. To assess the safety and efficacy of a larger than guideline-prescribed dose of magnesium sulphate (5g/20mmol), in non-mechanically ventilated asthmatics.
4. To identify the sub-group(s) of patients most likely to benefit from the addition of magnesium to standard optimal care.
5. To demonstrate sex differences in the β-2AR to β-2 agonist and magnesium.

It is hypothesised, that magnesium augments β-2 agonist induced cAMP, and that the addition of magnesium to β-2 agonist, will increase β-2 agonist stimulated cAMP production in human peripheral blood lymphocytes relative to β-2 agonist alone.

It is hypothesised, that intravenous magnesium will improve acute asthma outcomes, as indicated by a reduction in the admission rate to hospital and improved lung function, relative to standard care alone.

It is hypothesised, that there will be differences in outcomes from the use of magnesium between the sexes.

Chapter 2: Study of the effect of magnesium on lymphocyte β-2 adrenergic receptor function *in vitro*

2.1 Methods

2.1.1 Introduction

Clinical studies suggest that magnesium's mechanism of action is through augmented bronchodilator responsiveness [382, 468]. This could be mediated by an increase activation of the β-2AR and increased cAMP production, resulting in greater relaxation of airway smooth muscle. Therefore, it was hypothesised, that magnesium augments β-2AR stimulated induced ASM relaxation via an augmented cAMP response to β-2 agonist. The aim of this study was to examine whether magnesium augments β-2 agonist induced cAMP production and therefore, provide evidence for the possible mechanism of its effect.

Given the practical difficulties in obtaining HASM specimens, animal tissues could potentially be used to study the behaviour of the β-2AR, however, the predominant β receptor type in animal ASM varies between species [509]. Unlike in HASM, where the ASM β-receptors are entirely β-2, in animal ASM there is a predominance of one sub-type over another. In guinea pigs 85% [510], canines 80% [511] and pigs 70% [512] of the β receptors are of the β-2 sub-type. In rabbit ASM, β-receptors are predominantly β-1 [456]. It would, therefore, be difficult to extrapolate the findings in animal studies to HASM.

The β-2AR present in human lymphocytes is qualitatively, but not quantitatively, the same as the receptor in lung tissue [497]. The validity of their usage has been discussed in section 1.5 and therefore, lymphocytes provide a valid method to study the effect of magnesium on β-2AR responses. Lymphocytes have been frequently used to study the effects of asthma and β-2 agonists on β-2AR responses [488, 492, 496, 513, 514]. Only one other study reported the effect of magnesium on β-2 agonist stimulated cAMP in healthy patients [515].This is the first study to examine the effects of magnesium on β-2 agonist stimulated cAMP in asthmatic patients.

2.1.2 Study design

Selection of patients

Acute asthmatics were recruited from patients presenting to the Lyell McEwin Hospital Emergency Department with an exacerbation of asthma that required treatment with nebulisations of salbutamol.

An amendment was made to the clinical trial [TQEH/LMH Approval No: 2010076] to allow collection of blood for processing of lymphocytes from those patients recruited to the clinical trial. Patients who were retrospectively excluded from the clinical trial and those with a post-rescue treatment FEV1 ≥ 70% predicted, were also included. Healthy volunteers were recruited from the Lyell McEwin hospital staff. Stable asthmatics were recruited from Lyell McEwin hospital staff and from asthmatic patients presenting to outpatients for their routine visit at the Mater Hospital Brisbane. They were selected if they had medically diagnosed asthma and were not symptomatic at the time of recruitment. Males and females were selected in the same manner.

This study was approved by the Human Ethics Research Committee of the Lyell McEwin and Queen Elizabeth hospitals [Approval HREC/13/TQEHLMH/283] and [TQEH/LMH Approval No: 2010076]

Classification of patients

Healthy controls were defined by the absence of any medical condition, acute or chronic and not restricted to respiratory conditions. Asthma was defined by reversible airways obstruction demonstrated clinically and diagnosed by a medical practitioner, symptoms of asthma at any time during the patient's lifetime whether diagnosed or not (e.g. a patient giving a history of recurrent cough and wheeze but who had not sought medical assistance previously) or patients on asthma treatment as prescribed by a doctor. This treatment included regular inhaled corticosteroid, as needed inhaled salbutamol, or another bronchodilator.

Acute asthma

An acute attack was defined by the onset of symptoms in previously asymptomatic patients, acute worsening of symptoms irrespective of treatment or acute respiratory distress due to asthma.

Stable asthma

Stable asthma was defined by two criteria: an FEV1 >80% predicted or best if known and an Asthma Control Questionnaire (ACQ) score of ≤ 1.5. Both criteria were required for inclusion in to the study.

Inclusion criteria

Inclusion criteria were age 18–50 years and able to give written consent to participation. Patients who did not meet the above criteria or were unable to provide written consent or perform spirometry were excluded.

Withdrawal criteria

Patients could withdraw voluntarily from the trial at any time, for any reason.

Testing and treatment before consent.

Prior to consenting for inclusion in the study, an information sheet was given to patients and the study and its requirements explained. All asthmatics were assessed with an ACQ score to assess asthma control and spirometry to assess degree of airway obstruction.

Spirometry was performed with the EasyOne® hand held spirometer [n.d.d Medical Technologies US/Zurich]. If possible, the best value of the three attempts was recorded at the time of recruitment. For stable asthma ≥ 80% of the predicted value from NHANES (III) values or best if known, was required for study entry. For acute asthmatics, testing was performed at least 15 minutes after the last of the three rescue nebulisations however, many of the acutely exacerbating asthmatics were unable to perform 3 attempts and therefore, a single attempt was accepted, if it was adequate. An attempt was considered adequate if the forced expiration lasted at least 2 seconds, there was a sharp early PEF and the patient did not cough during the first 2 seconds of the manoeuvre. Repeated attempts were encouraged however, in cases where this was not possible a single effort was accepted. These patients were then consented after spirometry was obtained. This method has previously been acceptable in other studies of acute asthma, where repeated attempts to obtain "quality" spirometry were not possible [383, 384].

Sample collection and analysis

After written consent was obtained, an intravenous cannula was inserted and 30mLs of blood was drawn. Samples were divided and a 10mL aliquot was sent to the hospital's pathology laboratory – the Institute for Medical and Veterinary Sciences (IMVS), for routine blood cell counts, serum electrolytes and total serum magnesium. The remaining 20mL was reserved for isolation of PBMCs. Serum magnesium was measured using the "xylidil blue complexometric method" (MG2: Cobas-c) with a sensitivity 0.1-2.0mM.

Data collection

For each subject, base line demographic variable data (age, sex, ethnicity, BMI, smoking status and atopic status) were collected from all patients. Ethnicity data was not presented in the tables as all patients were of Caucasian ethnicity. Asthma medication use and blood for a serum magnesium level were collected from asthmatic patients and FEV1 was measured. An ACQ was also administered upon recruitment to the study as an indicator of asthma control in the past week. The questionnaire also provided information on the use of short acting β-2 agonist (SABA) in the preceding week. Atopic status was determined by self -reporting of the presence or history of eczema, hay fever or an allergic response such as hives, to specific medications or food.

Lymphocytes from individual patients were stimulated in separate experiments and data recorded separately for each subject. Mean cAMP values were determined from pooled data.

Statistical analysis

Statistical calculations were performed with IBM SPSS 24 (Statistical package software for students). For non-parametric data, a Mann Whitney U test was used for independent variables and a Wilcoxon signed rank test was used for dependent variables. To adjust for multiple comparisons, mean cAMP values were compared using a linear mixed effects model.

Power calculations for the linear mixed effects model were determined post-hoc, as neither the range of cAMP values nor the size of any effect of magnesium on cAMP production were known prior to experimentation. A University employed biostatistician performed the power calculations based on the cAMP values produced in healthy controls.

For salbutamol stimulated cAMP in the healthy control group (n=15), with a standard deviation = 4.6, intraclass correlation coefficient (ICC) = 0.286, α=0.05, the study had 26% power to detect the 2nM difference (28%) in cAMP production observed. To have 80% power would have required a sample size of n=60. For isoprenaline stimulated cAMP in the health control group (n=15), with a standard deviation= 8.7, ICC=0.92, α=0.05, the study had <50% power to detect the 1.5nM (9%) difference in cAMP observed. To have 80% power would have required a sample size of n=30.

Based on these calculations, a sample size of n=15 would have had an 80% power to detect a 4.5nM (70%) difference in salbutamol stimulated cAMP and a 3nM (30%) difference in isoprenaline stimulated cAMP. The data from groups where significant differences were detected are displayed in tables throughout the chapter.

The dose response graphs were constructed using mean agonist stimulated cAMP values, divided by the basal or un-stimulated cAMP values. The ratios of the means were then plotted on the y-axis against the concentration of the agonist.

2.1.3 Experimental procedures

Lymphocytes were isolated from the peripheral whole blood of healthy controls and asthmatic patients. Human peripheral blood lymphocytes were assessed for cAMP responses, to both a selective and a non-selective β-2 agonist, over a range of concentrations. The effect of a milli-molar concentration of magnesium on β-2 agonist stimulated cAMP response was also assessed.

Processing of lymphocytes

Isolation and cryopreservation of lymphocytes

For convenience, peripheral blood monocytic cells (PBMCs) were isolated by the Ficoll method, as detailed below, using the density gradient medium Lymphoprep™ supplied by Stemcell™ technologies. The population of the isolated monocytes is primarily lymphocytic and will henceforth be referred to as lymphocytes. For convenience, lymphocytes were required to be frozen immediately post-isolation from whole blood. Optimization experiments subsequently demonstrated that frozen cells provided optimal amount of cAMP.

Twenty millilitres (mL) of whole blood in 10mL aliquots was diluted with 10mL of Dulbecco's Phosphate Buffered Saline (DPBS: Sigma) and layered over 7mL of the separation medium (Lymphoprep™: Stemcell™ technologies) in a 20mL plastic V-bottom tube. The tubes were then centrifuged at 850g for 20 minutes at 20ºC (Haraeus X3R Multifuge centrifuge: Thermo Fischer Scientific). The lymphocytes were then carefully removed using a Pasteur pipette and re-suspended in 20mL of DPBS. The suspension was then centrifuged at 850g for 5 minutes at 20ºC. The supernatant was discarded, and the cell pellet was re-suspended in 20mL of DPBS, then centrifuged at 360g for 5 minutes at 20ºC. The supernatant was discarded.

Prior to freezing, the cell pellet was re-suspended in 950µL of freezing media, made from 50% each of foetal calf serum (FCS) and 30% Dimethysulphoxide (DMSO: Sigma) in plain media (RPMI: Sigma). Isolated lymphocytes were transferred to 1mL cryovials in 500 µL aliquots and stored in a Nalgene freezing container for 24 to 72 hours. Cells were then transferred to a plain container and stored at -80ºC for a period of up to 3 months, prior to use.

Thawing of lymphocytes

Plain RPMI solution (Gibco™) and thawing media (plain RPMI with 10% foetal calf serum) were pre-warmed to 37ºC in a water bath. The cryovial containing the frozen lymphocytes was then removed from the -80ºC freezer and transferred to the water bath. The vial was held in the water until most of the lymphocyte suspension was liquid, but a small solid pellet remained, then 1000µL of warm thawing media was slowly added to the cell suspension and gently mixed. The suspension was then transferred to a 1500µL reaction tube and spun at 1.6g for 3 minutes at 21ºC. The supernatant was then discarded. The cells were re-suspended with 950µL of plain RPMI for immediate experimentation, or 950µL of culture medium for overnight incubation, depending on the experiment to be performed.

Cell counts

A cell count was undertaken prior to freezing, subsequently to thawing and prior to experimentation, using an automated cell counter. Samples with a cell viability of >15% were included in the study. One µL of cell suspension was placed in a 500µl tube and 1µl of thymidine blue was added and mixed with the cells. The cells were then pipetted on to a glass slide and inserted into the cell counter. Dead cells were identified by uptake of thymidine blue. The total number of cells, the number of viable cells and the percent viable were displayed and recorded.

ELIZA assay for cAMP

Commercially available ELIZA kits, cAMP XP™ (#4339), were purchased from *Cell Signaling Technology Inc*. The assay had a sensitivity of 0.3 to 240Nm of cAMP.

Reagent preparation

Phenylmethylsulfonylfluoride (PMSF) was obtained as dry powder from Sigma laboratories. To make stock solution of PMSF, 0.174g of dry powder was dissolved in 1mL of anhydrous ethanol and then diluted in 9mL of anhydrous ethanol to make 10mLs of a 100mM solution, which was stored at -20ºC. As per the manufacturer's instructions, 10X cell lysis buffer was diluted to 1X in milli-Q purified water, to which 1mM PMSF was added. Lysis buffer was made in 2x 50mL aliquots at a time and stored at -4ºC for 2 weeks or until used.

Preparation of lysate as per manufacturer's protocol

Cells were plated in culture medium in a 96 well plate at a concentration of 10^4 cells per well and incubated overnight in a humidified incubator at 37ºC with 5% CO_2. Cells were then rinsed by adding 200µL of warmed DPBS to each well. The plate was then spun in the Haraeus X3R Multifuge centrifuge for 3 minutes at 360g at 20ºC. Supernatant was aspirated and test solutions along with warmed DPBS were added to each well. The cells were then incubated with desired reagents for 5 minutes. Cells were then rinsed by adding 200µL ice cold DPBS to each well and spinning and aspirating the supernatant as described. This step was repeated and 100µL of 1X lysis buffer was added to each well. The plate was then placed on ice for 10 minutes prior to cAMP assay.

Modified method for stimulation of lymphocytes

A modified protocol was developed, as an alternative to the manufacturer's protocol, to optimise cAMP production. A 1mL aliquot of thawed cells was diluted in 15mL of warmed DPBS and suspended in a water bath at 37ºC for 5-10 minutes, prior to stimulation. An aliquot of cells was added to 1.5mL reaction tubes, sealed and incubated in the water bath along with the desired reagents, to make the desired concentration of reagent in a final volume of 1000µL at an approximate

concentration of 10^5 cells per mL. The cells were incubated in the water bath for 5 minutes at 37ºC. Sealed reaction tubes were then immersed in an ice water bath for 5-10 minutes to terminate further cAMP production and minimise cAMP degradation.

Modified method for lysate preparation

For rinsing, the reaction tubes were spun in a small centrifuge at 1.6g for 3 minutes at 4ºC. The supernatants were discarded, the cell pellets re-suspended in 1mL of ice cold DPBS solution and centrifuged at 1.6g for 3 minutes at 4ºC. Supernatants were discarded and the procedure repeated. The cell lysate was then prepared by adding 450µL of lysis buffer to each reaction tube and standing on ice for 10 minutes. The cell lysate was frozen at -20ºC for 24 hours and the cAMP assay performed the following day. This was done for convenience, so that multiple samples could be processed at the same time.

cAMP Assay

All components of the ELIZA kit were brought to room temperature. Standards for interpolation were prepared as per the manufacturer's instructions. Cell lysates were prepared, except where the modified protocol was used, and frozen samples of lysate were thawed on ice. Lysates were assayed according to the cAMP XP™ manufacturer's protocol. Plates were read at 450Nm according to the manufacturer's instructions. Absorbance was recorded at 450Nm. Data were recorded in text file documents and Excel worksheets.

Calculation of cAMP production

Cyclic AMP values were interpolated from the standard curve using the Graph Pad Prism 7 program, and standards were distributed on sigmoidal 4pl logarithmic scale. For interpolation of the cAMP values, X values (standards) were transformed to logarithmic, absorbance was interpolated, and cAMP values transformed 10^X and recorded. The cAMP values were recorded in Excel form as nM and then transformed to nM per 10^6 cells using a simple mathematical equation;

$$[cAMP] \text{ per } 10^6 \text{ cells} = 10^6 \times \text{measured } [cAMP] / (\text{no of cells per mL})$$

Values were also expressed in ratios of cAMP over base level. Data were expressed in tabular and graphic form.

One of the acute asthmatic females produced insufficient cAMP to calculate ratios for all isoprenaline concentrations, so her data could not be included in the analysis. It was not clear whether this was a real phenomenon or due to a technical error in processing of the cells.

Reagents

Dulbecco's phosphate buffered saline solution (x1) (DPBS) Dimethysulphoxide (DMSO) and Forskolin 10mg dry powder were commercially prepared and supplied by Sigma laboratories. Plain medium (RPMI) was commercially prepared and supplied by Gibco^{TM}. Commercially available ampules of 10^{-3}M solution of the β-2 agonists, salbutamol and isoprenaline, 2M magnesium sulphate and 0.9% saline for human injection, were supplied by the hospital pharmacy.

Preparation of reagents

Culture medium was prepared from 10% foetal calf serum in plain RPMI with 1% penicillin-streptomycin (Gibco^{TM}) and stored at 4ºC for up to 12 months, prior to use.

Two hundred µL of 2M magnesium sulphate solution was diluted in 9.8 mL of 0.9% saline for human injection to make 10mL of 40mM solution, which was stored at room temperature. Fresh stock solution was made every three months.

Stock solution of forskolin 10^{-2}M was prepared and stored in accordance with the manufacturer's instructions. Ten mg of dry forskolin powder was dissolved in 1mL of DMSO to make a 2.4 x 10^{-2}M stock solution, which was stored at room temperature in the dark for up to 9 months. For each experiment, 10µL of forskolin stock solution was diluted in 90µL milli-Q to make 100µL of a 10^{-3}M forskolin solution. This was done immediately prior to commencement of the experiments as forskolin is unstable in aqueous solution.

Fresh β-2 agonist reagents were made on the day of the experiments and discarded at the end of the day. For each experiment, serial dilutions of the 10^{-3}M of salbutamol and isoprenaline solution were performed with 0.9% saline to make 1mL of each of a 10^{-4}M, 10^{-5}M, 10^{-6}M and 10^{-7} M solutions. For the experiments, 50µL of stock solution or diluted solution was added to each reaction tube to make final concentrations of 10^{-4}M, 10^{-5}M, 10^{-6}M, 10^{-7} and 10^{-8} M.

Optimisation

Optimisation experiments to determine the optimal conditions for stimulation and preservation of cAMP, were carried out prior to formal experimentation, using the investigators' own lymphocytes. Frozen lymphocytes were used initially for convenience and thawed prior to experimentation as previously described. The lymphocytes were frozen at an initial concentration of 2-4 x10^6 cells per mL and after thawing, had a concentration of 1-3 x10^6 cells per mL. Cells were incubated at 37ºC for 5, 15 and 30 minutes with 10^{-4}M, 10^{-6}M and 10^{-8}M of isoprenaline. Once the optimum incubation concentration and time was determined, a comparison of cAMP production between immediately

harvested fresh and frozen cells was then undertaken using the same concentrations of agonist as before.

Optimisation of experimental method

Thawed lymphocytes were diluted in a warm culture medium to a concentration of 10^5 cells per millilitre. Lymphocytes were then added in 200µL volumes to each well in a 96 well incubation plate to give approximately 10^4 cells per well. The plate was incubated overnight at 37ºC and the cells rinsed as per manufacturer's protocol described previously.

To each well, volume of warmed RPMI and isoprenaline solution was added for a final volume of 200µL. Isoprenaline to a final concentration of 10^{-4}M was added to the first 16 wells,10^{-6}M to the next and 10^{-8}M to the next. The plate was then incubated at 37ºC for 5 minutes to allow stimulation of cAMP. The cells were then processed as per the manufacturer's protocol as previously described.

The optimisation experiments were then repeated in a modified method, using 1.5mL sealed reaction tubes for each of the conditions tested for stimulation and processing of cells. Thawed lymphocytes were diluted in 10mLs of warm RPMI to a concentration of approximately 2.5 x10^5 cells per mL and left to stand in the water bath at 37ºC for 5-10 minutes. To each of four tubes, 900µL of lymphocyte suspension was added. To three of the tubes, 100µL of isoprenaline was added to a final concentration of 10^{-4}M, 10^{-6}M and 10^{-8}M. To the remaining tube, 100µL of saline was added. The tubes were sealed and placed in the water bath at 37ºC for 5 minutes. At the end of the stimulation period, the tubes were immersed in an ice-cold water bath for 10 minutes. The cells were then rinsed and lysed according to the modified lysate preparation protocol, and cAMP was assayed as per manufacturer's protocol described earlier.

Optimisation of time

The modified stimulation method described above was repeated for incubation times of 15 and 30 minutes. The cells were then rinsed and lysed according to the modified lysate preparation protocol, and cAMP was assayed as per the manufacturer's protocol.

Optimisation of preparation of lymphocytes

Lymphocytes were isolated according to the method previously described and re-suspended in thawing media. Frozen lymphocytes were thawed as described earlier. Both lymphocyte suspensions were then diluted in 10mL of warm plain RPMI and left to stand in a water bath at 37ºC for 10 minutes. The approximate final concentrations were 10^5 cells per mL for both samples. Both sets of lymphocytes were stimulated simultaneously for 5 minutes according to the modified optimisation

protocol described above. The cells were then rinsed and lysed according to the modified lysate preparation protocol, and cAMP was assayed as per manufacturer's protocol.

Beta-2 agonist stimulation of cAMP in peripheral blood lymphocytes

To determine differences in receptor function, lymphocytes were stimulated with salbutamol, the highly selective β-2 agonist and the non-selective β-agonist, isoprenaline. There were 15 different experimental conditions, and five different agonist concentrations were used for each agonist; 10^{-4}M, 10^{-5}M, 10^{-6}M, 10^{-7}M and 10^{-8}M. The effect of agonist alone was measured against the effect of agonist and 1mM, and agonist and 2mM of magnesium sulphate. Forskolin, a direct activator of Adenylate Cyclase (AC), was used as the positive control at a concentration of 10^{-4}M. To determine the baseline, un-stimulated cAMP values, measurements were taken from lymphocytes in suspension alone, without any additional reagent. Forskolin and baseline conditions were duplicated for each of the β-2 agonists, so that 34 conditions were tested for each subject.

For both β-agonists, 100µL aliquots of the various β-2 agonist dilutions were added to the reaction tubes. For agonist alone conditions (5 tubes), an additional 50µL of saline was added, for agonist and 1mM magnesium (5 tubes), 25µL of magnesium and 25µL of saline were added, and for agonist and 2mM magnesium (5 tubes), 50µL of magnesium stock solution was added to the required tubes. One hundred and fifty µL of saline alone was added to one tube as the basal or negative control. To the remaining tube, 50µL of saline was added. The 100µL of forskolin dilution was made up just prior to stimulation and added to the tube after the lymphocytes were added, as forskolin was known to be less stable once diluted in water.

Frozen lymphocytes were thawed as previously described and re-suspended in warmed RPMI. The lymphocytes were then counted and suspended in 15mL of pre-warmed plain DPBS and left to stand in a water bath at 37ºC for 5-10 minutes, prior to stimulation, while reagents were freshly prepared and added to the reaction tubes as described above. Once the lymphocytes had warmed and rested, 850µL of lymphocyte suspension was added to the 150µL of the reagent solutions in each of the reaction tubes, to make a final volume of 1000µL. Lymphocytes were stimulated under experimental conditions for 5 minutes as per the modified method described above. Cells were then rinsed and lysed according to the modified lysate preparation protocol, and cAMP was assayed as per manufacturer's protocol. The experiments were performed separately for salbutamol and isoprenaline agonists and repeated for each of the patients. For each patient, cAMP was assayed separately, but salbutamol-stimulated and isoprenaline-stimulated cAMP were assayed simultaneously.

2.2 Results

2.2.1 Optimisation of β-2 agonist stimulated cAMP

Lymphocytes incubated and stimulated in the 96 well culture plates, produced an insufficient amount of agonist-stimulated cAMP to be detected by the assay, i.e. only basal un-stimulated cAMP was detected in all cells. Repetition of the experiment at different incubation times produced the same result. The low cAMP level was thought to be due to rapid degradation of cAMP by cellular phosphodiesterase. A phosphodiesterase inhibitor was considered, but not utilised, as it was known to alter the dose-response relationship in β-2 agonist stimulated cAMP production, which would confound the magnesium experimental results. Rapid cooling was thought to be necessary to avoid undue loss of stimulated cAMP but was technically difficult and could not be performed using the 96 well plates.

The optimisation experiments were repeated using 1.5mL sealed reaction tubes, as described in the methods above, to enable the rapid cooling of the cells by immersion in an ice water bath and reduce the loss of cAMP through degradation by phosphodiesterase. Lymphocytes stimulated in 1.5µL reaction tubes and cooled in the ice water bath, produced measurable stimulated cAMP responses and an appreciable increase over baseline in relation to the dose of agonist used. The 1.5mL reaction tubes were used in the remainder of the optimisation experiments and the final protocol. The optimal time for stimulation was found to be 5 minutes (Figure 1). Freshly isolated lymphocytes failed to produce any measurable cAMP, independent of incubation times. The reasons for this are unclear and may reflect contamination of cells with bacteria.

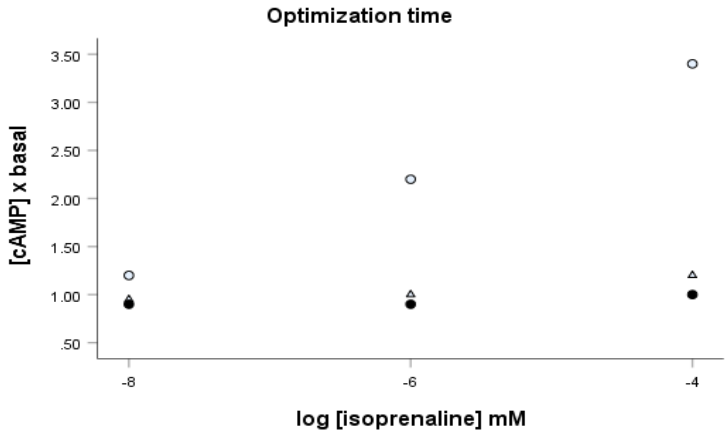


Figure 1. Optimization of cAMP production. Concentration of isoprenaline mM (millimolar) vs. [cAMP] expressed as fold increase over baseline; comparison of cell-agonist incubation times at 37°C.

30 minutes incubation ●, 15 minutes incubation Δ, 5 minutes incubation ○

2.2.2 Beta-2 agonist stimulated cAMP production

Lymphocytes were isolated from 15 healthy controls, 11 acute asthmatics and 14 stable asthmatics, and assessed for β-2 agonist responses in the presence and absence of magnesium as described in the methods section. Patients were all aged between 18 and 50 years-old and of Caucasian ethnicity.

Healthy Controls

There were 7 females and 8 males in the control group. There were no significant differences in age. The median age of males was 38.5 years (IQR 30-42.5) relative to 35 years for females (IQR 25-48) (p=0.96). There was one active smoker in each sex. Serum magnesium levels were within normal limits for both males and females. Baseline physiological parameters of heart rate, blood pressure, oxygen saturation, respiratory rate and FEV1% predicted, were not performed in healthy controls as they were assumed to be within the normal limits.

Lymphocytes from healthy controls produced 105% more cAMP when stimulated with isoprenaline than with salbutamol (p<0.01). In the lymphocytes from healthy controls, 1mM of magnesium increased the mean salbutamol stimulated cAMP by 28% relative to agonist alone (Wilcoxon Signed Rank; p<0.001), and 2mM of magnesium increased mean salbutamol stimulated cAMP by 25% relative to agonist alone (p<0.001). This was accompanied by a shift to the left of the dose-response ratios (Figure 2a). When adjusted for multiple comparisons, these differences were not significant.

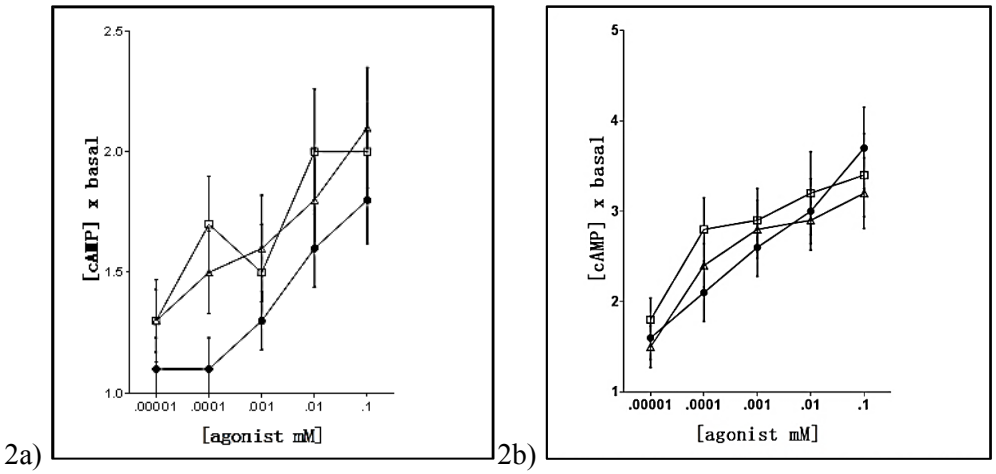


Figure 2. Dose response ratios cAMP healthy controls: the effects of different concentrations of magnesium on responses to stimulation with β-2 agonists. Effect of magnesium on increasing concentrations of β-2 agonist mM (millimolar) on cAMP production expressed as fold increase in cAMP over baseline.

2a salbutamol, 2b isoprenaline
No added magnesium ●, 1mM added magnesium □, 2mM added magnesium Δ.

In the lymphocytes of healthy controls, 1mM magnesium increased the mean isoprenaline stimulated cAMP by 9% relative to agonist alone ($p<0.01$) and produced a shift to the left of the dose response ratios, while 2mM of magnesium had no significant effect on mean isoprenaline cAMP production (Figure 2b).

Acute asthma

There were 4 male and 7 females with acute exacerbations of asthma. Lymphocytes of one of the females was not analysed as they failed to produce any measurable cAMP, despite repeating the experiments. There were 6 females included in the data analysis. The small sample size limited any meaningful statistical comparisons. There were no observed differences in baseline physiological parameters (Table 2).

Table 2. Baseline and demographic comparisons in acute asthmatic males and females.

Variables for acute asthma (n=10)	**males (n=4)**	**females (n=6)**
Age, median (IQR)	40.5 (26.5-47)	31 (27-42)
BMI, median (IQR)	30 (28-37)	38 (30-43)
Current smoker, n (%)	1 (25)	4 (57.1)
Serum magnesium (mM), mean (SE)	0.83 (.02)	0.79 (0.02)
Preventer, n (%)		
ICS only	1 (25)	
ICS+LABA	2 (50)	5 (71.4)
Other medications		
antibiotics		3 (42.9)
antihistamines	1 (25)	1 (14.3)
tiotropium		1 (14.3)
theophylline		1 (14.3)
SABA use mcg/day , median (IQR)	1800 (1400->1600)	1600 (1600->1600)
OCS use in 24 hours prior to arrival , n (%)	1 (25)	4 (57.1)
Asthma Control Questionairre (0-6), median (IQR)	4.3 (3.45-4.8)	4.5 (4.5-4.6)
Borg dyspnoea scale (0-10) , median (IQR)	7 (5-7)	6 (5-8)
FEV1 % predicted, median (IQR)	69 (40-73)	48 (44-53)
Respirations per minute , median (IQR)	23 (21-24)	28 (24-28)
Oxygen saturation %, median (IQR)	95 (93-97.5)	98 (96-98)
Pulse rate in beats per minute , median (IQR)	113 (97.5-131)	120 (101-127)
Systolic blood pressure mmHg , median (IQR)	127.5 (120-135)	125 (120-130)
Diastolic blood pressure mmHg, median (IQR)	70 (65-80)	80 (70-80)

IQR: interquartile range, SE: standard error, BMI: body mass index, ICS: inhaled corticosteroid, LABA: long acting β-2 agonist, SABA: short acting β-agonist, OCS: oral corticosteroid, FEV1: forced expiratory volume in 1 second.

Lymphocytes from acute asthmatic patients produced 74% more cAMP when stimulated with isoprenaline than with salbutamol ($p<0.01$). In the lymphocytes from acute asthmatics, 1mM magnesium had no effect on salbutamol stimulated cAMP and 2mM increased mean salbutamol stimulated cAMP by 18%, relative to agonist alone ($p=0.06$) however, there was no apparent relationship between the dose of agonist and cAMP response (Figure 3a).

In isoprenaline stimulated cells, 1mM magnesium increased mean cAMP production by 6% (Wilcoxon Signed Rank; $p<0.05$) and 2mM magnesium by 12% relative to agonist alone ($p<0.05$). Both 1mM and 2mM magnesium, produced a shift of the dose-response ratios to the left (Figure 3b). When adjusted for multiple comparisons these differences were not significant.

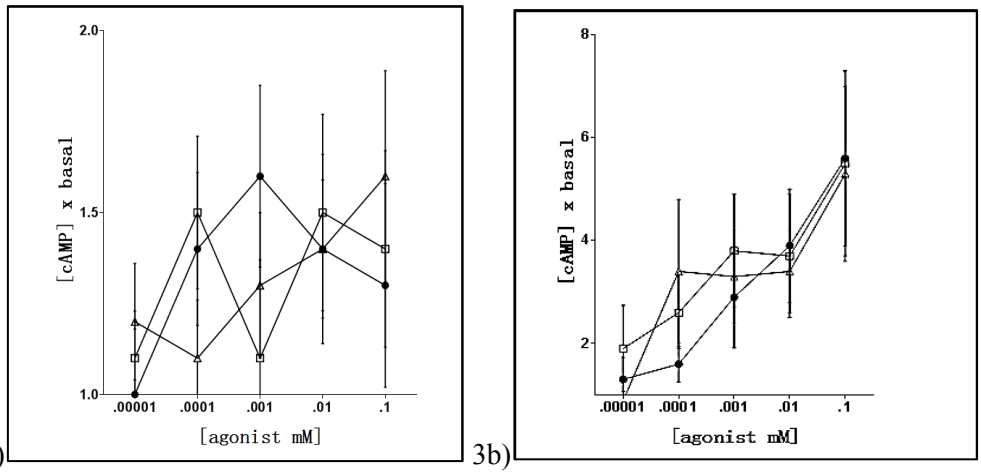


Figure 3. Dose response ratios cAMP acute asthmatics: the effects of different concentrations of magnesium on responses to stimulation with β-2 agonists. Effect of magnesium on increasing concentrations of β-2 agonist mM (millimolar) on cAMP production, expressed as fold increase in cAMP over baseline.

3a) salbutamol, 3b) isoprenaline
No added magnesium ●, 1mM added magnesium □, 2mM added magnesium Δ.

Acute asthma vs. healthy controls

There was greater variability in responses to β-agonists in acute asthmatics relative to controls. Lymphocytes from asthmatic patients, when stimulated with salbutamol, produced 52% less cAMP relative to healthy controls (Mann-Whitney-U; $p<0.001$). When adjusted for multiple comparisons, these were only significant for 1mM magnesium (Table 3). Lymphocytes from asthmatic patients produced 62% less cAMP when stimulated with isoprenaline relative to healthy controls ($p<0.001$). When adjusted for multiple comparisons, the differences were significant (Table 3). Basal and

forskolin stimulated cAMP were not significantly different in acute asthmatics relative to healthy controls (Table 3).

Table 3. Mean cyclic AMP production for each treatment condition by group; healthy controls vs. asthma (stable and acute), adjusted for multiple comparisons. Cyclic AMP values expressed as nM (nanomoles) per 10^6 cells.

			healthy controls (n=15)		acute asthma (n=10)			stable asthma (n=13)		
Agonist	[Mg]	[agonist]	Mean	SE	Mean	SE	p value	Mean	SE	p value
Salbutamol		*0.1mM*	7.8	1.13	2.5	0.4	***<0.01***	7.6	1.6	*0.83*
		0.01mM	7.1	0.92	2.7	0.6	***<0.05***	7.6	1.8	*0.56*
	no Mg	*1µM*	5.9	0.83	3.2	0.7	*0.15*	6.9	1.7	*0.37*
		0.1µM	4.5	0.63	3.0	0.6	*0.4*	6.6	1.8	*0.17*
		0.01µM	4.7	0.62	2.1	0.5	*0.19*	6.6	1.9	*0.22*
		0.1mM	8.6	1.1	2.6	0.4	***< 0.01***	7.7	1.7	*0.77*
		0.01mM	8.6	1.3	3.0	0.7	***< 0.05***	7.4	2.0	*0.74*
	1mM	*1µM*	7.3	1.2	2.2	0.4	***< 0.05***	7.2	1.9	*0.86*
		0.1µM	7.5	1.3	3.2	0.6	*0.07*	7.8	2.2	*0.71*
		0.01µM	6.3	1.2	2.6	0.6	***<0.05***	6.3	1.4	*0.89*
		0.1mM	10.3	1.5	3.8	0.8	***< 0.05***	9.7	2.3	*1.0*
		0.01mM	7.9	1.2	3.3	0.7	*0.1*	10.1	2.6	*0.28*
	2mM	*1µM*	7.5	1.2	3.4	0.8	*0.1*	8.6	2.2	*0.52*
		0.1µM	6.4	1.0	2.8	0.6	*0.07*	7.4	1.8	*0.47*
		0.01µM	5.8	1.0	2.6	0.5	*0.13*	6.7	2.0	*0.54*
Isoprenaline		*0.1mM*	19.3	2.4	9.0	2.0	***< 0.05***	18.7	3.7	*0.91*
		0.01mM	16.5	2.2	6.3	1.3	***< 0.01***	14.8	2.7	*0.78*
	no Mg	*1µM*	14.6	2.1	4.6	0.8	***< 0.01***	12.3	2.4	*0.59*
		0.1µM	11.8	1.7	3.1	0.6	***< 0.01***	10.3	1.9	*0.69*
		0.01µM	9.6	1.5	3.0	0.6	***< 0.05***	9.0	2.2	*0.98*
		0.1mM	18.5	2.5	8.3	1.6	*0.06*	19.9	4.6	*0.62*
		0.01mM	16.7	2.5	5.7	1.0	***< 0.01***	15.3	2.7	*0.85*
	1mM	*1µM*	16.2	2.3	5.8	1.0	***< 0.01***	12.9	2.9	*0.47*
		0.1µM	16.2	2.2	5.2	1.2	***< 0.01***	12.0	2.8	*0.29*
		0.01µM	10.5	1.5	3.2	0.8	***< 0.01***	8.0	1.8	*0.34*
		0.1mM	17.5	2.3	8.8	1.7	***< 0.05***	19.3	3.6	*0.47*
		0.01mM	16.9	2.3	7.0	1.6	***< 0.05***	15.2	2.9	*0.94*
	2mM	*1µM*	15.4	1.9	6.6	1.6	***< 0.05***	14.0	3.2	*.069*
		0.1µM	13.3	1.7	5.0	1.0	***< 0.01***	10.6	2.5	*0.43*
		0.01µM	8.6	1.4	2.4	0.8	***< 0.05***	6.4	1.7	*0.35*
Basal		*0*	5.2	0.8	3.5	1.0	*0.17*	5.5	0.8	*0.80*
Forskolin		*0.1mM*	10.4	1.6	6.6	1.9	*0.13*	12.1	1.7	*0.48*

SE: standard error, mM: millimolar, µM: micromolar.

Stable asthma

There were 7 male and 7 female stable asthmatics. One of the male patients was excluded, because although his ACQ score was =1.5, his FEV1 was below 80 % predicted, therefore, he did not fulfil the study criteria for stable asthma.

There were 6 males and 7 females included in the data analysis. ACQ scores were less than 1.5 (median 0.67; IQR 0.17-1.0) and the median FEV1 was 95% of predicted (IQR 87-105%) which correctly categorized the patient's asthma as stable. Short acting β-2 agonist use was less than 400mcg per day in the preceding week. ACQ scores ($p<0.01$) and SABA use were significantly less in stable asthmatics relative to acute asthmatics (<200mcg day vs. >1600mcg day; $p<0.01$). FEV1 was significantly less in acute relative to stable asthmatics (56% vs. 95%; $p<0.01$).

Males did not use any SABA on a prn basis in the preceding week however, females did, although usage was low relative to acute asthmatics ($p<0.05$) (Table 3). There was a non-significant trend for males to have lower FEV1 values (87 vs. 100) and lower ACQ (0.17 vs. 0.69) scores relative to females. Atopy status was only documented in 6 patients hence, a comparison of atopy prevalence was not made (Table 4).

Table 4. Baseline and demographic comparisons of stable asthmatics; males and females.

Variables for stable asthma (n=13)	males (n=6)	females (n=7)	p value
Age, median (IQR)	33.5 (32-35)	32 (27-39)	0.73
BMI, median (IQR)	28 (27-31)	27 (21-34)	0.37
Current smoker, n (%)	0	0	
Preventer, n (%)			
ICS only	1 (16.7)	0	0.26
ICS+LABA	4 (66.7)	6 (87.5)	0.51
Other medications	~	~	
SABA use mcg/day , median (IQR)	0 (0-0)	300 (200-400)	<0.05
OCS use in 24 hours prior to arrival , n (%)	0	0	
Asthma Control Questionairre (0-6), median (IQR)	0.17 (0- .17)	0.69 (.67-1)	0.13
Borg dyspnoea scale (0-10) , median (IQR)	0	0	1
FEV1 % predicted, median (IQR)	87 (83-91)	100 (90-105)	0.31

IQR: interquartile range, BMI: body mass index, ICS: inhaled corticosteroid, LABA: long acting β-2 agonist, SABA: short acting β-agonist, OCS: oral corticosteroid, FEV1: forced expiratory volume in 1 second, mcg: micrograms

Cyclic AMP production in lymphocytes of stable asthmatics was 75% greater with isoprenaline relative to salbutamol ($p<0.01$). In the lymphocytes from stable asthmatics, 1mM of magnesium had no significant effect on salbutamol stimulated cAMP, while 2mM of magnesium increased mean salbutamol stimulated cAMP by 20% relative to agonist alone ($p<0.001$) and produced a shift in the dose-response ratios to the left (Figure 4a). When adjusted for multiple comparisons, these differences were not statistically significant. In the lymphocytes from stable asthmatics, 1mM and 2mM of magnesium had no significant effect on isoprenaline stimulated cAMP production (Figure 4b).

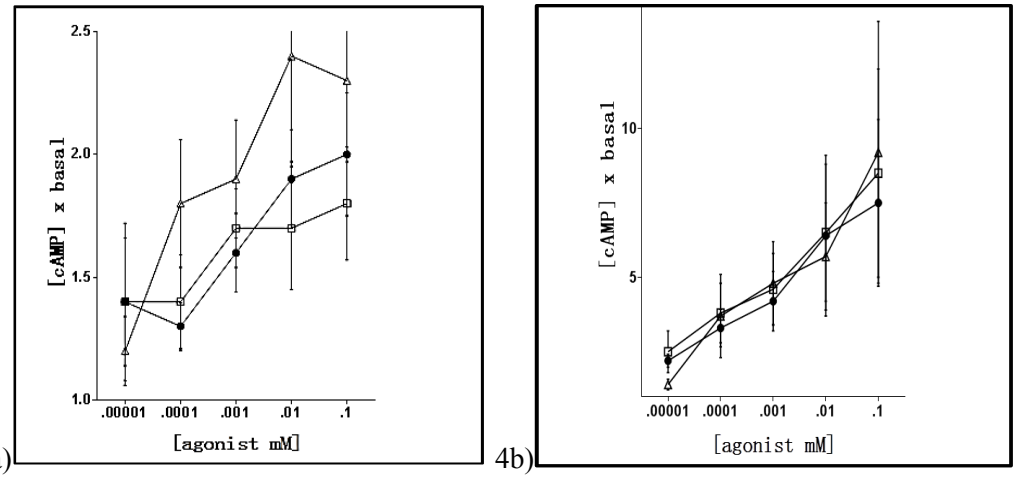


Figure 4. Dose response ratios cAMP stable asthmatics: the effects of different concentrations of magnesium on responses to stimulation with β-2 agonists. Effect of magnesium on increasing concentrations of β-2 agonist mM (millimolar) on cAMP production, expressed as fold increase in cAMP over baseline.

4a) salbutamol, 4b) isoprenaline
No added magnesium ●, 1mM added magnesium □, 2mM added magnesium Δ.

Stable asthma vs. healthy controls

There was greater variability in responses to the β-agonist in the healthy controls relative to stable asthmatics. There were no significant differences in salbutamol stimulated cAMP in stable asthmatics relative to healthy controls. Lymphocytes from stable asthmatics, when stimulated with isoprenaline, produced 10% less cAMP relative to healthy controls ($p<0.05$). When adjusted for multiple comparisons, these differences were not significant. There were no significant differences in mean baseline cAMP (5.53 vs. 5.3; $p=0.8$) or forskolin stimulated cAMP (12.06 vs. 10.4; $p=0.48$) between stable asthmatics and controls.

2.2.3 Beta-2 agonist stimulated cAMP production by sex

Healthy controls

When stimulated with salbutamol, lymphocytes of healthy females had a 38% greater increase in cAMP production relative to healthy males (Mann-Whitney-U; $p<0.01$). When stimulated with isoprenaline, lymphocytes of healthy females had a 79% greater increase in cAMP production relative to healthy males ($p<0.001$). When adjusted for multiple comparisons, the differences in mean cAMP between males and females were significant (Table 5).

Table 5. Mean cyclic AMP production for females; each treatment condition by group. Mean difference in cAMP production between acute/stable asthma and healthy controls adjusted for multiple comparisons. Cyclic AMP values expressed as nM(nanomoles) per 10^6 cells.

			healthy females (n=7)		acute asthma (n=6)			stable asthma (n=7)		
Agonist	[Mg]	[agonist]	Mean	SE	Mean diff	SE	p value	Mean diff	SE	p value
Salbutamol agonist	no Mg	*0.1mM*	9.53	1.58	-7.5	1.9	***<0.01***	-2.9	1.8	*0.12*
		0.01mM	8.42	1.30	-6.0	1.4	***<0.01***	-2.2	1.4	*0.12*
		1µM	7.11	1.17	-4.6	1.3	***<0.01***	-1.6	1.2	*0.20*
		0.1µM	5.58	0.87	-3.0	1.2	***<0.05***	-0.7	1.2	*0.55*
		0.01µM	6.28	0.79	-4.4	1.1	***<0.01***	-1.9	1.0	*0.08*
	1mM	*0.1mM*	9.88	1.62	-7.1	1.4	***<0.01***	-4.0	1.4	***<0.05***
		0.01mM	9.84	1.88	-7.1	2.1	***<0.01***	-3.2	2.0	*0.13*
		1µM	7.97	1.71	-5.8	1.7	***<0.01***	-2.0	1.7	*0.24*
		0.1µM	9.12	1.89	-6.6	2.2	***<0.01***	-3.5	2.1	*0.11*
		0.01µM	7.01	1.80	-4.9	1.8	***<0.05***	-2.3	1.7	*0.20*
	2mM	*0.1mM*	13.53	2.07	-10.9	2.3	***<0.01***	-6.3	2.1	***<0.01***
		0.01mM	8.96	1.81	-6.4	2.0	***<0.01***	-2.1	2.0	*0.30*
		1µM	7.91	1.84	-5.1	1.8	***<0.05***	-1.9	1.8	*0.29*
		0.1µM	7.28	1.46	-5.0	1.6	***<0.01***	-2.2	1.5	*0.16*
		0.01µM	6.28	1.50	-4.1	1.4	***<0.05***	-2.1	1.3	*0.14*
Isoprenaline agonist	no Mg	*0.1mM*	25.01	2.89	-17.5	5.2	***<0.01***	-7.6	4.7	*0.12*
		0.01mM	21.85	2.62	-16.7	4.0	***<0.01***	-8.6	3.7	***<0.05***
		1µM	18.92	2.79	-15.0	3.5	***<0.01***	-9.5	3.2	***<0.01***
		0.1µM	15.83	2.13	-13.6	2.9	***<0.01***	-7.6	2.6	***<0.05***
		0.01µM	12.83	1.87	-10.6	2.8	***<0.01***	-6.3	2.3	***<0.05***
	1mM	*0.1mM*	22.39	3.52	-15.1	5.0	***<0.01***	-7.1	4.6	*0.14*
		0.01mM	21.42	3.28	-16.8	3.9	***<0.01***	-9.4	3.6	***<0.05***
		1µM	21.74	2.83	-16.9	3.6	***<0.01***	-11.5	3.3	***<0.01***
		0.1µM	21.65	2.70	-18.3	3.2	***<0.01***	-13.4	2.9	***<0.01***
		0.01µM	13.62	1.98	-11.8	2.2	***<0.01***	-7.9	2.0	***<0.01***
	2mM	*0.1mM*	22.08	3.03	-15.2	5.2	***<0.05***	-6.8	4.8	*0.17*
		0.01mM	20.71	3.18	-15.8	3.5	***<0.01***	-9.8	3.2	***<0.01***
		1µM	21.05	1.92	-16.3	2.8	***<0.01***	-11.7	2.5	***<0.01***
		0.1µM	17.66	1.99	-14.4	2.5	***<0.01***	-10.6	2.3	***<0.01***
		0.01µM	12.25	1.59	-11.3	2.3	***<0.01***	-7.8	2.0	***<0.01***
	basal	*0*	6.44	0.85	-5.1	0.9	***<0.01***	-2.9	0.9	***<0.01***
	forskolin	*0.1mM*	14.74	1.26	-11.9	2.0	***<0.01***	-6.7	1.9	***<0.01***

SE: standard error, mM: millimolar, µM: micromolar.

There was no significant difference in mean baseline cAMP however, there was a difference in forskolin stimulated cAMP which was significantly higher in females (14.7 vs. 6.6; $p<0.001$).

In the lymphocytes from healthy females, 1mM of magnesium increased the mean salbutamol stimulated cAMP by 18% ($p<0.05$), and 2mM of magnesium increased mean salbutamol stimulated cAMP by 16% relative to agonist alone ($p<0.05$) (Figure 5a). In lymphocytes from healthy males, 1mM magnesium increased the mean salbutamol stimulated cAMP by 39% ($p<0.001$), and 2mM of magnesium increased mean salbutamol stimulated cAMP by 37% relative to agonist alone ($p<0.001$) (Figure 5b).

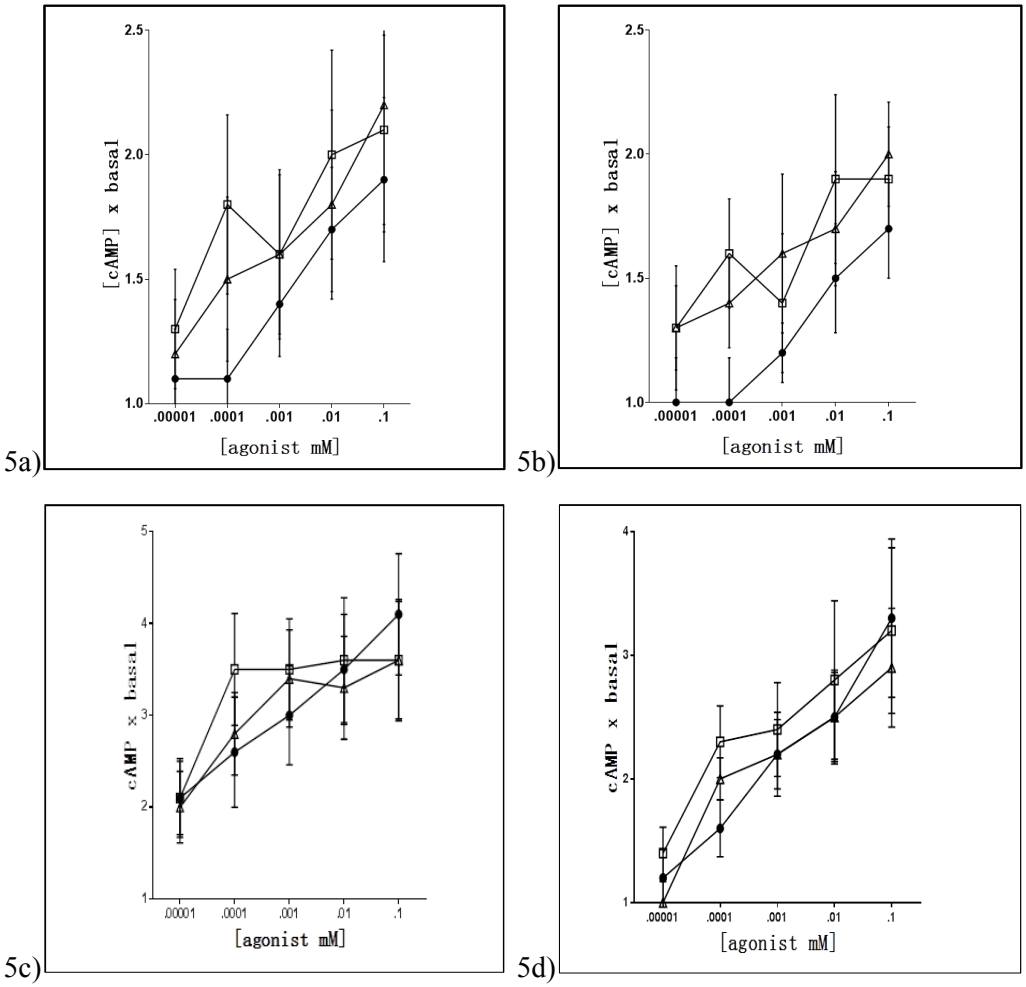


Figure 5. Dose response ratios cAMP healthy controls: the effects of different concentrations of magnesium on responses to stimulation with β-2 agonists. Effect of magnesium on increasing concentrations of β-2 agonist mM (millimolar) on cAMP production expressed as fold increase in cAMP over baseline. Comparison between the sexes.

5a) salbutamol females, 5b) salbutamol males, 5c) isoprenaline females, 5d) isoprenaline males No added magnesium ●, 1mM added magnesium □, 2mM added magnesium Δ.

In the lymphocytes from healthy females, 1mM and 2mM magnesium had no significant effect on the mean isoprenaline stimulated cAMP but produced a shift to the left of the dose-responses (Figure 5c). In lymphocytes from healthy males, 1mM magnesium increased the mean isoprenaline stimulated cAMP by 13% relative to agonist alone ($p<0.05$), and 2mM of magnesium had no effect on mean isoprenaline stimulated cAMP (Figure 5d).

Acute asthma

The subgroup sample size for males was small (n=4) therefore, the effect of sex on β-2 agonist stimulated cAMP, the effect of acute asthma on cAMP production and the effect of magnesium on β-2 agonist stimulated cAMP could not be formally assessed in males, however, there were some observed differences that are worthy of comment. When stimulated with salbutamol, lymphocytes of acute asthmatic males produced 46% more cAMP relative to asthmatic females. When stimulated with isoprenaline, acute asthmatic males produced 70% more cAMP relative to acute asthmatic females. Mean basal cAMP (6.2 vs. 1.4) and forskolin stimulated cAMP (11.8 vs. 2.9) were greater in asthmatic males relative to asthmatic females.

In lymphocytes from asthmatic males, 1mM magnesium had no effect, but 2mM of magnesium increased mean salbutamol stimulated cAMP by 27% relative to agonist alone (Figure 6b). In lymphocytes from asthmatic males, 1mM magnesium increased mean isoprenaline stimulated cAMP by 10%, and 2mM magnesium increased the mean cAMP production by 19% relative to agonist alone (Figure 6d).

In the lymphocytes from asthmatic females, 1mM and 2mM of magnesium had no significant effect on the mean salbutamol stimulated cAMP (Figure 6a). In the lymphocytes from asthmatic females, 1mM and 2mM of magnesium had no significant effect on the mean isoprenaline stimulated cAMP (Figure 6c).

When stimulated with salbutamol, lymphocytes from acute asthmatic males had a 40% less cAMP production relative to healthy male controls and when stimulated with isoprenaline, had 33% less cAMP production relative to healthy male controls. Basal and forskolin stimulated cAMP were similar between acute asthmatic and healthy male controls.

When stimulated with salbutamol, lymphocytes from acute asthmatic females had 71% less cAMP production relative to healthy female controls ($p<0.001$), and when stimulated with isoprenaline, produced 78% less cAMP relative to healthy female controls ($p<0.001$). When adjusted for multiple comparisons these differences were significant (Table 5). Basal cAMP (1.4 vs. 6.4; $p<0.01$) and forskolin stimulated cAMP (2.9 vs. 14.7; $p<0.01$) were both significantly lower in acute asthmatic females relative to controls.

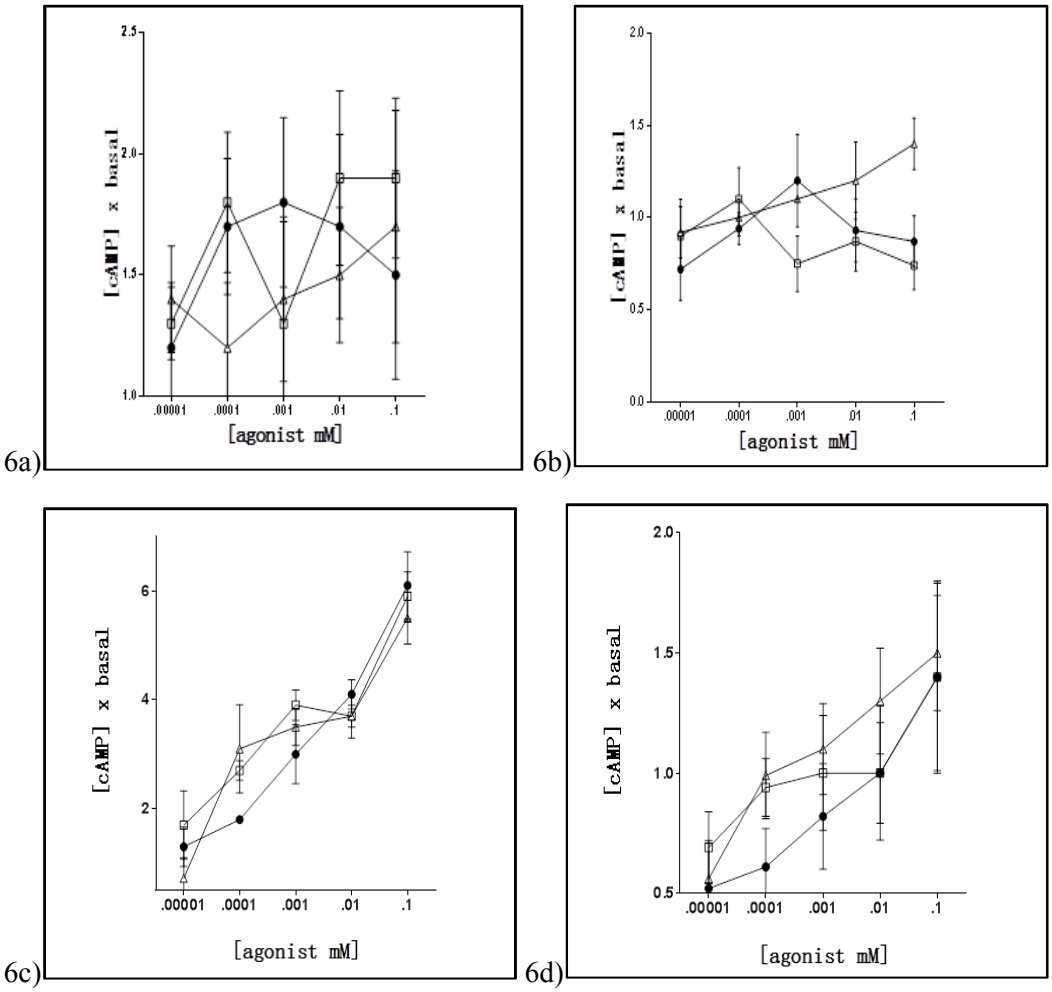


Figure 6. Dose response ratios cAMP acute asthmatics: the effects of different concentrations of magnesium on responses to stimulation with β-2 agonists. Effect of magnesium on increasing concentrations of β-2 agonist mM (millimolar) on cAMP production expressed as fold increase in cAMP over baseline. Comparison between the sexes.

6a) salbutamol females, 6b), salbutamol males, 6c) isoprenaline females, 6d) isoprenaline males No added magnesium ●, 1mM added magnesium □, 2mM added magnesium Δ.

Stable asthma

When stimulated with salbutamol, lymphocytes of stable asthmatic males had a 116% greater increase in cAMP production over baseline relative to females, but this was not significant. When stimulated with isoprenaline lymphocytes from stable asthmatic males had a 59% greater increase in cAMP production over baseline relative to females ($p<0.05$). When adjusted for multiple comparisons this difference was not significant. There were no significant differences in basal or forskolin stimulated cAMP between the sexes.

Magnesium had no significant effect on salbutamol stimulated cAMP in stable female asthmatics, and 2mM magnesium decreased the mean isoprenaline stimulated cAMP production by 17% relative to agonist alone ($p<0.05$) (Figures 7a, 7c*)*. In lymphocytes of stable male asthmatics, 1mM magnesium had no effect, but 2mM magnesium, increased the mean salbutamol stimulated cAMP by 27% relative to agonist alone ($p<0.01$) (Figure 7b). In lymphocytes of stable male asthmatics, magnesium had no significant effect of isoprenaline stimulated cAMP (Figure 7d).

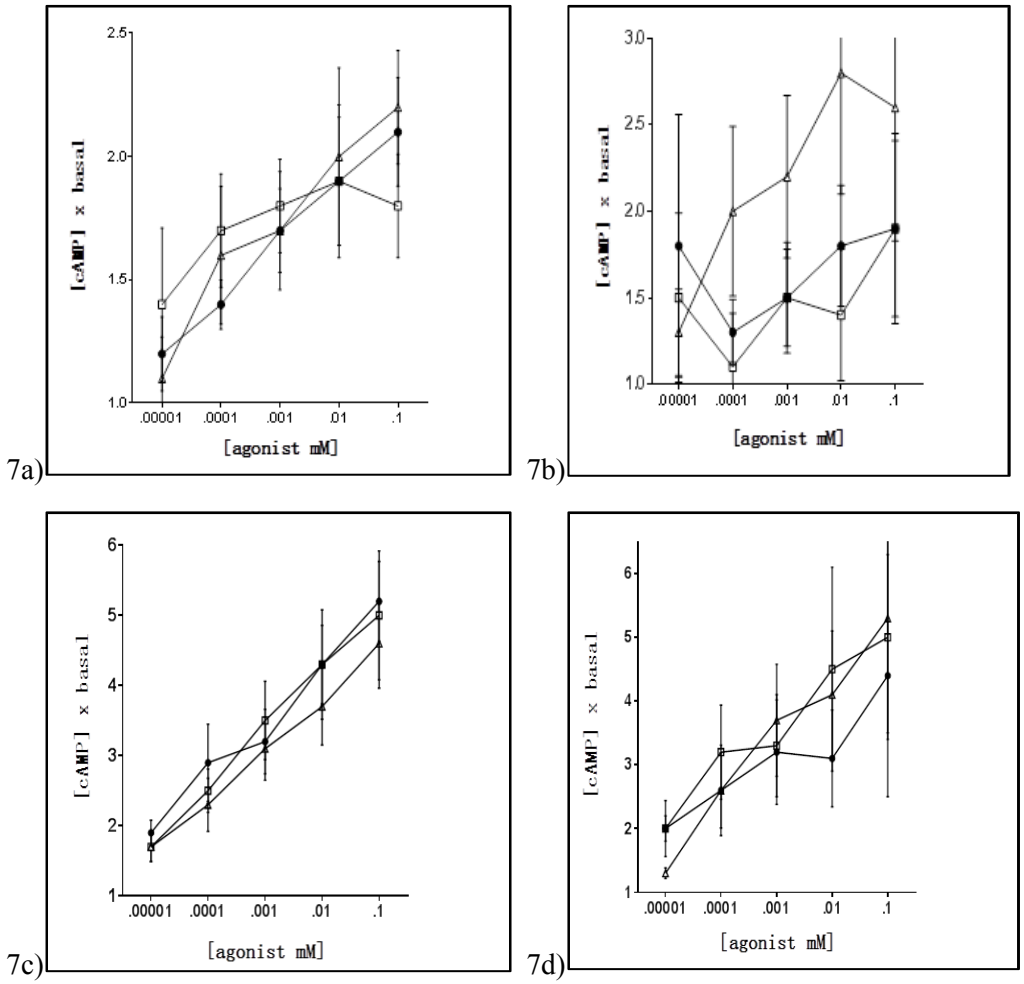


Figure 7. Dose response ratios cAMP stable asthmatics: the effects of different concentrations of magnesium on responses to stimulation with β-2 agonists. Effect of magnesium on increasing concentrations of β-2 agonist mM (millimolar) on cAMP production expressed as fold increase in cAMP over baseline. Comparison between the sexes.

7a) salbutamol females, 7b), salbutamol males, 7c) isoprenaline females, 7d) isoprenaline males, No added magnesium ●, 1mM added magnesium □, 2mM added magnesium Δ.

When stimulated with salbutamol, stable male asthmatics had a 113% greater increase in cAMP production over baseline relative to healthy males ($p<0.01$) and when stimulated with isoprenaline had a 50% greater increase in cAMP production over baseline relative to healthy males ($p<0.01$). When adjusted for multiple comparisons these were not significant. Basal cAMP was not significantly different, but forskolin stimulated cAMP was greater in lymphocytes from stable male asthmatics relative to male healthy controls (16.7 vs. 6.6; $p<0.05$).

When stimulated with salbutamol, lymphocytes from stable female asthmatics had 31% less increase in cAMP production over baseline relative to healthy female patients ($p<0.001$), and when stimulated with isoprenaline, had 47% less increase in cAMP production over baseline relative to healthy female controls ($p<0.001$). When adjusted for multiple comparisons, differences in cAMP were significant for isoprenaline-stimulated cAMP. Basal cAMP (3.6 vs. 6.4; $p<0.01$) and forskolin-stimulated cAMP (8.1 vs. 14.7; $p<0.01$) were lower in stable asthmatic females relative to healthy female controls (Table 5).

2.2.4 Other factors affecting β-2 agonist responses

Asthma control score (ACQ) and SABA use

ACQ scores and preceding SABA use were significantly greater in the acute asthmatics relative to stable asthmatics ($p<0.01$, $p<0.01$). There were significant positive correlations between ACQ and SABA use (R^2 0.790; $p<0.01$) and significant negative correlations between ACQ and FEV1 (R^2 -0.625; $p<0.01$) and FEV1 and SABA use (R^2 -0.530; $p< 0.05$).

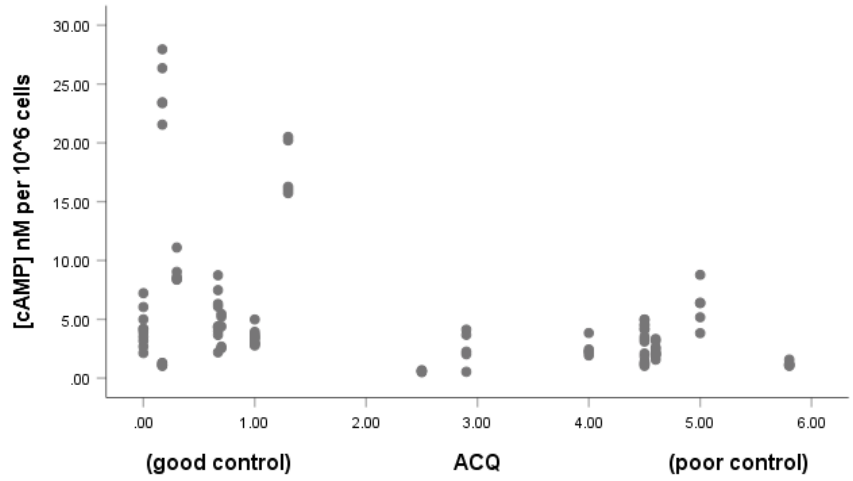
There were significant negative correlations between SABA use and the mean cAMP production in asthmatics. Salbutamol-stimulated cAMP (R^2 -0.508; $p<0.01$) and isoprenaline-stimulated cAMP (R^2 -0.464; $p<0.01$), were significantly negatively correlated with SABA use. ACQ score was significantly negatively correlated with salbutamol-stimulated (R^2-0.349; $p<0.01$) and isoprenaline-stimulated cAMP (R^2-0.393; $p<0.01$) (Figure 8a, 8b). FEV1 was significantly correlated with isoprenaline-stimulated cAMP (R^2 0.234; $p<0.05$) and more strongly correlated in acute asthmatics (R^2 0.660; $p<0.01$).

Use of oral corticosteroids

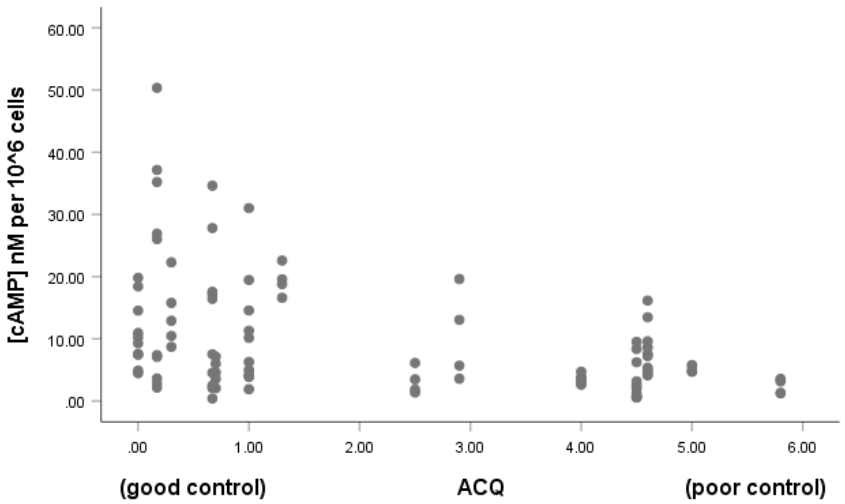
The sample size was too small (n=9) to allow a meaningful statistical comparison between those using OCS and those not however, an effect of OCS use on salbutamol stimulated cAMP production was observed. Asthmatics taking oral steroids had 30% less salbutamol stimulated cAMP relative to those

not taking OCS. This observed difference in cAMP may have been a confounding effect of sex, as there were more females using OCS relative to males.

There did not appear to be any differences in the mean isoprenaline stimulated cAMP between asthmatics taking OCS relative to those who were not, nor any differences in basal and forskolin-stimulated cAMP. Variability in salbutamol and isoprenaline stimulated cAMP was less in asthmatics taking OCS relative to those who were not. The use of OCS did not preserve the expected salbutamol dose response relationship.



8a)



8b)

Figure 8. The effects of ACQ score (asthma control questionnaire) on β-2 agonist stimulated cAMP production; β-2 agonist induced cAMP production, expressed nM (nanomoles) per 10^6 cells

8a) salbutamol stimulated cAMP, 8b) isoprenaline stimulated cAMP

Effect of atopy

Atopic status was missing for much of the stable asthmatics, and the numbers of atopic vs. non-atopic asthmatics in the acute group were small, therefore, a statistical comparison was not made. It was observed in the acute asthma group, that all the non-atopic asthmatics were female and tended to be more obese relative to atopic asthmatics, who were all male. There appeared to be a much greater response to β-2 agonist in the atopic group relative to the non-atopic asthmatics, irrespective of sex. This difference in atopy-sex distribution of the acute asthma group may explain the apparent sex differences in cAMP production.

Use of ICS/LABA

There were only 2 asthmatics not taking ICS or combination ICS/LABA therefore, the effects of ICS/LABA use on lymphocyte cAMP production was not assessed.

2.3 Discussion

2.3.1 Overview of findings

Interpretation of the findings in this study is limited by small sample sizes however, this study provides some information regarding the potential mechanism of magnesium's effects on β-2 agonist induced cAMP production and hence, magnesium's effect on bronchodilation. It also suggests that individual variation may influence treatment response.

In this study, magnesium appeared to increase β-2 agonist stimulated cAMP. This effect may occur via augmented β-2 agonist stimulation of the β-2AR, due to an increase in receptor affinity for the agonist and an increase in receptor-G-protein coupling efficacy. Elevated cAMP is the mechanism for airway smooth muscle relaxation via numerous but not well-defined pathways. Given the ubiquitous nature of the β-2AR, it is likely that this is the mechanism via which magnesium may increase beta-2 agonist induced smooth muscle relaxation. The serum levels at which Rolla and Bucca demonstrated augmented β-2 agonist bronchodilation [446], correlates with the in vitro environment of the lymphocytes and therefore, these results may be clinically significant.

This study found that lymphocyte β-2 agonist stimulated cAMP production varies in relation to sex, asthma status, severity, and salbutamol use for the treatment of asthma. These factors contribute to individual variation in β-2 agonist responsivity and could therefore, influence the response to magnesium.

For males and females in all groups studied, cAMP production over baseline was greater when cells were stimulated with isoprenaline than when stimulated with salbutamol. This is consistent with other studies in healthy lymphocytes [498]. The lymphocytes of stable male asthmatics appeared to be more responsive to salbutamol relative to isoprenaline when compared with healthy controls however, this could be explained by the preservation of β-2AR responsiveness and concomitant use of LABA which may have augmented salbutamol-stimulated cAMP production.

2.3.2 The effects of magnesium on β-2 adrenergic receptor responses

Magnesium in healthy cells

Magnesium appears to increase the mean β-2 agonist stimulated cAMP production in healthy controls and produce a shift to the left of the dose-response ratios. This suggests that magnesium may enhance cAMP production through changes in β-2AR affinity for agonists. Binding studies would confirm this hypothesis. Previous studies in isolated frog erythrocyte (β-2) [469] and rat lung (β-1) [516] membranes, report that the effect of magnesium on agonist stimulated cAMP was increased by improved binding of the β-agonists to the receptor, as indicated by a shift to the left of the competition binding curves. This increase in binding was in proportion to potency of the agonist [469]. This effect of magnesium was dependent on the ability of the receptor to stimulate AC [469, 516]. Magnesium increased isoprenaline binding with greater efficacy relative to the partial agonist soterenol and had no effect on the binding of the β-2 antagonist propranolol [469].

The mechanism by which magnesium could enhance binding of agonist and increase receptor affinity for agonist, may be via allosteric modification of the receptor. Previous studies have shown that magnesium can increase agonist affinity via allosteric modifications in a many G protein coupled receptors, including the μ,γ and κ opioid receptors in guinea-pig neurons [517] and M2 muscarinic receptors of the porcine heart [518]. This could be relevant to the β-2AR.

In this study, the observed effect of magnesium on increasing β-2 agonist stimulated cAMP was inversely proportionate to agonist efficacy. This suggests that magnesium also increases agonist ability to form a stable high affinity agonist-receptor-Gsα complex. This could also be accomplished via allosteric modification of the receptor. Conformational changes in the β-2AR at the agonist binding site, were found to result in marked alterations in the conformation of the receptors cytoplasmic Gs interacting domain [519]. This conformational change influences agonist ability to couple with Gsα [140].

A previous study of intact lymphocytes failed to show any effect of magnesium on cAMP production [515], however, the methodology used for this study was likely to have confounded their results. The

lymphocytes were incubated with a PDE inhibitor IBMX, prior to stimulation with β-2 agonist. PDE inhibitors increase cAMP by inhibiting cAMP degradation, which shifts the dose-response to the left [520] thus, use of a PDE inhibitor would mask any effect of magnesium on cAMP production. In this study the use of a PDE inhibitor was avoided, so as not to confound the results. These data taken together, suggest that magnesium may increase β-2 agonist stimulated cAMP in intact human β-2AR systems. The mechanism could be through the stabilization of an 'active' conformation via allosteric modifications, which increases the binding of the β-2 agonist to its receptor and/or coupling to its Gsα.

Magnesium in acute asthma

Lymphocyte β-2 agonist responses were reduced in asthmatics relative to controls and correlated with SABA use. In a study of peripheral lymphocytes, reduced β-2 agonist responses were demonstrated in asthmatic cells relative to non-asthmatic cells [488]. The differences in cAMP response could be attributed to increased exposure to β-2 agonist. Long term exposure to β-2 agonist stimulation *in vitro* [147-149] and *in vivo* [150, 521], resulted in a down regulation of lymphocyte β-2AR function as determined by isoprenaline stimulated cAMP, and receptor numbers as determined by radio-ligand binding studies. In exacerbating asthmatic patients, a reduction in the β-2AR response of lymphocytes to agonist stimulation was also shown to be proportionate to SABA exposure [491, 513]. In radio-ligand binding studies of the lymphocytes of exacerbating asthmatic, a reduction in surface receptors relative to stable asthma and healthy controls has also been demonstrated [492, 522, 523].

The effects of magnesium on β-2 agonist induced cAMP production were diminished in asthmatics. These data suggest that magnesium's effect on cAMP production may be limited by the proportion of receptors lost via down regulation. Magnesium may enhance β-2 agonist-receptor interactions and improve β-2 agonist responsiveness, but this effect may be limited by the degree of down regulation i.e. less effective where receptors have been lost from the surface. This may be clinically relevant. Tachyphylaxis as determined by a reduction in bronchodilator response to inhaled β-2 agonist, has been demonstrated *in vivo* in healthy subjects [524, 525] and asthmatic patients [526-528]. Meta-analysis has found that HASM tolerance to β-2 agonists occurs in asthma [529]. Studies have also correlated reduced responsivity with reduction in pulmonary β-2 receptors [530, 531].

Magnesium in stable asthma

In stable asthmatics, β-2AR function was well preserved. This would suggest that there was less down-regulation of β-2 receptor responses, which is consistent with the low SABA exposure, low severity scores and preserved FEV1, that was observed in the group of stable asthmatics in this study. The use of LABAs can also induce similar levels of tachyphylaxis as SABA [532]. Of the stable asthmatics, more than 70% were taking LABA however, 90% of LABA use was in combination with

an ICS. Corticosteroids have been shown *in vitro* to reduce the effects of β-2 agonist induced tachyphylaxis on uncoupling and receptor down-regulation [514, 533]. Clinical studies also suggest that a significant loss of response to β-2 stimulation is prevented by concomitant use of inhaled corticosteroid [327, 534]. These data suggest, that preservation of β-2 receptor responses in stable asthmatics was due to low exposure to down-regulating stimuli and concomitant ICS use.

Magnesium had almost no effect on β-2 agonist stimulated cAMP in this group of stable asthmatics. The reason for the efficacy of 2mM magnesium on salbutamol-stimulated cAMP is unclear. Magnesium's relative inefficacy may be explained by an interaction between LABA and the β-2 agonist, which may have masked any effect of magnesium in this group. The long acting β-2 agonist salmeterol has been shown to have extended receptor activation properties for the β-2AR and this may be due to binding to an 'exosite' on the receptor, thus, 'anchoring' salmeterol [535]. This is thought to be a property of other LABAs [536]. If magnesium were to increase agonist binding by allosteric modification of the receptor as proposed, the presence of another bound ligand such as a LABA, could inhibit magnesium's effect on the SABA ligand binding through the LABA ligands own effects on the β-2AR. A larger sample size would have enabled the assessment of the effects of ICS/LABA in acute asthma and enabled a comparison of the effect of ICS/LABA on the efficacy of magnesium in acute and stable asthmatics and explained these findings.

2.3.3 The effects of sex

Small sample size limited the full assessment of the effect of sex on response to magnesium however, there were some sex some differences in β-2 agonist stimulated cAMP production. These differences may influence responses to magnesium.

Healthy females produced more cAMP relative to males. In asthmatics the female sex was associated with a loss of β-2AR responses, as indicated by reduced cAMP production relative to healthy females. Stable asthmatic males had preserved β-2 agonist responsiveness. This effect was not confirmed in acute asthmatic males due to the small sample size. It is possible there are sex differences in β-2AR responses and a greater proportionate loss of surface receptors in females relative to males. This could limit magnesium's efficacy in female asthmatics relative to male asthmatics.

As discussed in chapter one, others have demonstrated sex differences in β-2AR stimulated cAMP in lymphocytes. Healthy females have been shown to have increased responses to β-2 agonist over time, associated with increased oestrogen and progesterone levels, relative to healthy males [300, 301]. Paradoxically, female asthmatics down-regulate β-2 responses in response to cyclic changes in sex hormones relative to non-asthmatic females [302]. Administration of exogenous progesterone during the follicular phase, decreased receptor density and lowered cAMP response to isoprenaline in

asthmatic females relative to non-asthmatics [537]. This difference between asthmatic and healthy females was not affected by the oral contraceptive pill [538]. These results are in keeping with the findings in this study and further support that there are sex differences in β-2AR function. These differences could influence magnesium's efficacy, and the loss of surface receptors may limit the efficacy of magnesium in female asthmatic patients. These apparent sex differences in susceptibility may be clinically relevant, as there are numerous phenotypic sex differences as discussed in chapter one.

In addition to β-2 agonist stimulated cAMP, there were sex differences in basal and forskolin stimulated cAMP, which would imply that the differences are not confined to the β-2AR but may also include post-receptor mechanisms. This data, however, may not be reliable due to the technical difficulties encountered in activating forskolin. Another study found that forskolin stimulated more cAMP in healthy females relative to males [301]. No other published studies comparing forskolin activation of cAMP between the sexes were found.

2.3.4 Other factors influencing response to magnesium

The effects of atopic status on magnesium's efficacy and β-2AR function were not able to be assessed because of the small sample size. Another study in lymphocytes showed heterogeneity in down regulation of response to β-2 agonist among asthmatics however, atopic status was not documented [539]. Pre-treatment with desensitizing agonist, receptor density was identical in both groups of asthmatics. Atopic status may influence β-2AR responses in asthmatics, in that atopy may provide some protection against loss of receptors by down-regulation. In a clinical study, *in vivo* desensitisation to inhaled β-2 agonist was only observed in non-atopic asthmatics and not in atopic asthmatics [525]. This protective effect may represent an intrinsic difference in β-2AR function. A larger sample size is required to adequately assess the effect of atopy however, these differences may be clinically relevant. Taken together with the observations in this study, these data suggest that a difference in susceptibility to down-regulation may exist between atopic and non-atopic asthmatics and therefore, atopic status may influence response to magnesium.

It is important to note in this study, that all male acute asthmatics were atopic, while all the non-atopic asthmatics were female. This is in keeping with relative frequencies of atopy among adult asthmatics described in the literature, as discussed in chapter one. Although atopic status may be another factor in determining the efficacy of magnesium in acute asthma exacerbations, there may be a sex-atopy interaction.

The effect of OCS use on responses to magnesium was not able to be assessed. Others have shown in lymphocytes of healthy and asthmatic patients, that corticosteroids mitigate the effects of β-2 agonist

induced tachyphylaxis but have no effect in in untreated lymphocytes [493, 514]. Corticosteroids were also found to shift the equilibrium towards a greater proportion of "high affinity" receptors [514].

The effects of ethnicity on β-2AR function and response to magnesium could not be assessed, as all the controls and asthmatics were Caucasian. Others have shown ethnic differences in β-2AR function. A study of lymphocytes from healthy adults found African-Americans to have greater isoprenaline stimulated cAMP relative to Caucasians [540]. Healthy female African-Americans also been found to have greater isoprenaline stimulated cAMP relative to healthy African-American males and relative to healthy Caucasian males and females [301]. A study in predominately males found isoprenaline stimulated cAMP to be greater in Caucasians relative to African-Americans [541]. Studies of males only with hypertension, found African-Americans had greater isoprenaline stimulated cAMP [542] and less down-regulation of β-2AR responses [543] relative to Caucasians. These data suggest an interaction of race and sex in determining β-2AR responses to agonist stimulation which could also influence response to magnesium.

2.3.5 Limitations

The small sample size and missing data prevented a complete assessment of the effect of sex, and the effects of atopy and corticosteroids, on magnesium's efficacy in acute asthma and a detailed assessment of any interactions between these factors. No distinction was made between self-reported atopy and doctor diagnosed atopy which may be an important factor in determining an effect of atopy.

The methodology used may have limited the accuracy of some of the data. The methodology for β-2 agonist stimulation of cAMP was subject to the influences of ambient temperature on enzyme activity, and there may have been a loss of cAMP by degradation prior to assay, causing potential errors in measurement. This is likely to have been consistent across all experiments.

The low cAMP levels produced may have limited the accuracy of the data. The determination of cAMP concentration required interpolation from standards. The interpolated values were often calculated as zero, despite an absorbance value that was greater than zero. This occurred because the assay was not sensitive at cAMP concentrations ≤0.3 nM, and in some of the experiments, cAMP production was ≤0.3 nM. This resulted in data loss in two patients.

Forskolin is a powerful direct activator of AC however, forskolin induced cAMP was much lower than expected. Some samples were exhausted due to poor cell viability, preventing repetition of the experiments. In many experiments where forskolin-stimulated cAMP levels were low, isoprenaline induced cAMP levels were preserved, suggesting that the low levels were not due to loss of cAMP

through degradation. It is more likely that forskolin activation was affected. Forskolin was stored as a stock solution in DMSO, which inhibits forskolin activation. This occurs at concentrations of ≥10% DMSO [544]. Given that the stock solution was diluted 1:100, it is unlikely that forskolin was inhibited by the DMSO. Stock solution was diluted in Milli-Q immediately prior to experimentation as forskolin is unstable in water. The amount of time taken to perform the experiments could have resulted degradation of forskolin.

2.3.6 Future directions

Further study of the effects of magnesium on the β-2AR is warranted. Larger studies are needed to confirm the findings of the effects of magnesium on β-2AR responses and to assess the effects of sex on the efficacy of magnesium. More sample material is required to allow for the need to repeat experiments, should insufficient cAMP be produced. Larger quantities of cells could be used to ensure generation of adequate cAMP for ELIZA assays to be performed more accurately.

Further study, with a larger sample size is required to assess the effects of atopy and corticosteroids with and without LABA, on β-2AR function and efficacy of magnesium. Larger studies would also allow assessment of any interactions between sex and these factors. Larger studies could also assess the effects of ethnicity on β-2AR function and efficacy of magnesium.

Future study should include radio-ligand binding studies to quantitatively and qualitatively assess the β-2AR. Correlation between these laboratory findings and clinical outcomes within individuals would confirm the clinical relevance of these findings.

2.4 Conclusions

Magnesium may increase β-2 agonist stimulated cAMP through increasing receptor affinity for the agonist, possibly via allosteric modification of the β-2AR. This mechanism may also contribute to increased coupling efficacy between the β-2AR and its Gsα sub-unit, and hence, further increase β-2 agonist efficacy. Magnesium's effect is decreased where receptors are lost through down-regulation.

Further work is required to confirm these results and to further assess the influence of sex, ethnicity atopy and asthma treatment on β-2AR function and magnesium's efficacy. The relevance of these findings to clinical outcomes is yet to be established.

Chapter 3: Clinical trial of intravenous magnesium in acute asthma exacerbations

3.1 Methods

3.1.1 Trial design and setting

This study was a randomised double-blind placebo-controlled clinical trial to assess the effects of the addition of 5g(20mmol) of intravenous magnesium sulphate as a single infusion, to standard treatment of nebulisations and oral steroids, in patients presenting to an emergency department with an acute exacerbation of asthma. The setting was an urban district emergency department of the Lyell McEwin Hospital in the northern suburbs of Adelaide, South Australia.

The clinical trial was retrospectively registered with the Australian New Zealand Clinical Trials Registry ACTRN: ACTRN12612000838819

Eligibility

Patients with an acute exacerbation of asthma that presented to the emergency department were considered eligible for inclusion in the study if they complained of acute difficulty breathing, had a known diagnosis of asthma and required immediate treatment for their symptoms.

Classification of patients

Asthma was defined by reversible airways obstruction demonstrated clinically, diagnosed by a medical practitioner, or in a patient with symptoms of asthma at any time during their lifetime whether diagnosed or not (e.g. patient giving history of a recurrent cough and wheeze but had not sought medical assistance previously), or on asthma treatment as prescribed by a doctor. This treatment included regular inhaled corticosteroid, as needed inhaled salbutamol, or another bronchodilator.

An acute attack was defined as the onset of symptoms in previously asymptomatic patient or an acute worsening of symptoms of diagnosed asthma, irrespective of treatment or acute respiratory distress due to asthma.

Inclusion Criteria

Inclusion criteria were age 18–50 years-old, the ability to give written consent to participation and an FEV1 of <70% predicted at least 15 minutes post ‘rescue treatment’. Those with features of life-threatening asthma as defined by British Thoracic Society Guidelines such as: altered mental state,

exhaustion, cyanosis, arrhythmia, hypotension, silent chest, poor respiratory effort, were excluded. Patients with an FEV1 of ≥70% predicted post rescue treatment were excluded. Patients were also excluded if; unable to perform a single forced expiratory manoeuvre for any reason, known to be pregnant, unwilling to provide written consent, unable to consent due to language barrier or intellectual impairment, known contra-indications to magnesium (hypermagnesemia, hypotension, heart block, allergy or sensitivity to magnesium, renal failure) were present, they had received magnesium in the previous 24 hours, or if they had been previously enrolled in the study. Patients were also excluded if they had a diagnosis of a pre-existing lung disease other than asthma at the time of enrolment (emphysema, chronic bronchitis, bronchiectasis, heart failure). Two patients were eligible for screening, however, were not screened, as they had already participated in the study.

Withdrawal criteria

Patients could withdraw voluntarily from the trial at any time for any reason. Involuntary withdrawal criteria were; a deterioration in the patient's clinical condition, requiring nebulisations of salbutamol more frequently then every half hour, the need for non-invasive ventilation, or impending or actual respiratory arrest. If withdrawn from the trial treatment, that subject's treatment was un-blinded for the purposes of determining further treatment including intravenous magnesium. Each bag of trial solution had a corresponding numbered sealed envelope with the information regarding the contents of the solution and was accessible to staff along with the trial solutions. This procedure was instituted to enable safe administration of magnesium should a patient deteriorate and be withdrawn from the trial, as our department guidelines included magnesium sulphate for treating severe-life-threatening exacerbations. The guidelines suggest 2.5g(10mmol) bolus for severe/life-threatening asthma which can be repeated up to 5g(20mmol) total whilst in the emergency department.

Patients were withdrawn if an alternative diagnosis was proven from the time of enrolment to the time of discharge from hospital. The electronic case record was used to review the results of radiology and pathology to confirm the diagnosis of asthma and exclude an alternate diagnosis. The decision to retrospectively exclude patients was made by the principal investigator.

Recruitment

Patients were recruited from the population of asthmatics presenting to the emergency department for the treatment of an acute exacerbation of asthma. Recruitment of patients occurred 24 hours a day, 7 days a week, provided that a nurse or medical officer who was accredited in the use of the 'EasyOne®' spirometer was available. The principal investigator was, therefore, contactable 24 hours/7 days a week, so as to be notified of any potential eligible recruits as per the inclusion criteria, and to recruit patients and collect data where onsite accredited staff were not available. This occurred on 7 occasions.

3.1.2 Testing and treatment before consent

Rescue treatment was given to those who were eligible for inclusion as part of the study protocol. Treatment consisted of three consecutive nebulisations of 5mg of salbutamol, one 500mcg nebulisation of ipratropium and 50mg of oral prednisolone, provided this had not been administered in the 24 hours prior to arrival. A baseline FEV1 measurement was then taken, prior to consent, to determine if the final of the inclusion criteria was met. Spirometry was performed with the EasyOne® hand held spirometer [n.d.d Medical Technologies US/Zurich]. The value of the best of three attempts was recorded although not all values were the best of 3 attempts. An FEV1 of <70% of the predicted value, from NHANES (III) values or best if known, was required for study entry.

Testing was performed at least 15 minutes after the last of the three rescue nebulisations however, many of the acutely exacerbating asthmatics were unable to perform 3 attempts, therefore, an attempt was accepted if considered adequate. An attempt was considered adequate if the forced expiration lasted at least 2 seconds, there was a sharp early PEF and the patient did not cough during the first 2 seconds of the manoeuvre. Repeated attempts were encouraged however, in cases where this was not possible, a single effort was accepted. This method of obtaining spirometry in acute asthma has been used in other published studies in acute asthma, where repeated attempts to obtain "quality" spirometry were not possible [383, 384].

Those patients with an FEV1 of ≥70% predicted were excluded from the trial and managed by the treating physician. Those who met the inclusion criteria were then consented and randomised.

Ethics and consent

Ethics was approved by the Human Ethics Research Committee of the Lyell McEwin, and Queen Elizabeth hospitals [TQEH/LMH Approval No: 2010076]. An amendment for the collection of whole blood samples was later approved for the laboratory component of this study, to enable the isolation of lymphocytes for cAMP analysis. The treating physician or the principal investigator obtained informed consent in writing. Written information sheets were provided to patients, prior to consent.

Randomisation and concealment of allocation

Patients were randomised to receive either magnesium sulphate or placebo. The randomisation sequence was numerical and computer-generated in lots of 30. Randomisation was carried out by the hospital's trial pharmacist, using a web-based free algorithm-based software program Urbaniak, G. C., & Plous, S. (2015): *Research Randomizer* (Version 4.0) [Computer software]; Retrieved on January 20, 2017, from http://www.randomizer.org/.

Trial solutions of placebo (0.9% saline) or magnesium sulphate (0.9% saline and 5g(20mmol) of magnesium) were made up in plain 100mL bags covered with an adhesive plain white label and allocated a number beginning with 1. Trial solutions in batches of 10 were distributed to the emergency department. These were stored at room temperature in accordance with pharmacy guidelines. Unused solutions were discarded after 3 months and replaced with new solutions to which new numbers were allocated. Each solution was allocated sequentially to each recruited patient in numerical order as labelled by the hospital pharmacy under direction of the trial pharmacist.

Interventions

After written consent was obtained, an intravenous cannula was inserted and 30mLs of blood was drawn. Samples were assayed for blood cell counts, serum electrolytes, serum magnesium and blood was set aside for isolation of PBMCs. Patients then received a 100mL intravenous infusion over 30-40 minutes of either 5g(20mmol) of magnesium sulphate in 0.9% saline or 0.9% saline alone. The experimental solution was administered concurrently with a further 5mg salbutamol nebulisation.

Further salbutamol nebulisations were administered on an as-needed basis, up to every half hour, for a further 3 hours. Supplemental oxygen was administered by the medical or nursing staff, as per usual practice, for a saturation below 92% on room air and titrated to effect. Other interventions and treatments were administered at the discretion of the treating medical officer during and after participation in the trial.

Four hours after randomisation, the patients' vital signs and FEV1 were assessed to determine disposition. Those fulfilling the requirements for discharge (n=6) and those that chose to leave against medical advice (n=2), were given standardised discharge medications and treatment. Discharge medications and follow-up were standardised and included 50mg oral prednisolone daily for 5-7days and salbutamol inhalations via an inhaler and spacer, 3-4 hourly as needed. A letter for GP follow-up and a respiratory nurse outpatient follow-up within 72 hours was provided to patients upon discharge or completion of the trial. Those who were admitted to hospital were reviewed by the hospital respiratory nurse and repeat FEV1 was performed within 24 hours of admission.

3.1.3 Trial end point

Discharge/admission

Disposition as defined in the trial protocol was determined by FEV1, taken at least 15 minutes after the last nebulisation and four hours post-randomisation. Those whose FEV1was ≥75% of predicted were considered to have been successfully treated and suitable for discharge, provided there were no

signs of clinical instability. These included an oxygen saturation <92% on room air, signs of increased work of breathing i.e. subcostal or sternal recession, subjective symptoms of dyspnoea as defined by Modified Borg Score ≥ 2, abnormal vital signs including but not limited to a respiratory rate ≥22. Patients were then discharged provided none of the following applied; concerns of the treating physician regarding compliance, social isolation or restricted access to medical care, or a history of previous mechanical ventilation or cardio-respiratory arrest as the result of an acute asthma attack. Those unable to be discharged for these reasons or at the patient's request, were further observed in the emergency department until the treating physician deemed it safe for the patient to be discharged.

Those patients whose FEV1was < 75% of predicted at 4 hours post randomisation were referred to the inpatient medical team for admission. Patients were then admitted to either a medical ward or intensive care, as determined by the treating physician. Those whose FEV1 was < 75% of predicted and refused admission to hospital, were discharged from the emergency department if clinically stable as described above. Patients who were not clinically stable were encouraged to remain in hospital however, some chose to leave against medical advice.

Patients who requested to be discharged prior to the four hours after randomisation, could attempt FEV1. Subjects with a FEV1 ≥ 75% of predicted were discharged from hospital, provided that there were no signs of clinical instability as described above. Patients with an FEV1 < 75% predicted at the time of request for discharge, were offered medical admission. Those whose FEV1 was < 75% of predicted and refused admission to hospital, were discharged from the emergency department if clinically stable as described above. Patients who were not clinically stable were encouraged to remain in hospital however, some chose to leave against medical advice.

Outcome measures

The primary outcome measure was discharge from the emergency department, which was determined on an intention-to-treat-basis by the treating physician at 4 hours after randomisation. At this time, the patient's participation in the trial was concluded.

Secondary outcome measures during participation were; adverse effects including flushing, injection site pain, hypotension and bradycardia, involuntary withdrawal from the trial and change in FEV1, respiratory rate and subjective dyspnoea score as measured by the Borg scale, from baseline at randomisation to 4 hours post randomisation. The patient's length of stay in hospital from the time of randomisation to discharge from hospital was also obtained from the clinical case record.

Data collection

Outcome data was recorded separately to the clinical case record. Data sheets were collated by the principal investigator. Data collected included; demographic data (sex, BMI, atopy, smoking status),

baseline physiological parameters taken at randomisation post rescue treatment (respiratory rate, oxygen saturation, blood pressure, heart rate and FEV1% predicted) and at 4 hours post randomisation, regular asthma treatment, use of oral steroid in the preceding 24 hours prior to presentation, previous intensive care admissions or need for invasive ventilation and other medication usage in the week prior to presentation (anti-histamines, anti-reflux, antibiotics, oral steroids). Additional data included FEV1% predicted at follow-up either 24 hours or 72 hours after completion of the trial. An abbreviated Asthma Control Questionnaire (ACQ) was administered by the treating physician or self- administered by the patient at the time of randomisation (i.e. post rescue treatment). The questionnaire contained 6 questions relating to asthma symptoms and answers were scored on a scale of 0 to 6. The ACQ score was determined by dividing the total score by 6 (the number of questions). The inclusion the ACQ score provided the additional information of asthma control/presence of symptoms of active disease and SABA use, that spirometry alone could not provide [545, 546].

Consent forms, patient information sheets, data collection sheets and a trial protocol flow diagram were available in the emergency department. Completed forms were sealed in envelopes that were provided by and collected by the principal investigator for safe storage in accordance with the Australian Code for the Responsible Conduct of Research 2007 (The Code).

FEV1 measurements were performed by emergency department nurses accredited for use of the EasyOne® spirometer [n.d.d Medical Technologies US/Zurich]. Nurses and the principal investigator were accredited by the respiratory nurse practitioner accredited in training staff for the use of the EasyOne® spirometer. Ten of the 280 nursing staff and one of the 80 medical officers (the principal investigator) were trained in its use. Due to staff workloads and competing priorities in staff education, no other persons were trained. This limited the number of patients that were recruited.

Consent form, data collection and patient information sheets are presented in the Appendix at the end of this document. Raw data is displayed in the Appendices at the end of this document.

3.1.4 Statistical analysis

The data analysis was on an intention-to-treat basis with the primary outcome variable of admission to hospital at first visit. A prior power calculation (X^2 test for a 2x2 contingency table) determined that a study of n=390 per group would have 80% power to detect a 10% difference in admission rates with α= 0.05. A post-hoc power calculation (X^2 test for a 2x2 contingency table) determined that with α=0.05 our study (n=24) had a 36% power to detect a 22% difference in outcomes. A sample size of n=60 per group would have an 80% power to detect a 22% difference in outcomes.

The secondary outcomes analysed were; re-admission to hospital within 72 hours, total length of hospital stay in hours, changes in FEV1, change in respiratory rate and subjective dyspnoea rating by 0-10 scale (Borg) and oxygen saturation from randomisation to 4 hours after randomisation. Differences in demographic variables and physiological parameters of heart rate and blood pressure were also compared between treatment groups. Primary and secondary outcomes were also compared between the sexes.

Statistical analysis was performed using SPSS 24 (Statistical package software for students, IBM). A Chi-squared test was used to compare differences in categorical variables of the primary outcome of admission to hospital, demographic parameters of age, sex, smoking status and asthma treatment at home. Continuous variables (vital signs, ACQ and Borg scores, SABA use) were non-normally distributed, so non-parametric tests were used. The Mann-Whitney U test was used to compare independent variables i.e. between treatment groups, disposition groups and between the sexes. For dependent variables i.e. repeated measures (difference in Borg score, respiratory rate, blood pressure and heart rate from randomisation to 4 hours post randomisation), a Wilcoxon-Ranked sign test was used. Correlations were performed with Spearman's Rho. Significance was considered at an alpha level of 0.05.

3.2 Results

Forty-one patients were enrolled in the clinical trial over a period of 5 years. Seventeen of these were excluded from analysis. Of the 17 excluded patients, one was consented without an FEV1 measurement, one was not randomised because the trial solution had expired, 7 patients were not randomised for reasons that could not be ascertained and 8 were withdrawn post-randomisation. One patient withdrew voluntarily for personal reasons, one was involuntarily withdrawn due to deterioration in asthma symptoms and six patients were withdrawn retrospectively post randomisation, due to an alternative primary diagnosis. Of the 6 that were retrospectively withdrawn, one had a diagnosis of bronchiectasis and 5 were excluded due to an alternative diagnosis of pneumonia. There were 24 patients suitable for inclusion and data analysis (Figure 9).

Demographics and baseline characteristics

There were 10 males and 14 females recruited. Twenty-two of the 24 patients were Caucasian. The remaining two were Aboriginal. The median age of patients was 35.5 years (interquartile range 26.3-46) with 45.8% (n=11) over the age of forty. The average BMI of patients was 29.4 (range 23.1-39.5) and 62.5% (n=15) were active smokers. The median FEV1 of the study group was 52% (IQR 38-

59%), which classified them as having mild to moderate asthma according to BTS/SIGN guidelines. ACQ scores were high (3.5), indicating poor control of asthma symptoms in the week prior to presentation. The median number of SABA inhalations per day in the preceding week was 12, which is equivalent to 1200mcg salbutamol per day (Table 6).

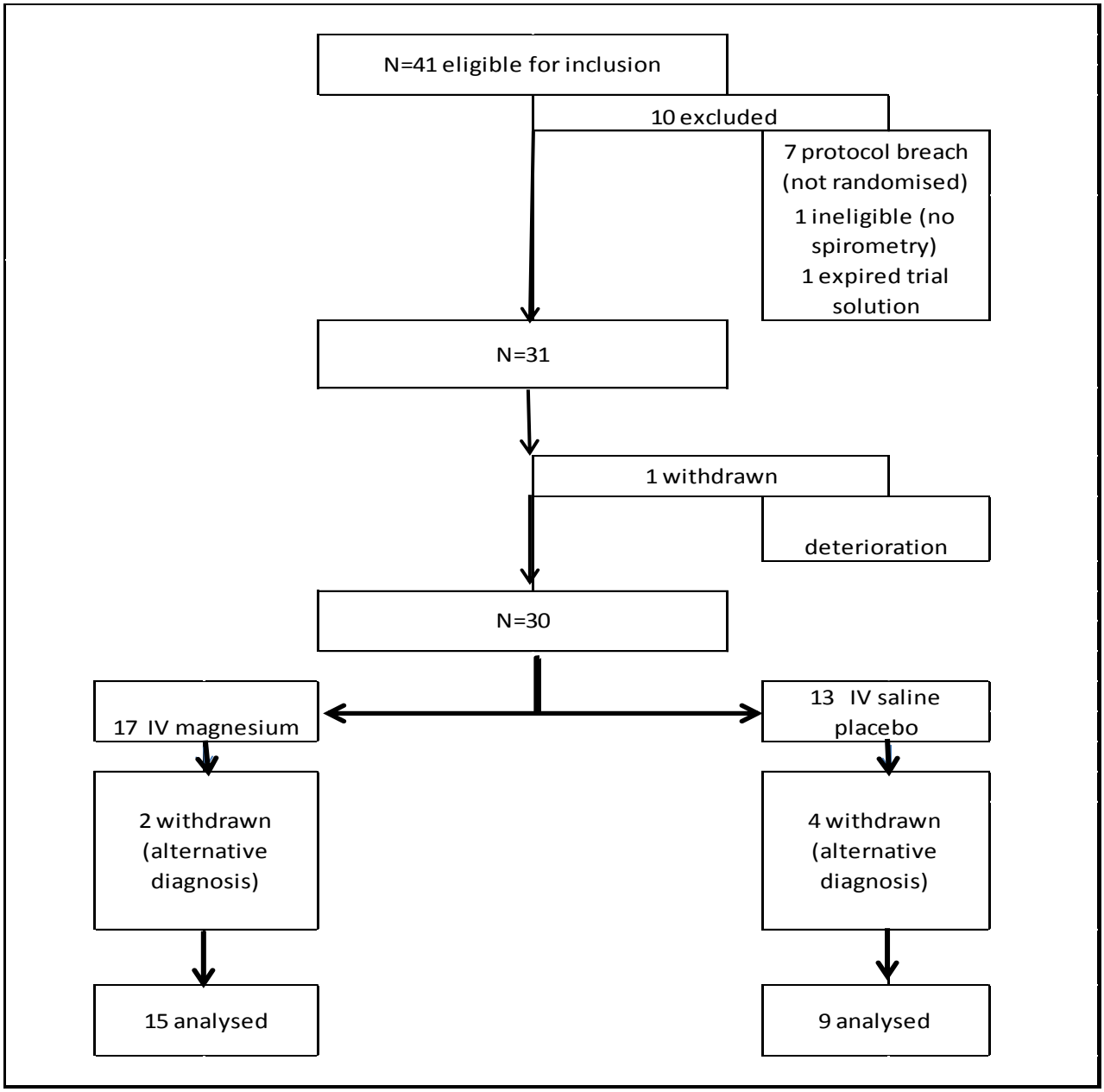


Figure 9. Flow diagram of the outcome of enrolment of patients recruited for the clinical trial subsequent to their consent.

Table 6. Demographic and baseline variables (at randomisation); comparison between treatment and placebo.

Demographic /baseline characteristic	magnesium (n=15)	placebo (n=9)	p value
Caucasian ethnicity	14 (93.3)	8(88.9)	0.70
Age, median (IQR)	38 (25-46)	31 (27-48)	0.86
Female , n (%)	8 (53.3)	6 (66.7)	0.52
BMI, median (IQR)	33.6 (23.0-37.9)	27.3 (24.0-41.2)	0.93
Current smoker, n (%)	9 (60)	6 (66.7)	0.81
Serum magnesium (mM), mean (SE)	0.84 (0.02)	0.87 (0.02)	0.48
Preventer, n (%)	8 (53.3)	5 (55.6)	0.92
ICS only	2 (13.3)	1 (11.1)	
ICS+LABA	6 (40)	4 (44.4)	
Other medications (antibiotics, antihistamines, others), n (%)	6 (35.7)	2 (16.7)	0.31
Atopy (hayfever/eczma/other), n (%)	6 (40)	5 (55.6)	0.47
OCS in 24 hours prior to arrival , n (%)	4 (26.7)	3 (33.3)	0.52
Asthma Control Questionairre (0-6), median (IQR)	3.1 (2.0-4.8)	4.5 (2.5-5)	0.34
Preceding SABA use (mcg/day), median (IQR)	1400 (500-1800)	1200 (800-1600)	0.92
Borg dyspnoea scale (0-10) , median (IQR)	6 (6-7)	6.5 (5-8)	0.85
FEV1 % predicted, median (IQR)	54 (44-61)	40 (34-51)	0.06
Respiratory rate in breaths per minute , median (IQR)	24 (22-26)	24 (22-26)	0.56
Oxygen saturation %, median (IQR)	94 (93-96)	95 (93-96)	0.77
Pulse rate in beats per minute , median (IQR)	121 (100-130)	112 (94-122)	0.19
Systolic blood pressure mmHg , median (IQR)	115 (110-140)	115 (110-120)	0.64
Diastolic blood pressure mmHg, median (IQR)	70 (60-80)	65 (60-80)	0.60

IQR: interquartile range, SE: standard error, BM: body mass index,
ICS: inhaled corticosteroid, LABA: long acting β-2 agonist, OCS: oral corticosteroid, SABA: short acting β agonist, FEV1: forced expiratory volume in 1 second, mM (millimolar).

Fifty four percent of patients (n=13) were taking inhaled corticosteroid (ICS), mostly in combination with a long acting β-2 agonist fluticasone 250-1000mcg/ salmeterol 25-100mcg daily, or budesonide 400-800mcg/eformoterol 6-24mcg daily. There was no difference in ACQ scores between ICS users relative to non-users. The prevalence of OCS use was 29% (37.5 mg-50mg daily for 1-7 days), and 45.8% (n=11) were atopic, as defined by having a history of allergic rhinitis (hay fever), eczema or hives, or a more serious reaction to another trigger such as eggs or latex. Serum magnesium levels were within normal limits.

Fifteen patients were randomised to magnesium and nine were randomised to placebo. There were 8 females and 7 males in the magnesium group and 6 females and 3 males in the placebo group. There were no statistically significant differences in any of the demographic or baseline physiological

parameters between the magnesium and placebo treatment groups (Table 6). There was a trend towards a greater baseline FEV1 in the magnesium group relative to the placebo group, which was near significant (p=0.06).

The overall admission rate for the study group was 75% (n=18). The average length of stay in hospital for admitted patients was 36 hours (IQR 15-48 hours). Only one patient fulfilled the discharge criteria as defined in the protocol as a post treatment FEV1 of ≥ 75% predicted. The remaining six patients were discharged as they were considered clinically stable. Retrospective analysis of the clinical case record found that those discharged had a Borg scores <2 and respiratory rates ≤ 20. These patients required salbutamol nebulisations less frequently than every 2 hours, and none of these patients returned to an emergency department within the 7 days following discharge. These patients were considered to have been successfully discharged, and the outcome of discharge from hospital was evaluated on an intention-to-treat basis.

Primary outcome

The difference in admission rates between the magnesium group (66.7%; n=10) and placebo (88.9%; n=8) was not significant (p=0.22). Five of those patients admitted, discharged themselves from the emergency department against medical advice (20%). They had persistently elevated respiratory rates of > 22 breaths per minute, a requirement for nebulisations more frequently than 2 hours and an average FEV1 of 57% predicted. Four of these were in the magnesium treatment group and three of these patients returned within 48 hours and were re-admitted to hospital.

Secondary outcomes

No patients from either the magnesium or the placebo group required an intensive care admission. None of the patients discharged from the emergency department by the treating physician required re-admission to hospital within 7 days.

Those in the magnesium group had a significant decrease in heart rate (HR) at 4 hours post-randomisation (p=0.02). The frequency of nebulisations 4 hours post-randomisation tended to be less in the magnesium group (2-4 hourly) relative to placebo group (1-2 hourly) (p=0.07) (Table 7). There were no significant improvements in FEV1 for either group, with a median improvement of 3% for both groups.

Adverse effects

There was a 20.8% (n=5) incidence of a sensation of body flushing in the magnesium group. In the placebo group, there was an 11.1% (n=1) incidence of pain at the cannula site and an 11.1% incidence of flushing. No other adverse effects were recorded.

Table 7. Secondary outcomes.

Secondary outcomes (4 hours post randomisation)	magnesium (n=15)	placebo (n=9)	p value
BORG score (0-10)	2 (9)	1 (3)	0.86
FEV1 % predicted (%)	56 (11)	53(5)	0.74
Heart rate in beats per minute (bpm)	98(13)	104 (7)	0.02
Respiratory rate (breaths/min)	22 (11)	22 (5)	0.59
Oxygen saturation (%)	96 (13)	95(8)	0.88
Length of stay (hours)	28 (5)	41 (5)	0.76
Time between nebulizations after randomization in hours (range)	2-4 (11)	1-2 (5)	0.12

Values given as median: (N), BORG: subjective dyspnoea ranking (0-10), FEV1: forced expiratory volume in 1 second.

Table 8. Sex differences in baseline variables and outcomes.

Demographic variable/outcome	males (n=10)	females (n=14)	p value
Admission rate , n (%)	6 (60)	12 (85.7)	0.15
Length of stay (hrs), median (IQR)	28 (12-48)	62 (38-145)	0.08
Atopy, n (%)	7 (70)	4 (28.6)	0.1
Asthma Control Questionairre (0-6), median (IQR)	3.0 (1.5 -4.2)	4.8 (2.5-5.0)	0.17
Preceding SABA use (mcg/day), median (IQR)	800 (200-1600)	1600 (1200-2000)	0.17

IQR: interquartile range, SABA: short acting β-2 agonist

Table 9. Outcome variables affecting disposition.

Factors affecting outcome of admission to hospital (baseline values at recruitment)	admitted (n=18)	discharged (n=6)	p value
Asthma control score (0-6) median (IQR)	4.7 (3.0-5.1)	0.6 (0.5-2.2)	0.001
Short acting B2 agonist use mcg/day , median (IQR)	1600 (900-1900)	200 (50-1600)	0.09
Initial FEV1 % predicted , median (IQR)	47.5 (37-56)	58.5 (44-63)	0.18
Oxygen saturations on arrival % , median (IQR)	94 (93-95)	95.5 (95-96)	0.14
Systolic blood pressure (mmHg) median (IQR)	110 (110-120)	135 (121-156)	0.02

SABA: short acting β-2agonist, FEV1: forced expiratory volume in one second, IQR: interquartile range

Other factors affecting outcomes independent of treatment

There were sex differences in admission rates, baseline and demographic characteristics but these were not significant (Table 8). The main factor affecting outcomes was severity as measured by ACQ score (Table 9). The ACQ score was correlated with the amount of SABA use prior to presentation (R^2-680; p=0.001). There were no significant correlations between ACQ score and improvements in secondary outcomes. ACQ score was negatively correlated with a degree of improvement in subjective dyspnoea at 4 hours post randomisation but this did not reach significance (R^2-.492; p=0.053). There was a negative association between SABA use and length of stay, which was significant (R^2 -568: p<0.043). Systolic blood pressure was significantly associated with admission, though the reason for this is unclear. The sample size was too small to detect any other associations with hospital admission however, we observed that all non-atopic asthmatics were admitted. There were no differences in baseline parameters between atopic and non-atopic asthmatics.

3.3 Discussion

When designing this study, it was identified that the Lyell McEwin emergency department had 90 patients admitted over a 12-month period for asthma presentation in the 18-50-year age range (SA. Health-HASS/EDIS 1/1/2009-31/12/2009), suggesting that the study was feasible for patient numbers. The primary objective was not met however, due to insufficient numbers of patients. Acute asthma patients were also required for the laboratory study and therefore, recruitment continued despite low numbers. There were no alternatives to obtaining "original" data for the purpose of the thesis. This study demonstrates the difficulties in conducting clinical research in a busy emergency department where the focus is service provision.

Potential benefits

This study identified some potential benefits of magnesium, such as a reduction in heart rate and possibly a reduced requirement for nebulisations. Heart rate is important parameter in acute asthma as heart rate is an indicator of severity of the exacerbation [2-4]. Reduction in heart rate therefore represents a clinical improvement.

A reduction in the requirement for nebulisations is important, as it reduces the risk of salbutamol toxicity and improves patient comfort. Only one other study has reported nebulised β-2 agonist use as an outcome and there was 10% reduction in nebulisations used in those treated with magnesium relative to placebo overall, which was not statistically assessed [378]. This suggests that the addition

of magnesium to the treatment regimen may contribute to a reduction in admission rates relative to standard care alone. A larger sample size may have detected a difference in this outcome.

The dose of magnesium used in this trial appears to be safe however, the small sample size may have been insufficient to detect any serious adverse effects. This is the first Randomised Controlled Trial to assess the effects of magnesium in adults with acute asthma at the dose of 5g(20mmol), although higher doses have been documented in case reports. Previous studies have used 1.2-2g(5-8mmol) of magnesium [377, 381, 382, 384, 387, 388]. Doses of 100mg/kg (0.4mmol/kg) have been used in paediatric asthma [414], and individual case reports in both males and females with life-threatening asthma have reported use of doses of up to 20g(80mmol) in one hour, without any serious untoward effects [398]. These data support the safety of the use of a 5g slow bolus infusion in acute asthma in adults however, care should always be taken in monitoring for potential adverse effects, such as respiratory muscle weakness.

Subgroup analyses and other factors influencing admission

The most significant contributing factor towards the primary outcome of disposition was a high ACQ score, which is indicative of poor control of asthma symptoms and instability. Two large international cohort studies also found poor asthma control to be a predictor of emergency department visits and hospital admissions [547, 548].

Due to the small sample size it was not possible to examine these data for other sub-group benefits, nor was it possible to assess the effect of other individual factors on admission rates and response to magnesium, such as atopy/sex. Phenotypic distinctions in asthma were traditionally made based on atopy [269], however, no studies comparing admission rates between atopic and non-atopic adult asthmatics have been published to the best of our knowledge. It would also have been useful to assess the effects of sex and assess for any sex-atopy interaction as women have more severe asthma [285, 289-294], are more likely to use oral steroids [291] and are more likely to be admitted to hospital relative to men [292, 296]. Additionally, the prevalence of non-atopic asthma is also greater in adult females relative to adult males [305].

3.3.1 Limitations

The main limitation of this study is the small sample size. The small numbers of recruited patients precluded an assessment of the effects of magnesium in this group. We were also unable to assess the effects of sex and atopy on outcomes.

The reliance on emergency nursing staff to participate in training in the use of the spirometer, and then to have them administer the lung function test at the time of patient presentation, was a

significant limitation that reduced recruitment rates. This reduced the potential number of patients recruited as it was only possible to recruit patients on days where a nurse trained in the use of the spirometer, was available. The principal investigator was available either directly during normal clinical duties, or remotely on call; however, being the sole investigator with no external funding provided, limited my ability to attend out of rostered work hours due to requirements for recovery, and time to conduct the laboratory component and further literature reviews towards this thesis.

The spirometry session quality varied greatly between individuals and the grading ranged from "A" to "D", with 2 sessions graded as "F". The inclusion of "poor quality spirometry" may have limited the accuracy of the data however, the rationale for including the low-quality data has already been discussed in the discussion in chapter 2, and the method of obtaining spirometry used in this study has been used in published studies in acute asthma where attempts to obtain "quality" spirometry were not possible [383, 384]. The rationale for including the "poor quality" data can be explained further.

The spirometry was graded for diagnostic mode, which requires a 6 second expiratory effort to obtain the FVC/FEV1 ratio. A grade of "A" or "B" indicates 3 acceptable manoeuvres and a "C" indicates 2 acceptable manoeuvres. A "D" indicates that 2 manoeuvres were adequate but non-reproducible, and "F" indicates there were no acceptable manoeuvres i.e. the patient was unable to sustain a 6 second effort. Thus, even if a patient was able to sustain a 2 second effort, the effort would not be acceptable and graded "F". Additionally, when an FEV1 is measured in a bronchial challenge test in non-exacerbating asthmatics, a best of three attempts is performed at only at baseline [549], and the FEV1 is only measured once during active bronchoconstriction [549, 550]. This active constriction could be compared to an exacerbation hence, a single manoeuvre would be acceptable.

There were 5 participants in this study, who on repeated attempts to obtain a 'best of three', showed a decline in FEV1 with each subsequent effort. This is due to deep inspiration-induced bronchospasm. In a study of acute asthma where FEV1 measures were made at 20-minute intervals, some were unable to perform three attempts, and some of the participants showed a decline in FEV1 values with repeated attempts [551]. This was thought to be due to worsening obstruction from the exacerbation or the effects of repeated inspiratory manoeuvres, rather than the interventions. The deep inspiration required for the manoeuvre can induce bronchoconstriction in asthmatics [196] and forced inspiratory/expiratory manoeuvre induced bronchoconstriction has been reported [552]. A decline in PEF with repeated attempts in acute asthma exacerbations has also been shown to occur [553]. Thus, repeated attempts at spirometry may reduce the accuracy of the measurements.

Some data, particularly data regarding the secondary outcomes was missing, such as a Borg score, which was not retrievable retrospectively from the case notes. The inclusion of this and other data may have shown some benefit from the use of magnesium.

Only one of the patients discharged from emergency, was discharged according to the protocol requirements. The treating physician discharged patients if they were deemed clinically stable. None who were deemed to be 'fit for discharge' required re-admission at any time within the 7 days following discharge, therefore, this is unlikely to have influenced the overall results.

3.3.2 Future directions

Clinical studies in acute asthma with sufficiently large numbers of patients to assess the effects of sex, recent asthma control as measured by ACQ score, smoking and atopy on outcomes would be useful. Larger numbers of patients would also be useful to confirm the safety in administration of a 5g(20mmol) dose of magnesium. A multicentre trial would be feasible; however external funding would be required to overcome many of the limitations that have been discussed. The use of PEF, as a means of selecting those with "severe" asthma, may be more appropriate for measurement of obstruction in acutely exacerbating asthmatics. Measurement of obstruction using a PEF meter is much simpler than FEV1 and does not require staff accreditation. This would improve recruitment rates.

3.4 Conclusions

This trial was unable to demonstrate a significant effect of intravenous magnesium on admission rates or other outcomes in acute asthma as the sample size was inadequate. Poor asthma control over the preceding week was the most significant contributing factor, and a high ACQ score was associated with a greater the likelihood of admission to hospital. Larger numbers are required to assess the efficacy of magnesium in acute asthma and to determine whether other factors influence response to treatment. Simplification of the trial protocol and funding for a multicentre study could allow recruitment of an adequate sample size.

Chapter 4: The effectiveness of 2g of intravenous magnesium for the treatment of acute asthma on admission rate: a re-analysis of 3M clinical trial data

Admission rates for acute exacerbations vary around the world [554], however, they are an important measure of outcome as hospital admissions represent a substantial cost to the health care systems. In Australia, $665 million dollars per year is spent on asthma with 20% of this total on hospital admissions [555]. In the UK, the most recent figure is £1.1 billion with 12% of this total on hospital care [556]. In addition to this, hospital admission has psychosocial implications for patients and families, including loss of work and isolation from family and friends. Treatments that reduce admission rates would have financial benefits for health systems as well as quality of life benefits for those individuals who are affected by asthma. Treatments or management practice change that consistently reduce ED presentations, would ideally produce maximum benefit for health systems and individuals with asthma.

The 3M trial was a randomised double-blind placebo-controlled trial in acute asthma, that compared the effect of nebulised or intravenous magnesium sulphate to a placebo, on admission to hospital [388]. It is the largest clinical trial of magnesium in acute asthma to date and was conducted at multiple centres around the UK. The patient demographic was reported as largely (90%) 'white', with the remainder, a mixture of Asian, 'Black' or 'other'.

Patients were given standardised treatment according to national guidelines for the treatment of an acute asthma exacerbation, alongside the experimental treatment. Standardised treatment included oxygen, 5mg of nebulised salbutamol, 500mcg of nebulised ipratropium and oral prednisolone. The primary outcome measure was admission to hospital and the main secondary outcome was a change in subjective measurement of breathlessness.

The group recruited 1109 (92%) of the 1200 proposed by the power calculation. They found that nebulised magnesium had no effect on the admission rate relative to placebo 79% (n=261) vs. 78% (281). The admission rate in the intravenous magnesium group was 72% (n=285), and intravenous magnesium was associated with an odds ratio of 0.73 (95% CI 0.51-1.04; p=0.083) for hospital admission relative to placebo. The overall admission rate in the combined placebo and intravenous magnesium groups was 74% (n=557). The prevalence of associated co-morbidity in this group was 17.8% (n=134). This is possibly due to the inclusion of older asthmatics up to the age of 88 years-old and could have had a significant influence on the overall outcome in the study.

In this analysis it was hypothesised that:

- Intravenous magnesium would significantly reduce admission rates relative to placebo in adults aged 50 years-old or less experiencing an acute asthma exacerbation.
- There may be differences in efficacy between categories of severity as defined by the predicted percent PEF values.
- There would be sex differences in response to magnesium and that magnesium would have a greater effect on reduction in admission rates in male asthmatics relative to female asthmatics.
- OCS use and active smoking would increase the likelihood of admission to hospital.

The aim of this study was; to re-examine the data from the 3M study's 'white' Caucasian population aged 18-50 years, who received either placebo or intravenous magnesium, to assess the effect of intravenous magnesium on admission rates and secondary outcomes, and to assess the effects of sex and severity of exacerbation on outcomes and response to treatment with intravenous magnesium. The sub-set data was chosen to remove any confounding variables, such as serious age -related co-morbidity, that may influence outcomes. Non-whites were excluded as race was thought to be a potential factor in the response to treatment with magnesium [376, 382].

4.1 Methods

The primary author was contacted via email and consented (via email) to a re-analysis of the data for this thesis. Data from a sub-set of 837 patients between the ages of 18 and 50 were obtained to correspond with the age of patients in my clinical trial. The data contained patient demographics of site recruited, age, sex, smoking status and ethnicity; physiological baseline parameters of heart rate, respiratory rate, systolic and diastolic blood pressure, temperature, oxygen saturations, oxygen flow rate, subjective dyspnoea score (VAS 0-100), repeated physiological parameters measured at 1 and 2 hours post treatment; airways obstruction as measured by % predicted and actual peak expiratory flow (PEF) in L/min at baseline and at 1 and 2 hours. The use of asthma medications such as inhaled and oral corticosteroids was also recorded. Outcome data included admission to hospital at first visit and admission to hospital within 7 days.

Data from patients in the intravenous magnesium and placebo treatment groups were selected and data from patients in the nebulised magnesium group were excluded, as the aim of this thesis was to study intravenous magnesium. Data from a total of 583 patients was then examined. There were 528 Caucasian patients, 35 of other ethnicities and 20 whose race was not stated. Caucasians only were

selected so that ethnicity corresponded to my clinical trial. The data from these 528 patients was assessed.

Primary outcomes assessed were admission to hospital at first visit and admission to hospital within 7 days. Secondary outcomes assessed were changes in respiratory rate at 2 hours, changes in VAS at 2 hours, changes in PEF at 2 hours as measured by the percent predicted.

Baseline and demographic parameters, primary and secondary outcomes were assessed between magnesium and placebo groups and between male and female patients. A separate sub-group analysis by severity and by sex was undertaken. Severity sub-groups were categorised, according to the British Thoracic Society guidelines of severity, into mild-moderate (PEF >50% predicted), severe (PEF 33-50% predicted) and life threatening (PEF <33% predicted) at baseline [4]. Sub-group analyses by severity and sex were also undertaken to determine if these factors affected response to magnesium treatment, as the data from the clinical and laboratory study suggested that they may influence the response to magnesium treatment.

Statistical analysis was performed using SPSS 24 (Statistical package software for students, IBM). Primary and secondary outcomes between treatment and placebo groups were compared. Direct comparison of primary and secondary outcomes between the sexes was undertaken. Chi squared analysis was used to compare outcomes between categorical variables. Continuous variables were analysed using t-test for independent samples. Significance was considered at an alpha level of 0.05. Logistic regression analysis was used to assess for other variables that may have had a significant impact on the primary outcomes.

4.2 Results

Of the 528 patients, 388 (73.5%) were female and 140 (26.5%) were male. There were 290 patients in the ‘active’ magnesium treatment group and 238 in the placebo group. There were no significant differences in age or frequencies of smoking status, or sex between active treatment and placebo groups. There were no significant differences in oral or inhaled corticosteroid between groups. The baseline percent predicted peak flow values were statistically greater in the magnesium group relative to the placebo group by 3% ($p<0.05$). There were no other significant differences in baseline parameters between the groups (Table 10). The overall admission rate for the group was 71.2% (n=376).

Table 10. Baseline parameters

Demographic /baseline characteristic	magnesium (n=290)	placebo (n=238)	p value
Age, mean (SE)	32.1 (0.5)	32 (0.6)	0.9
Female sex , n(%)	213 (73.4)	175 (73.5)	1
Current smoker, n (%)	115 (40)	86 (36)	0.7
ICS use	213 (73.4)	167 (70)	0.4
OCS in 24 hours prior to arrival , n (%)	103 (35.5)	75 (31.5)	0.3
Breathlessness by visual analogue scale ([VAS] (0-100) , mean (SEM)	62.6 (1.4)	64.4 (0.4)	0.4
Initial PEF , mean (SE)	55 (1.2)	52 (1.3)	***<0.05***
Respiratory rate in breaths per minute , mean (SEM)	25.3 (0.4)	25 (0.2)	0.7
Oxygen administration on arrival, n (%)	210 (72.7)	173 (73.6)	0.9
Oxygen saturation on air %, mean (SE)	96 (0.3)	96 (0.3)	0.7
Oxygen saturation on oxygen %, mean (SE)	98 (0.2)	98 (0.2)	0.6
Pulse rate in beats per minute , mean (SE)	112.9 (1.2)	111.7 (1.2)	0.5
Systolic blood pressure mmHg , mean (SE)	130 (1)	129 (1.2)	0.3
Diastolic blood pressure mmHg, mean (SE)	75 (0.8)	74 (1)	0.7

ICS: inhaled corticosteroid, OCS: oral corticosteroid, SE: standard error, VAS: Visual analogue scale. PEF: peak expiratory flow.

4.2.1 Overall effects

Primary outcome measures

There was a statistically significant greater admission rate of 8% in the placebo group relative to the magnesium group (χ^2 (1, N=528) = 4.13, p<0.05). When the outcome was adjusted for re-admission within 7 days, the difference in admission rate was reduced by 0.4% (χ^2 [1, N=528] = 3.78; p=0.052).

Secondary outcomes

There were no significant differences in VAS scores, respiratory rate or peak flow % predicted values between treatment and placebo groups. There was a trend towards a lower VAS in the magnesium group relative to placebo group (Table 11).

Effect of variables on outcome of admission to hospital

Direct logistic regression was performed to assess the effect of magnesium on the outcome of admission at first visit, correcting for other variables that may have affected the outcome of admission to hospital. Firstly, a model was constructed with all significant predictor variables. Bivariate logistic regression was performed separately for each variable, such as initial PEF, and any a-priori interactions such as sex-smoking, which were tested against the treatment variable.

Table 11. Secondary outcomes. Comparison of magnesium and placebo groups.

Secondary outcome	magnesium (n=290)	placebo (n=238)	p value
VAS @ 2 hours post treatment, mean (SE)	26.4(1.6)	30.2(1.9)	0.11
Respiratory rate @ 2 hours post treatment, mean (SE)	2.5(0.3)	20.0(0.4)	0.3
PEF % predicted @ 2 hours post treatment, mean (SE)	0(1.4)	68.4(1.6)	0.47

SE: standard error, VAS: Visual analogue scale. PEF: peak expiratory flow L/min

An initial multi-variate model was then tested by regressing outcome against treatment group and all variables and interactions where the p value was <0.2 in the bivariate regression. This model contained in addition to the treatment group, demographic variables of age, smoking status and sex and baseline physiological parameters of respiratory rate, heart rate, breathlessness (VAS), percent of predicted peak flow at baseline and use of oral and inhaled corticosteroid in the 24 hours prior to presentation. There were no significant interactions between any of the variables in the regression model. Backwards multivariate analysis of the model containing all significant factor variables was then performed to produce the most efficient and parsimonious logistic model. This was done by eliminating the variable with the highest p value and re-running the model. This step was repeated until only the variables with a p value of <0.05 remained.

The final regression model was significant, with an overall p<0.001(Table 12). The X^2 (N=483, 7) =69.1, and the model produced a –LLR=516.03. The model explained between 13.3% (Cox and Snell R^2) and 19% (Nagelkerke R^2) of the variance in admission rates.

Table 12. Logistic regression analysis: Acute asthma presentations aged 18-50. Effect of treatment on primary outcome of admission to hospital at first visit controlling for significant pre-randomisation variables.

							95% C.I.for EXP(B)/OR	
	B	S.E.	Wald	df	Sig.	Exp(B)/OR	Lower	Upper
Group (placebo)	-0.472	0.225	4.380	1	0.036	0.624	0.401	0.970
Age	0.033	0.013	6.732	1	0.009	1.034	1.008	1.060
Sex (Female)	0.638	0.248	6.649	1	0.010	1.894	1.165	3.076
Pulse rate (beats per minute)	0.013	0.006	4.220	1	0.040	1.013	1.001	1.025
PEF at baseline	-0.017	0.006	9.100	1	0.003	0.983	0.972	0.994
OCS use in 24hrs	0.778	0.248	9.821	1	0.002	2.177	1.338	3.541
Combined oxygen saturation	-0.213	0.055	14.842	1	0.000	0.808	0.725	0.901
Supplemental oxygen administration L/min	0.068	0.032	4.499	1	0.034	1.071	1.005	1.140
Constant	19.000	5.420	12.289	1	0.000	1.786E+08		

PEF: peak expiratory flow, OCS: oral corticosteroid

In this regression model controlling for other factors in the model, treatment was significant with OR 0.62 (CI 0.4-0.97), indicating a decreased likelihood of admission with magnesium treatment relative to placebo.

The strongest predictor of admission was the use of oral corticosteroid prior to arrival with an OR of 2.2 times increased risk of admission to hospital (CI 1.4 to 3.5), controlling for other factors in the model. Female sex was associated with an OR of 1.9 times increased risk of admission to hospital (CI 1.16-3.1), controlling for other factors in the model. Oxygen saturation was associated with admission with an OR 0.81 (CI 0.7.2-0.9) reduced likelihood of admission for each percent increase in saturation. Age was associated with an OR of 1.03 times increased risk of admission (CI 1.01 to 1.06) for every year older than 18, controlling for other factors in the model. Higher PEF at baseline was associated with a OR of 0.98 times decreased risk of admission (CI 0.97 to 0.99) when controlling for other factors in the model. Supplemental oxygen flow rate was associated with an OR of 1.07 times increased risk of admission to hospital (CI 1.0-1.14).

When the outcome was adjusted for re-admission within 7 days, the model was significant with an overall $p<0.001$. The X^2 (N=483, 7) = 67.14 and the model produced a –LLR =503. The model explained between 13% (Cox and Snell R^2) and 18.7% (Nagelkerke R^2) of the variance in admission rates. Magnesium decreased the likelihood of re-admission with an OR 0.62 (CI 0.4-0.98).

4.2.2 Sub-group analyses

Severity defined by PEF% predicted

Admission rates increased according to severity. For those with life-threatening asthma, the admission rate was 81.8% (n=66), for those with severe asthma 74.3% (n=187) and mild to moderate asthma 64.5% (n=242). The proportion of cases within each of the severity categories was significantly different between the sexes. Females were more likely to have life-threatening asthma (53%, n=192) relative to severe asthma (36.5%, n=132) or mild-moderate asthma (10.5%, n=38). Males were more likely to have severe asthma (41.4%, n=55) relative to life-threatening asthma (37.6%, n=50) or mild –moderate asthma (21.1%, n=28) ($p<0.01$). There were no differences in frequency of OCS use between categories of severity (p=0.3).

In the life-threatening sub-group, there were no significant differences in admission rates in the magnesium group (84.8%, n=28) relative to the placebo group (78.8%, n=26). There were no significant factors in determining the primary outcome of admission to hospital. In this sub-group there were no significant differences in secondary outcomes between treatment and placebo (Table 13).

In the severe category, magnesium significantly reduced the admission rate by 14.8% relative to placebo ($\chi 2$ [N=187, 1]=7.01; p<0.01). Significance was retained for the outcome of admission to hospital within 7 days ($\chi 2$ [N=173, 1] =5.22; p<0.05). There were no other significant factors identified in determining admission. There was a trend towards greater subjective improvement in VAS at 2 hours and a lower respiratory rate in the magnesium group relative to placebo group (Table 13).

Table 13. Secondary outcomes by severity, magnesium vs. placebo.

Secondary outcome (mild-moderate PEF >50% predicted)	magnesium (n=143)	placebo (n=99)	p value
VAS @ 2 hours post treatment , mean (SEM)	25.1(2.2)	25.2(2.5)	1
Respiratory rate @ 2 hours post treatment , mean (SEM)	20.5(0.5)	20.2(0.5)	0.7
PEF% predicted @ 2 hours post treatment , mean (SEM)	81.4 (1.5)	81.5(1.5)	0.95
Secondary outcome (severe PEF 33-50% predicted)	**magnesium (n=102)**	**placebo (n=85)**	**p value**
VAS @ 2 hours post treatment , mean (SEM)	26.2(2.8)	33.0(3.2)	0.11
Respiratory rate @ 2 hours post treatment , mean (SEM)	20(0.5)	21.6(0.7)	0.12
PEF% predicted @ 2 hours post treatment , mean (SEM)	60.1(2)	61(2.2)	0.8
Secondary outcome (life-threatening PEF <33% predicted)	**magnesium (n=33)**	**placebo (n=33)**	**p value**
VAS @ 2 hours post treatment , mean (SEM)	36.2(5.6)	39.7(5.6)	0.7
Respiratory rate @ 2 hours post treatment , mean (SEM)	21.6(1.2)	21.8(0.9)	0.9
PEF% predicted @ 2 hours post treatment , mean (SEM)	47.8(4.3)	46.5(3)	0.8

SE: standard error, VAS: Visual analogue scale, PEF: peak expiratory flow.

In the mild-moderate sub-group, there was a lower admission rate in the magnesium group (61.4%, n=88) relative to placebo (68.7%, n=68), but this was not significant (p=0.25). Logistic regression identified the predictor variables of age, PEF % predicted at baseline, OCS use and oxygen saturation (X^2 [N=242, 4] =48.17, –LLR =265.9; p<0.01) (Table 14).

Table 14. Logistic regression analysis: Acute asthma presentations aged 18-50, mild-moderate severity. Effect of treatment on primary outcome of admission to hospital at first visit, controlling for significant pre-randomisation variables.

	B	S.E.	Wald	df	Sig.	Exp(B)/OR	95% C.I. for Lower	Upper
Age	0.056	0.017	10.521	1	0.001	1.058	1.022	1.094
OCS use in 24 hours	1.462	0.349	17.589	1	0.000	4.314	2.179	8.542
PEF at baseline	-0.177	0.068	6.900	1	0.009	0.837	0.733	0.956
Combined oxygen saturation	-0.023	0.011	4.670	1	0.031	0.977	0.957	0.998
Constant	17.318	6.542	7.007	1	0.008	3.32E+07		

OCS: oral corticosteroid, PEF: peak expiratory flow

The model explained between 18.1% (Cox and Snell R^2) and 24.9% (Nagelkerke R^2) of the variance in admission rates. The strongest predictor of outcome was OCS use with an OR of 4.3 times increased risk of admission to hospital (CI 2.18- 8.5) controlling for other factors in the model. There were no significant differences in secondary outcomes between treatment and placebo groups.

Effect of sex

There were 388 female and 144 male patients in the analysis. Baseline respiratory rate ($p<0.01$), pulse rate ($p<0.05$), breathlessness by VAS score ($p<0.05$) and baseline PEF ($p<0.01$), were greater in females relative to males. There were significantly more active male smokers relative to females ($p<0.01$). The frequency of OCS use was 6% greater in females relative to males but was not significant ($p= 0.2$). Other demographic variables were similar between the sexes (Table 15).

Table 15. Difference in parameter variables between sexes.

Baseline characteristic	males (n=140)	females (n=388)	p value
Age, mean (SE)	32.6 (0.8)	31.8 (0.5)	0.37
Current smoker,n(%)	65(47.8)	136(35.6)	***<0.01***
ICS use, n (%)	109(77.9)	271(69.8)	0.07
OCS in 24 hours prior to arrival, n (%)	41 (29.3)	137 (35.3)	0.2
Breathlessness by VAS (0-100), mean (SE)	60(2.1)	64.6(1.1)	***<0.05***
Initial PEF, mean (SE)	49.2 (1.6)	55.3 (1.0)	***<0.01***
Respiratory rate in breaths per minute, mean (SE)	23.8 (0.4-6)	25.7 (0.3)	***<0.01***
Oxygen saturation on air, mean (SE)	95.8(0.4)	96.1(0.3)	0.5
Oxygen saturation on oxygen %, mean (SE)	97.7 (0.2)	98.1 (0.1)	0.09
Pulse rate in beats per minute, mean (SE)	109 (1.6)	114(1)	***<0.05***

SE: standard error, ICS: inhaled corticosteroid, OCS: oral corticosteroid, VAS: Visual analogue scale, PEF: peak expiratory flow

When outcomes between the sexes were compared, the admission rates were 8.5% greater in females relative to males, but this was not statistically significant ($p=0.058$). The improvements in secondary outcomes were greater in males relative to females (Table 16).

Table 16. Differences in secondary outcomes between sexes

Secondary outcome	Males (n=140)	Females (n=388)	p value
VAS @ 2 hours post treatment, mean (SE)	24 (2.4)	29.6 (1.4)	<0.05
Respiratory rate @ 2 hours post treatment, mean (SE)	19 (0.3)	21.4 (0.3)	<0.01
PEF% predicted @ 2 hours post treatment, mean (SE)	66(2)	70(1.2)	0.07

SE: standard error, VAS: Visual analogue scale. PEF: peak expiratory flow

Female Sex

There were 213 in the magnesium group and 175 in the placebo group. PEFs were lower in the magnesium group (53.3%) relative to placebo group (56.9%) but not significantly. There were no other differences in demographic and baseline parameters between treatment groups. There was a 5.7% greater admission rate in the placebo group relative to the magnesium group, but this was not statistically significant (p=0.21). When adjusted for re-admission within 7 days, the effect of magnesium was slightly greater with a 6% reduction in admission rate relative to placebo (p=0.18). There were no statistically significant differences in secondary outcomes between treatment and placebo groups in female patients.

The logistic regression model identified 5 predictor variables of admission. These were age, supplemental oxygen flow rate, oxygen saturation, oral corticosteroid use and PEF at baseline. The model was significant (χ^2 [N=352, 5] =61.08; p<0.001) with a –LLR 349.459 and explained between 15.9% (Cox and Snell R^2) and 23.1% (Nagelkerke R^2) of the variance in admission rates (Table 17).

Table 17. Logistic regression analysis: Female sex: Effect of treatment on primary outcome of admission to hospital first visit controlled for significant pre-randomisation variables.

							95% C.I.for EXP(B)/OR	
	B	S.E.	Wald	df	Sig.	Exp(B)/OR	Lower	Upper
Age	0.041	0.016	7.058	1	0.008	1.042	1.011	1.075
Supplemental oxygen flow rate L/min	0.084	0.039	4.591	1	0.032	1.087	1.007	1.174
PEF at baseline	-0.028	0.007	15.649	1	0.000	0.973	0.960	0.986
OCS use in 24 hrs	1.000	0.304	10.838	1	0.001	2.719	1.499	4.931
Combined oxygen saturation	-0.234	0.066	12.521	1	0.000	0.792	0.695	0.901
Constant	23.349	6.391	13.348	1	0.000	1.382E+10		

PEF: peak expiratory flow, OCS: oral corticosteroid

The strongest predictor of outcome in females was oral corticosteroid use which was associated with an OR of 2.7 times greater risk of admission (CI 1.5 to 4.9) when controlling for other factors in the model. For each 1% increase in oxygen saturation, the risk of admission to hospital decreased by 79% (OR 0.79, CI 0.6 to 0.9). PEF% at baseline was associated with a slightly decreased risk of admission (OR.97, CI.96 to 0.99) and with each percent increase in PEF, there was a 97% decreased risk of admission. Age was associated with a 4% increase in admission for each year over 18 years (OR 1.04 CI 1.01-1.07). When the outcome was adjusted for admission within 7 days, a similar model was produced.

Male sex

There were 77 males in the magnesium group and 63 in the placebo group. There were no significant differences in baseline parameters or demographic variables between treatment groups. PEFs were lower in the magnesium group relative to placebo but not significantly.

There was a 14.6% greater admission rate in the placebo group relative to the magnesium group which was not significant (p=0.072). When adjusted for re-admission within 7 days, this effect decreased slightly to 12% (p= 0.14). There were no other significant factors contributing to admission. For males, there was a significantly lower respiratory rate at 2 hours in the treatment group relative to placebo group (Table 18). The sex difference in the effect of magnesium on admission rates was significant (5.7 vs 14.6, p<0.05).

Table 18. Secondary outcomes: males only, comparison between magnesium and placebo

Secondary outcome (males)	magnesium (n=75)	placebo (n=60)	p value
VAS @ 2 hours post treatment, mean (SE)	20.3 (3)	28 (3.6)	0.1
Respiratory rate @ 2 hours post treatment, mean (SE)	18.2 (0.4)	19.8 (0.5)	<0.05
PEF @ 2 hours post treatment, mean (SE)	67(3)	65(3)	0.67

SE: standard error, VAS: Visual analogue scale, PEF: peak expiratory flow.

4.3 Discussion

The sub-set analysis of the 3M study was similar to my clinical study in ethnicity of the population, demographics, age and treatment, which was standardised according to the same acute asthma treatment guideline [4]. Thus, this data, potentially provided a similar patient group in which to further assess the effects of sex and severity on response to magnesium. Severity was also assessed by the degree of airways obstruction, but PEF was used, and patients were included irrespective of the degree of airways obstruction. This resulted in asthmatics with a wide range of severities included in the 3M study who would have been excluded from my study. These patients could have been excluded from my sub-set analysis however, a large sample size was needed to assess the effects of sex on response to magnesium. Current OCS use at presentation, an indicator of severity and poor asthma control, and smoking status were documented. A subjective dyspnoea assessment was also included in the secondary outcome measures.

This 3M study therefore provided a comparable but larger sample of 'white' Caucasian adult asthmatics from which sub-group analyses could be performed. The limiting factor, however, was inclusion of all participants, irrespective of initial β-2 agonist responses.

4.3.1 Main findings

Magnesium's effect on admission rates

This sub-analysis of the 3M trial has identified that magnesium can reduce admission rates in white Caucasian adults aged 18-50 years, presenting to emergency with an acute asthma exacerbation. The finding of statistical significance in this cohort differs from that of the original 3M study, but the difference in admission rates between magnesium and placebo was only slightly greater. The exclusion of those over 50, would have removed many of those participants with significant co-morbidities that may have had a significant impact on the outcome. By excluding these participants in re-analysis, it was expected that there would be a greater influence of magnesium on admission rates hence, a greater reduction in admissions. Data from a national health survey in the USA found that asthmatics over 54 years-old, had worse outcomes in terms of the number of hospital admissions and mortality relative to younger asthmatics [557]. The overall admission rate decreased by only 2.8% relative to the original study, and the overall effect of magnesium on decreasing admission rates in the re-analysis was only 1% more than in the original study, which is surprisingly small.

The original 3M study also included recruitment site in their logistical regression analysis and found a small effect of site on outcomes and effect of magnesium (unadjusted OR 0.72, CI 0.51-1.0, p=0.051; adjusted for site OR 0.73, CI 0.51-1.04, p=0.083). My subset contained some sites with very small

numbers which precluded the inclusion of site as a covariate. Exclusion of this covariate may have influenced the result in this analysis. Admission rates varied greatly between sites (45-95%), with the greatest variations in admission rates between centres with the least recruits. This is likely to reflect non-standardized admission policies; therefore, this covariate may have been a confounder in the original analyses. Local admission policies may have influenced outcomes irrespective of the effect of magnesium. This illustrates the importance of having well-defined admission/discharge criteria in clinical studies.

The ability to conclude definitively that magnesium is effective in this subset analysis is limited. It is likely that a larger data set is needed to confirm magnesium's overall effect on admission rates.

Other variables that influenced admission rates

The use of OCS prior to arrival and female sex were the strongest predictors of the outcome of admission to hospital. Most subjects in this study were female (>70%), consistent with the literature on emergency department presentations [287, 289]. Large cohort studies in adult asthmatics in the USA and Canada, have also shown that female asthmatics relative to male asthmatics are more likely to be admitted to hospital [285, 287, 291, 292]. A small study in Spain found the same increase in admission rates in females relative to males [558].

In this analysis, prior OCS use was associated with the greatest increase in odds of admission, controlling for all other factors including magnesium. Current OCS use is another marker of severity and indicates initiation of an asthma action plan. Regular and current OCS use has also been associated with an increased risk of hospital admissions in studies in the USA [559, 560], Canada [561] and Australian [562]. Females tended to use more OCS relative to males, which is also consistent with the literature [291]. There may have also been a significant interaction between sex and OCS use, which a larger sample size may have been able to detect.

In this study, a 1% decrease in oxygen saturation increased admission rate by 23%. In a small study in Canada of those aged 18 to 55 years, who presented to emergency departments with asthma exacerbations, a saturation of <95% was associated with an almost two-fold increase in admission rate [561]. BTS guidelines classify a saturation of <92% with life-threatening disease [4]. Hypoxia is life threatening and an indicator of severity of disease; hence, it increases the risk of hospital admission.

Secondary outcomes

PEF values

The re-analysis did not find magnesium to have significant effect on improvements in lung function as measured by changes in PEF values. Magnesium was given alongside optimal standard care as prescribed in the relevant guideline [4], not based on 'non-response', thus, the inclusion of

participants with good initial bronchodilator responses may have masked any benefit from magnesium.

4.3.2 Subgroup analyses

Severity

Despite magnesium's small overall benefit, the results of the subgroup re-analysis suggest that magnesium may be beneficial in those with severe exacerbations as defined by PEF 33-50% predicted. The reduction in admission rates was accompanied by small improvements in VAS but not in PEF.

This contrasts with other studies where magnesium was most effective in those with predicted values <25-30%. Magnesium was shown to be effective in reducing admission rates in those with a FEV1 <25% predicted [381], FEV1 <30% predicted [383] or PEF <200L/min [382]. The reductions in admission rates were large at 42% [381] and 45% [382]. Studies of the severe sub-groups also found between 10 and 25% greater improvements in airways obstruction relative to placebo [381-384]. The study populations were largely female. The reason for the difference in outcome in this subset may relate to ethnic differences. The 3M subset in this analysis was entirely white" whereas, the populations in these studies were "non-white" The possible influence of ethnicity on response to magnesium has already been discussed in detail in chapter one. The reason for the apparent benefit on admission rate in this subgroup is not clear.

Sex

The effect of magnesium on reduction in admission rates was greater in males relative to females; however, the effect of magnesium on admission rates in male asthmatics was not statistically significant, due to small sample size. There was, however, an improvement in VAS and respiratory rate. Whether these sex differences in efficacy are independent, or confounded by severity, is important given the sex differences in severity. The small sample size however, precluded our assessment of this. A larger sample size may also have been able to detect an interaction between sex and severity.

Previous clinical trials of intravenous magnesium in acute asthma have not assessed the effects of sex on response to magnesium, and sample sizes would have been insufficient in most studies. Sex differences in adult asthma phenotypes have been well characterised and may account for the differences in efficacy. These data suggest that there are intrinsic sex differences in phenotypic expression of asthma, and this may account for the sex differences in response to magnesium.

4.3.3 Potential clinical benefits of magnesium in acute asthma

Magnesium's apparent effect on outcome of admission to hospital was not associated with any improvements in airways obstruction as measured by improvements in peak flow. It may be that any effect was masked by the inclusion of "treatment responders".

It is also possible that magnesium has other benefits in acute asthma that are not measurable with spirometry. In HASM, the increase in cAMP is also associated with alteration in tension due to alteration in cytoskeletal dynamics, as demonstrated in HASM where isoprenaline induced actin de-polymerisation [167]. Clinically this would result in a reduction in 'stiffness' via alteration in the dynamic properties of HASM. A pharmacological effect of a reduction in stiffness has been demonstrated in healthy porcine ASM [163]. A reduction in stiffness could reduce the sensation of chest tightness and dyspnoea experienced. This would not be measurable using PEF but would result in a subjective improvement in dyspnoea as measured on a VAS scale; however, magnesium had no effect on VAS overall and the subgroups were likely to have been too small to detect the differences observed.

Beta-2 agonists have also been shown to have anti-inflammatory effects *in vitro* through inhibition of TNF-α from mast cells [323] via an increase in cAMP [565]. Cyclic AMP elevation has also been shown to have anti-inflammatory activity in other cell types, including human neutrophils [566], monocytes [567] and cultured HASM cells [568]. Thus, magnesium may reduce inflammation via augmented β-2 agonist induced cAMP production. This may be another mechanism whereby magnesium could decrease admission rates without an improvement in PEF.

4.3.4 Limitations

Despite the relatively large sample size relative to previous studies, the data sample size was insufficient for a complete assessment of sub-groups and differences in baseline demographic parameters, such as ICS and OCS use, oxygen saturation, and secondary outcome of respiratory rate, between treatment groups in the sub-groups. A larger sample size may have altered the outcomes. The sample size was too small to compare the effect of severity within each sex, which may have revealed important differences in outcomes. The sample size of this sub-set analysis was also too small to assess the effects of magnesium in life threatening asthma or the need for non-invasive or invasive ventilation and intensive care or high-dependency admissions. Given that this was a sub-group analysis, there was increased risk of a type I errors; however, the findings in this analysis are consistent with the findings of others where comparisons can be made.

There was missing data from the original data set with regards to ethnicity, therefore, these data were not included in the sub-set analysis. These data could have influenced the results. There was also missing sub-set data for variables such as smoking, and secondary outcome data including PEF, respirations and VAS at 2 hours, which could have influenced the results. The data used in this re-analysis was collected across multiple centres and hence, there may have been variability in the reliability of PEFs and other observations, relative to the experience of researchers across these centres.

Asthma control scores and atopic status were not documented, hence, the effects of these variables on outcomes could not be ascertained. Information on the length of stay was available, however, was not obtained from the original 3M data, due to an oversight on my behalf.

4.3.5 Future directions

As this was a sub-group analysis, further prospective studies should be undertaken to confirm these findings. The effects of race and sex have already been recognised as influencing the disease expression, however, the effects of race and sex in determining a response to magnesium, appear to be relevant and warrant further study. The effect of atopy on disease expression is also well recognised and should be further assessed in terms of response to magnesium. Retrospective analysis of previous studies may be useful; however, any future studies should clearly document ethnicity and atopic status, as they are important demographics for further understanding of the effects of magnesium in asthma. Studies assessing the effects of severity should be consistent in classification methods. The PEF reference ranges used in the BTS guideline appear to be appropriate for stratifying patients based on severity.

The reason for magnesium's reduction in admission rates has not been clearly illustrated. The use of PEF is limited in its ability to measure lung function and requires a forced expiratory manoeuvre, which can alter airway tone. Inhaled β-2 agonist has been shown to abolish deep inspiration induced bronchoconstriction in asthmatics [198]. Augmentation of β-2 agonist responses by magnesium, could enhance this effect and influence airway tone and AHR and may improve asthma symptoms and contribute to a reduction in admission rate. This effect would be difficult to assess by standard spirometry however, other measures of lung function may detect an alteration in airway tone.

Studies have found the forced oscillatory technique (FOT) to be a useful tool for study of lung function in asthma [567-569]. FOT was able to detect differences in levels of airway obstruction, abnormalities in dynamic compliance and airways reactivity between healthy and asthmatic subjects of different levels of severity, as determined by FEV1 [567]. FOT was also able to detect differences

in the level of airway obstruction between healthy and asthmatics subjects with normal FEV1 values [567]. FOT has been shown to be sensitive in detecting bronchodilation in children, but sensitivity in adults varies [568]. This may be due to differences in methodology, as a time lag between pressure and flow measurements can reduce accuracy [570] which can be corrected with the appropriate choice of a FOT machine. Further studies using additional outcome measures as determined by FOT, such as dynamic compliance and airways reactivity in addition to obstruction, may reveal the mechanism of benefit from the use of magnesium in acute asthma. Recently, there have been recommendations for targeting regulatable stiffness in the treatment of asthma [571], suggesting that these measures may be valid for determining treatment outcomes.

4.4 Conclusions

Although my findings in this sub-set analysis support the role of magnesium in treating acute asthma exacerbations, in severe asthma, the ability to fully analyse subgroups was limited by sample size within these subgroups. The findings suggest that the benefits of magnesium are modest overall; however, there may be a greater benefit in males, as they are likely to present with more 'acute' presentations and those with severe exacerbations as defined by presenting PEF 33-50% predicted. These sub-group findings are relevant to Caucasian populations in whom the overall efficacy may differ from other ethnicities. The reasons for these differences may relate to bronchodilator responsiveness or genetic variation. Further study could be warranted to explore the effects of individual variation and to confirm these findings.

Chapter 5: Final discussion

5.1 Summary

This thesis was unable to conclusively show that magnesium was an effective in the treatment in the management of acute asthma exacerbations. By re-examining selective data from a larger study, it was possible to show that magnesium reduced hospital admission rates in the subgroup of severe asthma.

Asthma is a heterogeneous condition with individual variations in immuno-pathological and phenotypic manifestations. It follows that clinical presentation and treatment responses also vary and are also influenced by individual factors such as sex, race and atopy. When assessing magnesium's efficacy, these factors should be taken in to consideration as they may influence treatment response.

Individual variation in response is likely to account for the modest overall effect. This means that larger sample sizes are required to fully assess the utility of magnesium in acute asthma. Magnesium may be effective in certain populations, despite a lack of conclusive evidence in the literature. Larger studies are needed to fully assess the influence of individual factors such as sex, race and atopy on response to magnesium in the treatment of acute asthma.

The laboratory study illustrated a potential mechanism of action for magnesium in acute asthma and some explanation for its variable efficacy. This needs further study with larger numbers to confirm these observations and to fully explore the effects of individual factors, such as sex, ethnicity and atopy, on treatment response. Magnesium may increase β-2 agonist stimulated cAMP in a dose dependent manner and proportionate to agonist strength. The likely mechanism for the increase in cAMP, is via an increase in an agonist binding to the β-2AR. This requires ligand binding studies for confirmation. There were sex differences in lymphocyte responses to magnesium and overall cAMP production, and others have also demonstrated sex and racial differences in lymphocyte cAMP production as discussed in chapter two. This could explain the mechanism whereby the variation in response to magnesium occurs and hence, explain the heterogeneity of the results of previous studies of magnesium in asthma. Magnesium, when used as an adjunct to standard care, may improve clinical outcomes in some sub-groups of patients presenting with acute exacerbations.

Magnesium is inexpensive and easy to administer, with few if any serious adverse effects at modest doses. The literature supports this, at least in theory. The overall effects of its usage may be difficult to appreciate, however, a small overall reduction in hospital admission rate would be beneficial due to the high demands on limited health care resources around the world. The impact of an 8% reduction in asthma admissions on hospital resources in the UK would be £10.5 million per-year, based on the

national figures on health expenditure [556]. One ampule (2.5g) of magnesium costs around £2 and therefore, if found to be efficacious, would be a cost-effective addition to the treatment of acute asthma exacerbations. The benefits are likely to be more significant in a targeted population.

5.2 Recommendations regarding the use of magnesium in acute asthma exacerbations

Magnesium could be considered as an adjunct to standard treatment of an acute exacerbation in those that present with acute, sudden, severe symptoms. It may reduce admission rates and/or improve symptoms of breathlessness. It is unlikely to do any harm and may be of benefit. Guidelines recommend 2g however, a dose of 2.5g could be given and repeated, if there is limited response to the initial dose. Theoretically, an initial dose of 5g could be given in those with normal renal function.

5.3 The mechanism of action of magnesium and its modification of efficacy by sex

Beta-2 agonist stimulation of the β-2AR induces HASM relaxation and bronchodilation through production of cAMP [317] and causes a reduction in passive tension or 'stiffness'[163] (Figure 10). If magnesium were to augment β-2 agonist stimulated cAMP, it could potentially increase HASM relaxation, reduce stiffness and perhaps, reduce airway hyper-responsiveness. Magnesium's efficacy is, therefore, dependent on the properties and function of the β-2AR.

Sex differences in lymphocyte β-2ARs were demonstrated in stable asthmatics, where female asthmatics showed a greater degree of down-regulation of receptor response to SABA exposure relative to male asthmatics, as shown by the greater loss of β-2 agonist induced cAMP. Down-regulation of β-2 responses in lymphocytes also occurs in mast cells [563], and the greater loss of receptor function could also occur in these cell types. A greater loss of responsiveness may result in the inability of magnesium to increase β-2 agonist stimulated cAMP in female asthmatics relative to male asthmatics. An increase in binding to the β-2 adrenergic receptor would have minimal effect where a greater loss of surface receptors reduces the capacity to generate cAMP. This lack of capacity to generate cAMP translates to a reduced ability to decrease HASM tone (Figure 10).

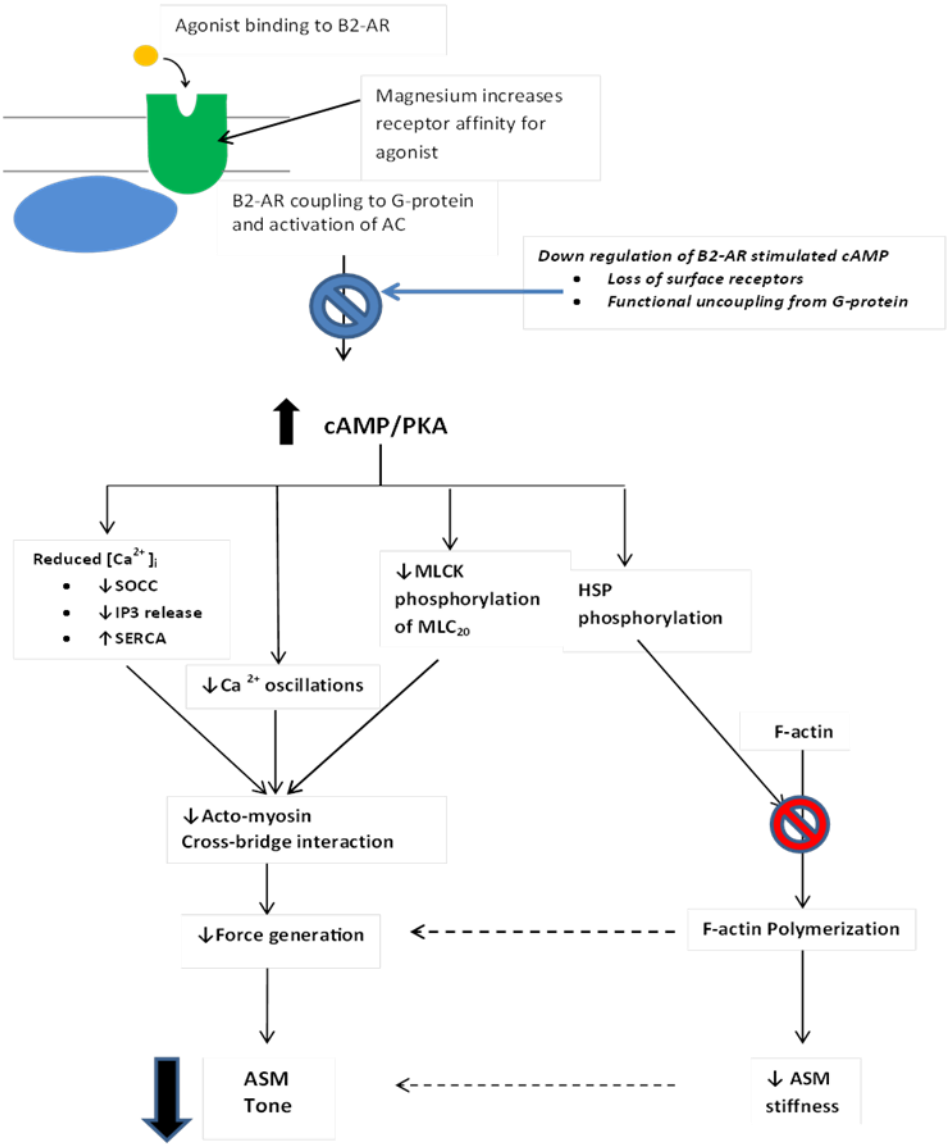


Figure 10. Proposed mechanism of action of magnesium. Increased binding of β-2 agonist to β-2AR. Consequent increase in cAMP/PKA pathways and cAMP/PKA independent pathways. Reduction in intracellular calcium via inhibition of store operated calcium channels; IP3 induced release from sarcoplasmic reticulum and increased SERCA activity. Reduction in calcium oscillation frequency, reduced phosphorylation of myosin light chain. These mechanisms decrease acto-myosin cross bridge cyclic and hence reduce the force of contraction.

5.4 Future directions

Magnesium appears to increase β-2 agonist stimulated cAMP however, a larger study is needed to confirm this. The potential mechanism appears to be that of increased affinity for the receptor. The use of radio-ligand binding assays may confirm the mechanism responsible for the observed effects of magnesium on cAMP production. Results of such studies may also explain the sex differences in cAMP response and further explain the sex differences in the effects of magnesium. Further study of the anti-inflammatory effects of magnesium and its effects on airway dynamics could also provide information about its mechanism of effect.

Response to magnesium may vary with atopic status, and there may be an interaction between atopy and sex. Further studies in lymphocytes, comparing the effects of magnesium on β-2 agonist stimulated cAMP in male and female atopic, and male and female non-atopic asthmatics, may provide important information regarding differences in efficacy and further optimise the selection of asthmatics most or least likely to benefit from magnesium.

Further clinical studies would be helpful in ascertaining the effects of sex, atopy and ethnicity on response to treatment with magnesium. Further clinical studies should document racial background, atopic status and an asthma control score. Studies should be adequately powered for the outcomes, such that small but clinically significant differences may be measured. It is also important to consider ethnic differences in response to treatment and it would be reasonable to report results of further studies based on race or ethnicity. Statistical advice should be sought to determine the sample sizes needed and as such, is it likely that larger numbers would be required. This would also require a multicentre approach and adequate funding. Further study into the effects of magnesium may provide evidence to support its more widespread use, and its potential to improve outcomes for those with a greater risk of adverse outcomes.

5.5 Clinical recommendations

Magnesium is safe, inexpensive, and easy to administer. It is included in current acute asthma treatment guidelines and therefore, may be considered as an adjunct to standard treatment of an acute asthma exacerbation, in an acute, sudden severe exacerbation, when the patient does not respond to standard treatment or deteriorates. It could also be considered early in the course of treatment of acute exacerbations in males and those of non-white ethnicity, as it may be more effective in these situations.

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