Repurposing of Statins via Inhalation to Treat Lung Inflammatory Conditions

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Abstract

Despite many therapeutic advancements over the past decade, the continued rise in chronic inflammatory lung diseases incidence has driven the need to identify and develop new therapeutic strategies, with superior efficacy to treat these diseases. Statins are one class of drug that could potentially be repurposed as an alternative treatment for chronic lung diseases. They are currently used to treat hypercholesterolemia by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which that catalyses the rate limiting step in the mevalonate biosynthesis pathway, a key intermediate in cholesterol metabolism. Recent research has identified statins to have other protective ~~pleotropic~~pleiotropic properties including anti-inflammatory, anti-oxidant, muco-inhibitory effects that may be beneficial for the treatment of chronic inflammatory lung diseases. However, clinical studies have yielded conflicting results. This review will summarise some of the current evidences for statins pleiotropic effects that could be applied for the treatment of chronic inflammatory lung diseases, their mechanisms of actions, and the potential to repurpose statins as an inhaled therapy, including a detailed discussion on their different physical-chemical properties and how these characteristics could ultimately affect treatment efficacies. The repurposing of statins from conventional anti-cholesterol oral therapy to inhaled anti-inflammatory formulation is promising, as it provides direct delivery to the airways, reduced risk of side effects, increased bioavailability and tailored physical-chemical properties for enhanced efficacy.

Keywords: Statins, anti-inflammatory, inhalation, respiratory diseases, anti-oxidant, muco-inhibition

1.0 Introduction

Statins are a class of drug widely used to lower cholesterol levels, consequently reducing the rates of stroke, myocardial infraction, vascular death, cerebrovascular events and mortality from coronary artery disease [1]. In the past decade, mounting evidence have shown that statins provide greater protection than predicted from just cholesterol lowering effects, possibly mediated by other pleiotropic actions. This includ~~ing~~es anti-inflammatory, anti-oxidant, immunomodulatory effects, improved endothelial cell function and anti-thrombotic actions [2, 3]. The exploitation of these pleiotropic statin-induced protective effects ~~combination of these statins protective functions~~ could be used therapeutically to manage chronic lung diseases with inflammatory and oxidative stress components such as asthma, chronic obstructive pulmonary disease (COPD) and pulmonary hypertension [4-7].

In recent years, despite the many therapeutic advancements, the frequency of chronic inflammatory lung diseases has continued to rise ~~despite many therapeutic advancements~~ due to air pollution, tobacco smoke and occupational chemicals [8]. Currently, hundreds of millions of people suffer every day from chronic respiratory diseases (>235 million living with asthma and >64 million have COPD) and in fact, the World Health Organisation predicts that chronic lung disease will be the third most common cause of death by 2020 [9]. Chronic lung diseases are typically characterised by airway inflammation, thick hyperviscous mucus production and chronic infection. Taken together, these lead~~ing~~ to the disruption of the mucociliary clearance mechanisms, bronchoconstriction and airflow limitation that subsequently manifest to debilitating symptoms such as cough, wheeze and breathlessness. ~~Available~~ Current treatments available to manage this condition are limited to bronchodilators, corticosteroid, non-steroidal anti-inflammatory drugs and antibiotics, all of

which are focussed on symptom elevations and not the disease [10]. In addition, long-term exposure to these therapies could lead to ~~drug resistance (~~reduced responsiveness (drug resistance) over time~~)~~ with a proportion of patients that do not respond well to these standard treatments [11, 12]. Therefore, statins may provide an important new alternative to treat chronic lung diseases. This review will outline current evidences for statins pleiotropic effects that could be used for the treatment of chronic inflammatory lung diseases~~.~~. We will discuss in detail the ~~their~~ mechanisms of actions by which statins exert their actions and the potential to repurpose statins as inhaled therapeutics, including a detailed discussion on their different physical-chemical properties that could ultimately affect treatment efficacies.

2.0 Mechanisms of actions

Originally, statins were used to lower cholesterol levels by inhibiting cholesterol synthesis. Statins block and completely inhibit the action of the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) Reductase enzyme, the rate-limiting enzyme responsible for the conversion of HMG to mevalonate, termed the mevalonate pathway [13]. By inhibiting HMG-CoA Reductase, statins~~,~~ stop~~ping~~ the downstream synthesis of cholesterol (*Figure 1~~Figure 1~~*), ~~and~~ resulting in a transient decrease of cholesterol. Importantly, inhibition of HMG-CoA Reductase also prevents the synthesis of pyrophosphate intermediates, namely farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), from which prenyl groups (also known as prenoids) are derived [14-17]. Prenoids are important post-translational modifiers that covalently bind to specific proteins to facilitate protein association with the lipid membrane and also downstream protein-protein interactions [14,

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15, 17]. Thus, while ~~the use of~~ statins ~~to~~ directly block the ~~activity of~~ HMG-CoA Reductase activity ~~not only directly~~ to inhibit~~s~~ cholesterol synthesis, statins ~~but~~ also ~~importantly,~~ indirectly inhibit~~s the synthesis of~~ preniods synthesis. As a result, statins act to modulate a number of intracellular signalling pathways that regulate inflammation, oxidative stress and immunity [18-20]. Despite decades of research, ~~T~~the exact molecular mechanism(s) ~~by which~~that statins act to exert these pleiotropic effects remains unclear ~~despite decades of research~~; this in itself, is a testament to the pleiotropicity of statins. The aim of this review section is to summarise and critically analyse the literature, focusing specifically on the molecular mechanisms and signalling pathways influenced by statin treatment, to better understand statin induced protection.

Currently, there are 8 statins available for *in vivo* and *in vitro* studies, atrovastatin, cerivavastatin (withdrawn from the market), lovastatin, simvastatin, fluvastatin, pravastatin, pitavastatin, and rosuvastatin. These can further be sub-grouped into one of two categories, lipophilic or hydrophilic (***Table 1*** ~~***Table 1***~~). Generally, lipophilic statins are passively diffused across the membrane, while the hydrophilic statins require active transport (***Figure 2*** ~~***Figure 2***~~). Several studies have reported significant differences in the efficacy of lipophilic and hydrophilic statins [21-24], resulting in differences ~~is~~ in tissue permeability and metabolism, which will be discussed in detailed in section 4.0 of ~~the~~ this review. ~~However, t~~The most commonly ~~investigated~~ studied statins are the lipophilic statins, specifically simvastatin, as ~~they are considered more likely~~it is thought to effectively ~~to~~ enter the vascular cells by passive diffusion enabling ~~to have more~~the ~~pleotropic~~pleiotropic effects. In stark contrast, ~~compared to~~ hydrophilic statins ~~, which~~are primarily~~y~~ target~~s~~ed to the liver and undergo degradation, that could potentially diminish their pleiotropicity [25-27]. Thus, when

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comparing the effects of statins or determining the molecular pathway of a specific statin-induced pathway, it is important to understand the properties of the particular subgroup [21].

Protein prenylation, also referred to as lipidation, is the addition of a prenyl group to the carboxyl~~C~~-terminus of a protein. Specifically, protein geranylgeranylation occurs ~~at carboxyl-terminal *CaaX* (where C represents cysteine, *a* is an aliphatic amino acid, and *X* is a terminal amino acid) tetrapeptide motifs~~ to the GTPase superfamily of proteins: Ras, Rac, Rho, and Cdc42 [28]. While protein farnesylation ~~occurs at either the CXC or CXXC motifs (two cysteine in sequence) and~~ is limited to the Rab proteins of the endocytic recycling pathway [14, 17]. A number of studies have identified that statin-induced protective effects are a direct result of geranylgeranylation inhibition and not farnesylation [29, 30], highlighting a ~~the~~ role ~~of~~ for GTPase's in mediating the statin induced effects. The GTPase superfamily of proteins acts as a 'molecular switch' for a diverse range of signalling pathways that regulate cell migration, proliferation, and growth, and relies on the tethering of the GTPase to the cell membrane [16]. As such, treating cells with statins decreases the prenylation of GTPase family members and therefore their association with the lipid membrane, consequently regulating GTPase activity [30-32] (**Figure 2**~~***Figure 2***~~).

2.1 The Statin Mode of Action: A combination of inhibiting cholesterol synthesis and prenylation

One of the key questions in the literature is to define and articulate the mechanism of action by which statins exert their pleiotropic effects. This has proved difficult due to the multitude of pathways that statins have been implicated in- and affect. Thus, there is much

debate in the literature as to whether the pleiotropic statin effects (anti-inflammatory, anti-oxidative and immunomodulation) are a direct result of the inhibition of cholesterol synthesis, the indirect result of the loss of prenyl group or a combination of the two. Most likely, it is a combination of the two, however many studies do not consider this when defining their mechanisms. ~~In this review, the role and mechanisms outlined in the literature to date are discussed in details.~~

2.1.1 Statins regulate inflammation ~~via lowering~~by decreasing intracellular cholesterol levels.

As statins act to inhibit cholesterol synthesis, cells will respond by compensating for the loss of cholesterol. Cells have been shown to ~~increasing~~ increase the expression and enzymatic activity of HMG-CoA Reductase [33-35] and are also~~the~~ suspected to increase the transcription of low-density lipoproteins (LDL)-receptors ~~to compensate for the loss of cholesterol and~~ to maximise cholesterol uptake from serum [36]. However, ~~L~~later studies ~~showed~~ found that the increase in LDL-receptor mRNA did not correspond to an increase in LDL-receptor protein levels [23, 37]. Further investigation of this pathway found that statin treatment had increased the turnover of the LDL-receptor [37], suggesting that statins may play a role in endosomal-mediated degradation of the LDL-receptor. ~~However~~Interestingly, ~~the Ness *et al.* study did not investigate~~ the role prenylation of Rab endocytic vesicles played in LDL-receptor recycling have not been investigated in the study.

Reverse cholesterol transport is a mechanism by which cells actively efflux intracellular cholesterol via ATP-binding cassette (ABC) transporters to maintain intracellular lipid homeostasis [38-40]. In the lung, two ABC transporters have been identified~~;~~, ABCA1 and

ABCG1 ~~, and both are transcriptionally regulated by liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPAR) α~~ [40-43]. Statins act to regulate ABC transporter expression via two mechanisms. In the first, the statin induced decrease in oxysterol formation inhibits the extracellular-signal regulated kinase (ERK)1/2 – cyclooxygenase (Cox)-2 signaling axis, resulting in the decreased transcription of the ABCA1 and ABCG1 transporters [43-45]. Importantly, ERK1/2 is downstream of GTPase activity [46] (***Figure 3*** ~~***Figure 3***~~) In the second pathway, the decreasing abundance of intracellular cholesterol triggers the transcription of sterol regulatory element binding protein (SREBP)-2 gene, containing the microRNA miR33, a known inhibitor of ABC Transporter transcription [47, 48]. Thus, the simultaneous expression of SREBP-2 and miR33 negatively regulate the transcription of the ABCA1 and ABCG1 transporters [23, 49, 50]. Statins have been shown to play a regulatory role in the expression of ABCA1 and ABCG1 transporters, albeit a controversial role, with numerous studies citing conflicting results. Some studies state that statins decrease the expression of ABC transporters [23, 51], others have identified that statins increase ~~transcription and translation of~~ ABC transporter expressions~~s~~ and therefore increase cholesterol efflux [52, 53], while another study states no change to ABC transporter expression [34]. ~~It is important to note that all of these studies used either human or primary murine derived macrophages and used statins from both statin subgroups at comparable concentrations, suggesting the differences cannot be attributed to cell line specificity or the diffusive properties of statins. A study conducted by Wong *et al.* found that the reason for the discrepancies was a result of the extent of macrophage differentiation. Importantly, the Wont et al. study also found that statin-induced ABC transporter expression is significantly influenced by the initial concentration of intracellular levels of cholesterol and therefore may account for some of the discrepancies outlined above~~

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~~discrepancies was a result of the extent of macrophage differentiation. Importantly, the Wong *et al.* study also found that statin-induced ABC transporter expression is significantly influenced by the initial concentration of intracellular cholesterol and therefore may account for some of the discrepancies outlined above [54].~~

The statin-induced regulation of ABCA1 and ABCG1 transporter expression has been associated with the increase production and secretion of inflammatory cytokines, highlighting a role for statins in mediating cellular inflammation [54-57]. This occurs as a direct result of the statin-induced loss of ABC transporters as by blocking the efflux of cholesterol from cholesterol-loaded cells (mimicking hypercholesterolemia), creating~~—~~ a build-up of intracellular cholesterol. This increase in intracellular fatty acid is then trafficked to the endoplasmic reticulum creating a 'fatty acid overload' at the reticulum membrane [58], suggesting ~~the~~a possible ~~involvement of~~role for Rab proteins. Importantly, this 'overload' activates a number of downstream signalling pathways, including mitogen activated protein kinase (MAPK) p38, ERK1/2, and the nuclear factor (NF)-κB, resulting in the increased production and secretion of both pro- and anti-inflammatory cytokines (tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, IL-12p20) [20, 55, 56, 58, 59] (***Figure 3*** ~~***Figure 3***~~).

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~~To properly outline and confirm mechanism by which statins induce cytokine production, a number of ABCA1 transporter knockdown and knockout studies have been conducted. In thioglycollate-elicited peritoneal macrophages derived from ABCA1 transporter knockout mice, NF-κB activity, and phosphorylation of ERK1/2, c-Jun N-terminal kinase (JNK) and MAPK p38 were significantly higher in knockout cells when compared to the knockout [58,~~

~~61]. This hypersensitivity of ABCA1 knockout cells was then followed by the increased transcription, and later the secretion, of the pro-inflammatory cytokines TNF-α and IL-12p20. Additional studies have confirmed the hypersensitivity of the ABCA1 transporter knockout models, with Yvan-Charvet *et al.* reporting that the knockout of ABCA1 transporters increased the secretion of granulocyte colony stimulating factor (G-SCF) and TNF-α, while the double knockout of ABCA1 and ABCG1 transporters increased IL-6 secretion [57]. Interestingly, this same result was not observed in ABCA1 transporter knockout bone marrow derived macrophages or RAW 267.4 macrophages [56, 57], with these cells displaying increased secretion of anti-inflammatory IL-10 cytokines, and a decrease in pro-inflammatory markers, TNF-α, IL-12p20, G-CSF, highlighting the cell specific effects of statins.~~

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2.1.2 Statins increase anti-inflammatory cytokines and anti-oxidative mediators via regulating GTPase activity.

An important aspect of statin induced anti-inflammatory effects - that is not often studied in parallel with the mechanism outlined above - is the role of prenylated GTPase proteins. Specifically, the GTPase superfamily of Rac, Rho, Ras, and Cdc42 that are upstream of MAPK p-38, ERK1/2, c-Jun N-terminal kinase (JNK) and NF-κB activation [24, 60-62]. As statins reduce the synthesis and therefore amount of prenyl groups available for prenylation, statins inhibit the association of GTPases with the membrane resulting in the functional inactivation of these molecules [30-32].

The GTPase superfamily regulates multiple pathways including cellular architecture, proliferation and migration. Important to this review is the role GTPases play in regulating

downstream signal transduction of inflammatory mediators. In response to extracellular inflammatory signals, GTPases are recruited and anchored to the membrane [31]. GTPase family members Rac and RhoA, initiate downstream signalling that involves numerous intermediates (Figure 3~~*Figure 3*~~), most notably via the MAPK/~~–~~NF-κB signalling axis to initiate synthesis of pro-inflammatory cytokines TNF-α, IL-6, IL-8 and pro-inflammatory intermediates intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 to name a few [29, 32, 53, 61, 63-66]. Importantly, ~~the treatment of~~ cells treated with a variety of hydrophilic and lipophilic statins treatment ~~has shown to~~show decreased Rac/RhoA-activity and therefore decreased downstream cytokine synthesis and secretion [32, 61, 65]. ~~Subsequent~~ Additional studies have then further confirmed the role of preynlated GTPases play in ~~the~~ regulating cytokine synthesis ~~of inflammatory mediators by~~as chemical~~ly inhibiting~~ inhibition of prenylation ~~in a variety of cell types~~resulted in similar results [18, 29, 30, 62, 67].

Of equal importance is the role statins play in modulating the oxidative stress pathway. Statins are known to reduce cellular oxidative stress via two mechanisms: 1. By inhibiting Rac mediated activation of the nicotinamide adenine dinucleotide phosphate (NAPDH)-oxidase pathway [68]. Activation of NAPDH must occur at the membrane, indicating Rac must be prenylated to initiate the signalling cascade that ~~resulting~~ results in the generation of reactive oxygen species (ROS) and the subsequent activation of NF-κB-mediated transcription [29, 69]. To initiate the signalling pathway, Rac must interact with NAPDH at the membrane and thus Rac must be prenylated [32]. 2. By inhibiting the prenylation of the Ras family member, Rap-1 [70]. Unprenylated Rap-1 has been shown to activate the mitogen-activated protein kinase kinase (MEK)-5/extracellular signal-regulated kinase (ERK)-

5 signalling axis [29, 70] (*Figure 3*~~*Figure 3*~~). Interestingly, MEK5 is a member of the MAPK family, yet the structure and signalling events that occur downstream of the interaction MEK5/ERK5 are distinct to that of MAPK-mediated signalling [71, 72]. MEK5 is the only known kinase to phosphorylate and activate ERK5 [73, 74]~~ while Rap-1 works upstream of MEK5 to activate the pathway [72]~~. As such, statins have been shown to increase ERK5 kinase activity [22, 32, 75] leading to the downstream activation of transcription factors, monocyte enhancing factor (MEF)2 and krupple-like factor (KLF)2 [76]. MEF2 and KLF2 both inhibit endothelial inflammation via independent pathways: MEF2 inhibits the expression of a known pro-inflammatory protein, cytokine-mediated adhesion molecule, [77], while KLF2 induces transcription of endothelial Nitric Oxide Synthase (eNOS), a potent anti-inflammatory mediator [24, 77-79]. Interestingly, overexpression of KLF2 was shown to attenuate expression of the pro-inflammatory mediator VCAM-1 [24] by muting the cytokine-mediated activation of Rac and subsequent downstream signaling [76, 80]. ~~Further to this, increased RhoA activation has been shown to inhibit the activation of KLF-2 [24], reinforcing the notion of synergy between Rac-mediated expression of pro-inflammatory mediators and the Rap-1 mediated eNOS synthesis.~~

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3.0 Potential role of statins in the treatment of respiratory diseases

In light of the pleiotropic actions of statins, there is the potential for statins to be used to treat a number of inflammatory-mediated respiratory diseases.

3.1 Cellular inflammatory and oxidative stress processes in the airways

In chronic lung diseases, the cascade of uncontrolled inflammatory processes is the main driver of disease pathogenesis and progression. Exposure of the respiratory system to a variety of stimuli such as infection (bacteria or virus), allergens (pollens, house dust mites) and airborne pollutants (dust, combustibles, cigarette smoke) play an important role in causing and perpetuating inflammation in the airways ~~after long-term exposure~~ [81]. During the inflammatory processes, epithelial cells and alveolar macrophages are activated as the first line of defence~~, which~~ and results in the subsequent sequestration of other inflammatory cells from the systemic circulation (i.e. neutrophils, eosinophils, monocytes and lymphocytes) in to the respiratory bronchioles and airway lumen to release pro- and anti-inflammatory cytokines (IL-1, IL-4, IL-5, IL-6, IL-8, IL-13, IL-1β, TNFα, IFN-γ). The release of these cytokines ~~creates an amplifying~~amplifies the production ~~loop to produce more~~of cytokines and activation of additional inflammatory cells via~~through~~ the MAPK/ NF-κB signalling pathway exaggerating the inflammatory responses further in chronic respiratory diseases [82].

In addition, inhalation of airborne pollutants also creates an exogenous, as well as endogenous (through nitric oxide, NO production) oxidant load, ~~creating~~ generating reactive oxygen species (ROS) and nitrogen oxygen species (such as superoxide anion and hydroxyl radical) that are capable of initiating oxidative stress in the airways. Overproduction of these reactive species contributes to tissue damage and lung injury that are associated with DNA, RNA and protein damage as well as lipid oxidation [81]. Specifically, these reactive species ~~they~~ also inactivate many of the anti-protease ~~mediators~~ mediator that are important in maintaining respiratory homeostasis, such as α_1-antitrypsin, serine

antiproteases and α_1-anti-chymotrypsin and consequently resulting in an acquired anti-protease deficiency, important in maintaining respiratory homeostasis. Activated macrophages also produces a number of matrix metalloproteases (notably MMP1, MMP2, MMP9, MMP12 and MMP15) ~~which has~~that have the ability to degrade both elastin and collagen fibres and therefore destroy the structural integrity of the lung. The altered protease/antiprotease balance, skewed in favour of ~~that increases~~ proteases activity, leads to the development of emphysema, poor elastic recoil of tissues, excessive ~~healing~~ remodelling (airway fibrosis), further damaging the lung parenchymal ~~damage~~ and the ~~increases~~ increased susceptibility to respiratory infections [4, 83, 84].

Another hallmark characteristic of chronic lung diseases is the excessive mucus production. This ~~that~~ contributes to the cycle of airway obstruction, inflammation and infection if not cleared from the lung. Hypermucus secretion is due to goblet cell hyperplasia and upregulation of the various inflammatory cytokines (i.e. neutrophil elastase, TNFα, IL-1β, EGF, IL-13) that ~~is~~ are concurrently associated with the increased transcription and translation of two mucin genes: ~~expression of~~ MUC5AC and MUC2 proteins [85]. These mucin proteins in turn are responsible for increased mucin production, which is the major component of mucus and is highly regulated in the airway epithelia via the RhoA/p35 pathway [86].

Of direct relevance to the discussed ~~pleotropic~~pleiotropic mechanisms, statins have been shown *in vitro* and *in vivo* to exert its beneficial protective effects for the treatment of chronic lung disease through 2 main actions in the airways:

(1) Reducing airway inflammation and oxidation through their roles in regulating NOS, decrease inflammatory cytokine production and reduction of inflammatory cells influx into the airways [87-93], as well as;

(2) Attenuating airway remodelling by regulating cellular architecture and function and reducing airway epithelial changes. Studies have shown that statin treatment could reduce contractile and proliferative properties of airway smooth muscle cells during airway hyperresponsiveness [94, 95], regulate MMP expressions [96, 97], and decrease MUC gene expression leading to decreased mucin production and suppression of goblet cell hyperplasia [98-104].

Hence, given the diverse pathways affected by statins through their inhibition of cholesterol synthesis and intracellular prenylation and subsequent GTP-binding proteins that underlie key pathway components of chronic inflammatory lung diseases, it is not surprising that repurposing of statins provides an exciting new opportunity for the treatment of these diseases.

3.2 Clinical trials on statins

The overwhelming positive results derived from preclinical studies involving statins have fuelled the interest of clinical studies over the past decade. Key clinical trials on the use of oral statins to treat chronic respiratory diseases such as asthma, COPD, acute lung injury and pulmonary hypertension that have been performed over the past 10 years have been summarised in ***Table 2*** ~~***Table 2***~~. Although numerous *in vitro* and *in vivo* animal studies have demonstrated beneficial effects of statins to treat chronic respiratory diseases, the overall results and conclusions from clinical trials were not consistent. ~~with s~~Several studies ~~demonstrating~~ demonstrated improved outcomes from statin therapies, and other studies

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~~that have found~~reported otherwise. These conflicting results may be attributed to several issues, but mainly is due the oral delivery of statins. Oral delivery of statins result~~ing~~ in low systemic bioavailability and subsequent varying sub-therapeutic levels of statins in the airways for its pharmacological efficacies. The low levels of statins in the lungs are unfavourable since the main pharmacological targets of statins are the airway epithelium, alveolar macrophages, fibroblasts, endothelial cells and inflammatory cells which are responsible for the inflammatory cascade in chronic airway diseases. ~~Other~~ Additional issues that need to be considered include the different types of statin which may exert various anti-inflammatory efficacies, the required optimal dose to be delivered to the airways for effective therapeutic ~~effect~~outcomes, the duration of statin treatment required before therapeutic effects ~~could~~ can be observed, whether statins are better as an alternative sole therapy or as an adjunct/combination with other therapies, and finally if statins are only e~~a~~ffective in a ~~particular~~specific patient population phenotype [105].

4.0 Inhalation of statins

One of the major problems ~~of administrating~~in using statins ~~for the treatment~~to treat ~~of~~ chronic respiratory diseases, is the ~~requirements~~ need for high doses of drugs to be administered to reach therapeutic concentrations in the airways for its clinical efficacies. This has resulted in conflicting results and observations in clinical trials investigating the protective roles of oral statins in patients with various respiratory diseases (Table 2). To complicate matters further, statins have low bioavailability following oral administration owing to the different lipophilic properties of the molecules, high first–pass metabolism in the liver and intestines, as well as rapid systemic clearance with short half-lives. The

resultant adverse effects from high oral doses of statins include hepatotoxicity, nephrotoxicity, myalgia, rhabdomyolysis and multiple drug interactions with other concomitant therapies and food intake due to its extensive metabolism by hepatic microsomal cytochrome P450 isozymes [106, 107]. Therefore, delivery of statins via the inhalation route could provide a unique opportunity and an important alternative to circumvent these problems. Ideally, inhaled statins will deliver high drug concentrations directly to the lungs using relatively lower doses ~~compared to oral administration~~ with minimal drug degradation and systemic absorption compared to oral administration. This will~~,~~ provid~~e~~~~ing~~ efficient and effective therapeutic effects while simultaneously reduc~~ing~~~~e~~ the risk of unwanted side effects.

There are a number of ways to classify statins including: (1) how they are synthesised (natural or synthetic), (2) physico-chemical properties according to their aqueous solubility (hydrophilic or lipophilic), (3) chemical structure- linked to pharmacological activity (active open ring acid or inactive lactone forms), and (4) statin generations based on their increasing safety profiles, potency and efficacy in lowering plasma low-density lipoprotein cholesterol (LDL-C) concentrations [107, 108]. Consequently, statins may also exert differential anti-inflammatory efficacies for treatment of lung diseases based on their physico-chemical properties that will in turn affect absorption, intracellular uptake mechanisms and activation of statins *in vivo*. ***Table 1*** ~~***Table 1***~~ summarises the different classifications of statins based on their physico-chemical properties, pharmacokinetic profiles and efficacies on various lipid factors. There have been several studies that have shown promising anti-inflammatory properties for statins to be formulated for pulmonary administration for chronic respiratory diseases rather than its conventional oral anti-

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cholesterol activity. These studies will be discussed in the following review sections in relation to statins physicochemical characteristics and pharmacokinetics parameters.

4.1 Simvastatin

Simvastatin is one of the most effective HMG-CoA reductase inhibitor for lowering cholesterol and associated cardiovascular risk. Combined with its affordability and availability of simvastatin generics drugs on the market, simvastatin~~this~~ is the most prescribed and well-studied statin molecule in terms of its anti-inflammatory potential and ~~pleotropic~~pleiotropic actions in literature. However, there are several issues to consider when formulating simvastatin as an inhaled formulation, which includes its low water solubility and chemical instability when in solution [103]. The lactone ring of the simvastatin structure renders this lipophilic prodrug susceptible to hydrolysis and oxidative reactions, forming ~~into~~ four metabolites: simvastatin hydroxy~~l~~ acid (SVA), 6'-exomethylene SV, 6'-ß hydroxy~~l~~- simvastatin, and 3'-5'-dihydrodiol simvastatin [109]. Yet, SVA is the active metabolite of simvastatin that is responsible for its therapeutic effects and is found to be 400 times more hydrophilic than simvastatin (log P of 4.68 at pH 7) [110]. Although the lipophilic simvastatin can passively diffuse across the cell membranes without the need for specific transporter uptake, ~~whereas the~~ hydrophilic SVA ~~becomes~~ is poorly absorbed by~~in to~~ the body, ~~as well as~~including ~~in to the~~ respiratory cells, ~~that could ultimately~~ possibly limiting its pharmacological action [100, 111]. Therefore, simvastatin ~~has to~~must be delivered in its stable pro-drug form to the airways to achieve maximum therapeutic efficacy to treat chronic airway diseases. Once in the lungs, simvastatin is ~~subsequently~~ activated into SVA by cytochrome P450 (CYP) isozymes and microsomial carboxylesterases

(CES), specifically CYP3A4/5 and CES1A1 ~~and CYP3A4/5 that are identical to~~ the same enzymes responsible for simvastatin metabolism ~~found~~ in the liver. Previous studies by Munger *et al.* [112] and Anttila *et al.* [113] have found that these CES and CYP enzymes are expressed ~~o~~in the membrane surface~~s~~ of alveolar cells ~~membrane~~, ~~in~~ bronchiolar cells, lung epithelial cells and alveolar macrophages. Hence, it is assumed that after deposition and dissolution of simvastatin into the airway lining fluid, the lipophilic drug molecule will then diffuse across the lipid bilayer membrane ~~into~~ of the various respiratory cells and ~~will~~ be metabolised into its active SVA metabolite by the CES and CYP enzymes ~~within the cells for its~~ to initiate its pharmacological effects.

To date, there are only a handful of studies that have investigated the potential of delivering simvastatin directly to the airways. ~~, with most~~The majority of these studies ~~being~~ are performed using intraperitoneal administration of simvastatin ~~to~~ in different animal models of various respiratory diseases including asthma, emphysema and ventilator induced injury [93, 114, 115]. The earliest study to investigate ~~on~~ inhaled simvastatin was conducted by Tschernig *et al*. [116] that compared the effects of ~~a~~ single dose of intraperitoneal and intratracheal administration of simvastatin prior to ovalbumin challenge in allergic airway inflammation in a rat model of asthma. In this study, different dosages were used for the two different routes of simvastatin administration with a higher dose of simvastatin administered intraperitoneally (10 mg, 1 mg and 0.1 mg) compared to intratracheal administration (1 mg and 0.1 mg). It was found that both administration routes ~~were able to~~ partially reduced the numbers of neutrophils, eosinophils and lymphocytes with increasing doses of simvastatin, ~~but~~ with the most notable effect ~~was~~ observed on CD4 T cell numbers, ~~which plays~~ an important ~~role~~ mediator of ~~in~~ asthma pathophysiology.

~~Interestingly~~Importantly, the authors noted that intratracheal administration of 0.1 mg simvastatin ~~was able to~~ significantly reduced the number of eosinophils to ~~similar~~ levels comparable ~~of~~to the intraperitoneal administration of 10 mg simvastatin dose.

Similar findings were also observed in a study by Xu *et al.* [117] that investigated the anti-inflammatory properties of inhaled simvastatin compared to intragastric injections of dexamethasone corticosteroid. The study also compared~~, as well as~~ the pharmacokinetic ~~properties~~ profiles of simvastatin delivered locally to the airways by inhalation, intratracheal injection or systemically by intraperitoneal injection and gavage in a mice model of asthma~~, compared to intragastric injections of dexamethasone corticosteroid~~. The~~A~~ pharmacokinetic study was performed using a single dose of simvastatin (5 mg/mL), while the anti-inflammatory efficacy study was evaluated with simvastatin treatment once a day for 7 days at 1, 5, or 20 mg/mL (dissolved in 20% ethanol and 80% saline) dose delivered by a jet nebuliser for 10 minutes. Local administration of simvastatin showed significant reductions in airway inflammation, remodelling and hyper-responsiveness in a dose dependent manner similar to corticosteroids dexamethasone. ~~-~~Concurrently, ~~observations~~ the study reported that~~of~~ higher drug concentrations were detected in local lung tissues ~~and~~ with lower plasma drug concentration compared to the drug administered systemically (100 mg/kg). The pharmacokinetic profile of a single dose of nebulised simvastatin (with 10% ethanol) in the lungs and systemic circulation was also confirmed in a recent study that ~~by Zeki *et al.* [120]~~ used~~ing~~ healthy rhesus macaque non-human primates to determine the metabolism and distribution of ~~the drug~~simvastatin [118]. Simvastatin and SVA were able to achieve maximum concentration inside the airway epithelial cells at time 0 and 1 hour post exposure and were both~~as~~ cleared within 6 hours. ~~Simultaneously~~Additionally, simvastatin

appears to enter the systemic circulation immediately after drug administration but was rapidly cleared by 3 hours, with no evidence of harm or injury to the animals. These evidences further support~~ed~~ the benefits of using inhalation strategies to reduce the oral doses required to obtain enhanced therapeutic efficacy in the airways and reduce the incidence of side effects.

The efficacy of localised delivery of simvastatin by inhalation (0.06, 0.6 or 6 µg/kg) ~~for~~ over a 2-~~-~~week period~~s~~ has also been demonstrated in additional ~~series of other~~ studies [119-121] that used~~ing~~ house dust mite allergen challenged mice models of airway inflammation and hyper-reactivity. Furthermore, t~~T~~hese studies also demonstrated that intranasal administration of simvastatin was able to suppress allergic airway inflammation and hyperactivity. The mice in these studies ~~as demonstrated by the~~displayed reduced numbers of inflammatory cells such as eosinophils and neutrophils~~,~~ in the bronchoalveolar lavage and lung histology samples ~~of the mice models~~ in a simvastatin-dose dependent manner. However, considering the lipophilic nature and instability of the simvastatin molecule *in vitro* and *in vivo*, a critical issue with these studies is the lack of information on the simvastatin formulation that was used to deliver the drug to the lung. ~~in terms of~~This also includes drug stability, aerosol performance, solubility in the various vehicle used, physical-chemical properties and whether a dry powder or solution based formulations were utilised, all of which could ultimately result in differential pharmacokinetic, toxicity and efficacy profiles when translated to human studies.

More recently, several inhalation formulations of simvastatin that have addressed the issues of stability, dosages and aerosol performance have been developed including a low dose

pressurised metered dose inhaler (pMDI, 150 µg/actuation) [101, 122], dry powder for inhalation (DPI) [102] and nebulised nanoparticles [103], as potential treatment of mucus overproduction and inflammation for respiratory diseases. These formulations have been shown to possess favourable physical and chemical properties with a demonstrated good chemical stability of simvastatin up to 3-9 months in various storage conditions, and suitable aerosol performance to reach the deep lungs. More importantly, these inhaled simvastatin formulations ~~were able to~~ demonstrated anti-inflammatory activities, muco-inhibitory efficacy, anti-oxidant properties and superior safety profiles over a wide range of drug concentrations. ~~The~~ Ttherapeutic efficacies of the formulations were investigated using a physiologically relevant *in vitro* model set-up shown to be predictive of *ex vivo* and *in vivo* activity, that combined~~s~~ an air interface Calu-3 bronchial epithelial cell model with a twin stage impinger to simulate realistic airway drug deposition ~~, which have been shown be predictive of *ex vivo* and *in vivo* activity~~ [123-126]. The study ~~results~~ showed significant inhibition of mucus production, inflammatory cytokine release (IL-6 and IL-8) and oxidative stress (~~reactive oxygen species~~ROS) i~~e~~n cells ~~that were~~ treated with simvastatin formulations when compared to untreated cells stimulated with lipopolysaccharide to ~~simulate~~ mimic the inflammatory process~~es~~ in the airways. ~~In addition,~~T ~~t~~he nebulised simvastatin nanoparticles encapsulated with a poly(lactic-co-glycolic) acid (PLGA) and Pluronic F-127 formulation [103], had an added benefit of controlled release properties and superior cytotoxicity profile, compared to the other simvastatin formulations. In turn, t~~T~~hese properties ~~which in turn~~ resulted in sustained anti-inflammatory actions and potentially better patient compliance for up to 48 hours. In these studies, the d~~D~~irect administration of the pro-drug simvastatin into the lung as pMDI, DPI or nebulised nanoparticle suspension ~~in these studies~~ was ~~also able to be activated~~converted into the

active SVA metabolite ~~for its therapeutic effects.~~, with almost ~~A significant proportion (approximately~~ 40%~~)~~ of the simvastatin delivered being ~~was~~ converted ~~in~~to SVA intracellularly~~, as well as on the apical surface of the epithelium after treatment~~. This reaffirms the ability of inhaled simvastatin to diffuse and penetrate into the airway epithelium and be subsequently converted to SVA metabolite ~~in the presence of the metabolising enzymes~~. Hence, the repurposing of simvastatin in these inhaled formulations could potentially be used to treat chronic pulmonary diseases locally where hyper-mucus production and uncontrolled inflammation are key features of the disease. However, ~~but~~ further *in vivo* and clinical studies are required to translate these treatments to the clinical setting as well as to determine a safe and effective therapeutic dose.

4.2 Hydrophilic statins: Pravastatin and Rosuvastatin

Another potential statin therapy that has shown ~~to have~~ promising anti-inflammatory properties ~~for treatment of asthma~~ when delivered by inhalation is pravastatin. Unlike simvastatin, which is, the most lipophilic statin molecule used clinically, pravastatin is the most hydrophilic, with partition coefficient of -0.84 at pH 7.4. ~~Its~~ The pharmacokinetics of inhaled pravastatin, as well as efficacy to reduce airway inflammation and mitigate structure damage ~~of inhaled pravastatin~~, was investigated ~~by~~ in a recent study ~~by Zeki *et al.* [129]~~ in a murine allergic model [127]. ~~While~~T ~~t~~he authors found that intratracheal instillation of water-soluble pravastatin was able to achieve relatively high concentrations of drug in the bronchoalveolar lavage fluid and lung tissue with minimal systemic absorption ~~with~~ and no damage to the airway epithelium. However, ~~,~~ no statistically significant anti-inflammatory effects on inflammatory cell influx in the lungs were~~as~~ ~~observed~~ reported nor ~~did~~ was pravastatin shown to attenuate~~d~~ airway hyper-reactivity or preserve lung compliance.

~~However~~Importantly, inhaled pravastatin was ~~only~~ effective in reducing goblet cell hyperplasia and metaplasia as well as secretion of selected cytokines (TNF-α and keratinocyte chemoattractant, KC), but not other cytokines/chemokines including IL-13, IL-4, IL-5, IL-1α, IL-1β, IL-17, interferon gamma-induced protein 10 (IP-10), IFNγ, or regulated on activation, normal T-cell expressed and secreted (RANTES). The lack of anti-inflammatory efficacy with pravastatin is most likely due to it hydrophilic properties, ~~that~~ preventing~~s~~ efficient entry ~~of the molecule~~ via passive diffusion into respiratory cells such as epithelium and immune cells.

The active form of all statins is the 'open ring' hydroxy acid (such as simvastatin and lovastatin hydroxy acid) that binds to the active site of the HMG-CoA reductase enzyme to inhibit its enzymatic function. P~~P~~ravastatin is administered as the active hydroxy~~l~~ form ~~that~~ and is not readily absorbed by cells when~~,~~ ~~as~~ compared to the more lipophilic inactive ~~form~~ statins, ~~such as~~ simvastatin and lovastatin. ~~This was also~~C ~~c~~onsistent ~~to~~ with this, the~~e~~ findings of Marin *et al.*[100], ~~which~~ demonstrated that the transport of the open ring SVA into and across Calu-3 bronchial epithelial cells was significantly lower compared to its inactive 'close lactone ring' simvastatin counterpart. Hence, intracellular uptake of pravastatin requires membrane transporters such as organic anion transporting polypeptide (OATP), primarily OATP1B1 to facilitate transport into the effector cells [128, 129]. Importantly, the OATP1B1 transporter is ~~, which is~~ not known to be expressed in the human lung tissues [130, 131]. Although pravastatin can be easily delivered directly to the airways in solution form, the lack of mechanisms to enter the cells could have prevented the drug from reaching sufficient intracellular concentrations to exert its therapeutic anti-inflammatory effects. Therefore, statin polarity properties (i.e. hydrophilicity, lipophilicity)

not only determine experimental designs with respect to drug delivery and solubility, but are also ~~are~~ equally important considerations for cell penetration that ~~will~~ in turn affect bioavailability, potency and efficacy of the various statin molecules as anti-inflammatory agent in the airways.

Another hydrophilic statin molecule that has the potential for inhalation delivery is rosuvastin, which is a newer generation of statin also termed superstatin, delivered in its active open ring form [107]. Rosuvastatin is a fully synthetic HMG-CoA reductase inhibitor, whereas the other statins are natural, mevinic derived or synthetic, heptenoic acid derived. While the characteristics of all statin pharmacophores remain similar, the addition of a stable polar methane-sulphonamide group and fluorinated phenyl group to the rosuvastain molecule provide low lipophilicity and enhanced binding affinity with the HMG-CoA reductase enzyme. The strong ~~through~~ ionic interactions between rosuvastatin and HMG-CoA reductase enzyme are~~,~~ responsible for its superior efficacy, ~~and~~ potency and ~~also~~ slow HMG-CoA reductase enzyme recovery ~~after~~ following statin ~~removal of rosuvastatin the statin~~ clearance [132]. ~~Consequently,~~ T~~t~~he affinity of rosuvastatin for the HMG-CoA reductase active site is four times greater compared to the native HMG-CoA reductase ~~for the enzyme~~ and has the greatest affinity amongst the other older generations of statin molecules [133]. However, since rosuvastatin is a hydrophilic statin, it relies on the OATP1B1 transporters as the key mechanism for active transport into the various cells [134], which may be absent in the pulmonary tissues as previously discussed. Nevertheless, Patil-Hadhe, A. *et al.* [135] have successfully formulated rosuvasatin loaded nanostructure lipid carrier in the form of solid lipid nanoparticles using a mixture of lauric acid and capryol 90 (7:3). This mechanism of rosuvasatin delivery has the~~a~~ potential as an anti-inflammatory

Commented [PB2]: What does this mean? For the enzyme? Im a little confused

therapeutic for pulmonary application to attain high and sustained drug concentrations in the lung tissue. The dry powder formulation was evaluated for its physical-chemical characteristics, *in vitro* aerosol performance and *in vivo* pharmacokinetics in a murine model. The study found that the formulation ~~was able to~~ produced particles of <200 nm in size, ~~have~~ high drug encapsulation efficiency, ~~,~~ sustained drug release ~~over an extended period of time~~ over an extended 24-hour period~~s~~ as compared to the 3 hours for the free drug with aerosol performance ~~that is~~ suitable for the deep lung when lyophilized with mannitol as a cryoprotectant-carrier. In addition, the *in vivo* pharmacokinetics of the rosuvasatin nanostructure lipid carrier formulation ~~study~~ demonstrated higher Cmax, improved elimination half-life, and an improved~~ment in~~ area under the plasma concentration-time curve (AUC) ~~AUC for~~when ~~the rosuvasatin nanostructure lipid carrier formulation~~ compared to pure drug solution, indicating significant bioavailability of rosuvastatin in the lungs. It was postulated that the lipid~~ic~~ nature and smaller size of the nanoparticles ~~facilitate the~~enabled the particles to bypass~~ing of~~ macrophage clearance leading to lower lung clearance and ~~may~~ help facilitate entry of rosuvastatin molecule into pulmonary airway epithelial cells. However, the anti-inflammatory and antioxidant effects of the rosuvastatin formulations were yet to be explored, which is critical for it to be an effective therapy.

4.3 Pitavistatin

Pitavastatin is another third generation superstatin ~~that is a~~and a very potent inhibitor of the HMG-CoA reductase ~~inhibitor~~enzyme. Its chemical structure with a quinolone ring at the core and a flouro-phenyl group is similar to the other statins, especially fluvastatin and rosuvastatin. The presence of a cyclopropyl side chain is unique to pitavastatin, ~~which~~

making~~es~~ it moderately lipophilic. ~~and C~~Consequently, the addition of this side chain confers several distinct pharmacokinetic and pharmacodynamics properties to the drug molecule when compared to other statins [136]. This structure makes pitavastatin a stronger inhibitor of HMG-CoA reductase activity than other statins, including rosuvastatin~~,~~. As such, ~~and hence~~pitavastatin requires a lower dose ~~compared to other statins~~ to inhibit cholesterol synthesis and~~s well as~~ elevat~~e~~ing high-density and apolipoprotein A1.~~,~~Pitvastatin undergoes minimal metabolism resulting in higher bioavailability and extended duration of action, and has a unique metabolic profile that reduces the potential for drug-drug and drug-food interactions [136]. The metabolic properties of pitavastatin are similar to that of the~~e~~ other hydrophilic statins, having a clinically insignificant metabolism occurring via the CYP2C9 pathway [137, 138].

In terms of its pleiotropic effects, pitavastatin behaves similarly to other statins as ~~appears to be a~~a modulator ~~for~~ of a variety of vascular, inflammatory markers, endothelial nitric oxide synthases and endothelial cells ~~similar to other statins~~. *In vitro* studies ~~It was~~have found that pitavastatin ~~has the~~displayed the most potent ~~angiogenic~~ effects on proliferation of pulmonary artery smooth muscles and endothelial cells when compared to other marketed statins ~~in *in vitro* studies~~ [139, 140]. ~~Subsequently~~Further to this, Chen *et al.* [141] investigated the use of inhaled ~~the potential of~~ pitavastatin formulated as PLGA polymeric nanoparticles to improve the efficacy and reduce the side effects when treating pulmonary artery hypertension ~~as inhaled delivery approach to treat pulmonary artery hypertension with the aim to improve efficacy and reduce side effects~~. Using a rat model ~~of model~~ of monocrotaline-induced pulmonary artery hypertension, the delivery of pitavastatin polymeric nanoparticles directly to the lung was found to me more effective than

intratracheal delivery of pitavastatin alone or systemic delivery of pitavastatin in reducing the development of pulmonary artery hypertension. Nanoparticle formulation of pitavastatin ~~was also able to~~ induced the regression of established pulmonary artery hypertension and improved the survival rates of the ~~murine~~ rat model. ~~However~~Interestingly, no therapeutic effect was observed ~~with~~ following the inhalation of pitavastatin suspension alone, although the concentrations of pitavasatin in the lung and systemic circulation were similar ~~between~~ to animals treated with the same dose (100 µg) of intratracheal pitavastatin suspension alone and ~~those treated with~~ the nanoparticle formulation. In addition, the study ~~also~~ found that oral daily doses of pitavasatatin over 21 days at 1, 3, and 10 mg/kg per day (cumulative doses = 84, 252 and 840 mg per animal, respectively) showed similar therapeutic benefits ~~similar~~ to a single dose of 0.1 mg inhaled pitavastatin nanoparticle formulation, a dose ~~which is~~ equivalent to ~850 times lower ~~dose required compared to~~ than thee cumulative systemic doses. These observations suggest an ~~particular~~ added benefit of using inhaled nanotechnology to mediate intracellular delivery of pitavastatin ~~at the target site~~directly to the lung to induce greater therapeutic effects. The very limited solubility of the pitavastatin in aqueous solutions (0.426 mg/L) [142, 143] ~~could have~~may potentially limited drug exposure and subsequent uptake ~~into the~~by target cells and could have been further complicated by the requirements for cellular uptake mechanisms, which are mediated by various transporters including OATP1B1, OATP1B3, organic anion transporter 3 (OAT3) and sodium(Na)-taurocholate cotransporting polypeptide (NTCP)[144-146]. Interestingly, encapsulating pitvastatin within the nanoparticle negates this challenge. The findings in this study emphasises the importance of targeted delivery, as well as formulation technologies, to circumvent certain physical-chemical properties of the drug molecule to improve drug solubility into the limited airway

surface liquid. ~~and to~~Nanotechnologies could also bypass the requirements for facilitated transporter through endocytic mechanisms [147] for intracellular drug uptake leading to~~for~~ improved drug efficacies.

4.4 Atorvastatin

Atorvastatin is another second generation statin that is administered as the active hydroxy~~l~~ acid and not as the lactone prodrug [148]. *In vivo*, atorvastatin is converted to its lactone form and appears to have the same ~~area under the plasma concentration time curve (~~AUC~~)~~ as its active hydroxy~~l~~ acid form after oral administration. Interestingly, the acid and lactone forms of atorvastatin have different partition coefficient octanol/water (log D values) at pH 7.4 of 1.53 and 4.2 respectively, which consequently have an impact on the pharmacokinetics in terms of partition into biological membranes and diffusion across ~~the~~ membranes [149-151]. Atorvastatin ~~The drug~~ is subjected to extensive metabolism by CYP3A4 ~~to~~ and is converted into two active forms of the metabolite~~s~~, 2-hydroxy-atorvastatin acid and 4-hydroxy-atorvastatin acid, both of which are in equilibrium with their inactive lactone forms [152]. Hence, intracellular uptake mechanisms of atorvastatin are mediated by both passive and carrier mediated processes depending on its form. ~~with T~~the lactone form is transported ~~through~~ via passive diffusion ~~due to~~as it~~s~~ is more lipophilic in nature, while the atorvastatin acids are substrates for cellular membrane transporters of P-glycoprotein (P-gp) and OATP C [148].

As with most statins, there ~~are~~ is increasing evidence to show that ~~the~~ atorvastatin also exerts pleiotropic properties, independent of the lipid-modifying properties, ~~B~~by inhibiting the synthesis of ~~nonsteroidal~~ isoprenoid compounds, atorvastatin ~~that is able to~~ modifies ~~y~~

inflammatory responses, endothelial cell function and smooth muscle cell proliferation [153, 154]. A recent study by Pinho-Ribeiro, M. *et al.* [155] investigated the efficacy of inhaled atorvastatin versus inhaled simvastatin on inflammation, oxidative stress and lung repair in a murine model of chronic obstructive pulmonary diseases with emphysema caused by long term exposure to cigarette smoke. ~~The outcome measures were then compared to inhalation of simvastatin.~~ This was the first study to our knowledge to compare the ~~pleotropic~~pleiotropic efficacies of different inhalation statins. In the study, mice were exposed to smoke from 12 cigarettes per day for 60 days to induce pulmonary emphysema. Mice were then ~~and were subsequently~~ treated with either atorvastatin (1 mg/mL), simvastatin (1mg/mL), or a vehicle control ~~vehicle~~ that were administered in aerosol form for 15 minutes once per day for a further 60 days. Both atorvastatin and simvastatin ~~were able to~~ improved the pulmonary morphologies and function due to the restoration of extracellular matrix, decreased influx of ~~if~~ inflammatory cells ~~influx that~~and the subsequently ~~led to~~ reduced~~tion in the~~ release of inflammatory mediators and oxidative stress damage. The most notable difference between the two statins wa~~i~~s that atorvastatin showed better anti-inflammatory ~~effects~~ properties when compared to simvastatin. Mice treated with ~~,~~a~~-~~torvastatin ~~as~~ demonstrated a~~by higher~~ significant reduction in the number of inflammatory cells (i.e. macrophages and neutrophil) in the bronchoalveolar lavage fluids ~~from the animal~~ and associated ~~-~~cytokine levels ~~when treated with inhaled atorvastatin~~. On the other hand, the ability of simvastatin to improve lung repair and airway function is most likely related to its anti-oxidative stress. In the study, ~~I~~inhaled simvastatin demonstrated superior anti-oxidant effects compared to atorvastatin, and seems to be the pharmacological major action against inflammation with lower levels of lipid peroxidation and reduced redox marker formation (such as reactive oxygen species, superoxide

dismutase, catalase activity). The mechanisms of how the statins elucidate its differing actions were not discussed. While the findings provided some insights into the differing efficacies of the different statins that could potentially guide therapeutic approaches for treatment of chronic lung diseases, a major limitation with the study is the lack of information ~~on the inhaled statin~~regarding the formulations of the inhaled statins ~~used~~. Details regarding the stability/form of the statins, vehicle used to deliver the statin solution and physical-chemical properties were not reported, which have been shown in previous studies (discussed in this review) to have an effect on intracellular drug uptake, pharmacokinetics and ultimately efficacy in the airways.

5.0 Conclusions

Despite therapeutic advancements, incidence of chronic inflammatory lung disease continues to increase every year due to air pollution, tobacco smoke and occupational hazards. These debilitating diseases do not only affect a patient's quality of life but also creates huge burden on the health care system, as well as a country's social-economic sector. Furthermore, current treatment options are limited and may not be effective for all patient populations. Hence, new treatment options with superior efficacies to treat these diseases are urgently required as potential substitution, alternative or adjunct therapy to currently available therapies. However, the discovery of new molecules and development of formulation that are safe and effective has become increasingly challenging in recent years. To address this issue, the repurposing of conventional older drug molecules for different therapeutic applications either by changing its treatment indication, route of administration or formulation properties are ~~is~~ a highly feasible solution~~,~~. A comprehensive understanding

of the pharmacological mechanisms, drugs physical-chemical characteristics, safety and efficacies are therefore fundamental to the repurposing of drugs prior to market approval ~~which requires a comprehensive understanding of the drugs physical-chemical characteristics and biological activities~~. There are also several other potential advantages of conventional drug repurposing compared to the discovery of new drug molecule, which include: 1) a well-established commercial manufacturing scale and quality control of the active pharmaceutical ingredient, 2) lower ~~cost of~~ manufacturinge costs, 3) known systemic pharmacokinetic and safety profiles leading to the use of lower drug concentrations for the proposed new purpose that will ultimately reduce the risk of systemic side-effects and drug-drug interactions, and 4) ~~the~~well documented active pharmaceutical ingredient properties ~~including stability in various formulations are well understood~~. Nevertheless, the repurposing of drugs still requires the development of a suitable formulation and delivery system with long term stability, manufacturing capability ~~for the product~~, good long term inhalation safety and carcinogenecity profiles in animals, as well as proven safety and efficacy studies in humans.

This review has highlighted the potential of statins as an effective therapy for the treatment of chronic lung diseases that have conventionally been used for the treatment of hypercholesterolemia. Statins have been shown to have multiple ~~pleotropic~~pleiotropic effects other than its lipid lowering activity ~~though~~ by modulating~~ation of~~ multiple signalling pathways ~~including~~ that govern ~~anti~~ inflammatory, muco-inhibitory, ~~anti~~ oxidant stress and ~~anti~~ proliferation~~ve effects~~. While it is clear that statins have a protective role that could be exploited for the treatment of chronic lung diseases, the use of oral statins in clinical trials

to treat these diseases have been non-conclusive. This controversy is most likely due to the statin delivery routes used in the trial, i.e. oral versus inhalation, which continues to be the main limiting factor in directly assessing the beneficial effects of statin as potential anti-inflammatory agents. No clinical studies have been published to date on the use of inhaled statins. Furthermore, studies on reformulating statins as an inhaled therapy are still in ~~its~~ their infancy and further investigations are required to ~~to gain a~~ better understand~~ing of~~ the efficacy, toxicity and mechanism of action of these statin molecules in the airways.

~~Nevertheless~~N~~,~~ ~~n~~umerous *in vitro* and *in vivo* studies have demonstrated the protective effects of statins for the treatment of chronic inflammatory lung conditions. Clearly, the treatment efficacies of statins will be greatly enhanced compared to oral delivery when delivered directly to the airways by inhalation, ~~which will also~~ reduc~~e~~ing the~~e~~ incidence of side effects and potential for drug-drug interactions. Therefore, the repurposing of statins from conventional anti-cholesterol oral therapy to inhaled anti-inflammatory formulation provides a novel therapeutic avenue and an exciting future challenge that will be beneficial for patients with chronic inflammatory lung diseases, and potentially as an anti-proliferative treatment for other neo-plastic diseases like lung cancer and lymphangioleiomyomatosis.

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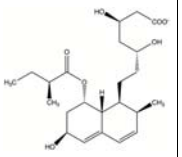
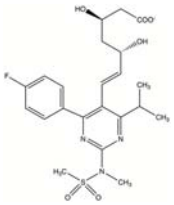
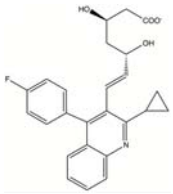
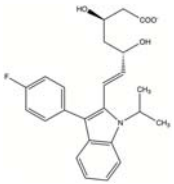
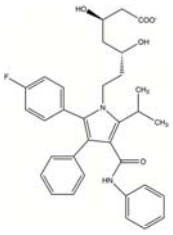
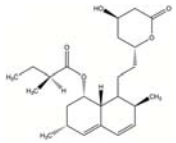
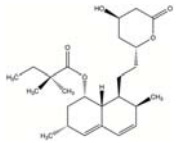
Figure Captions

Figure 1: *Effects of inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme by statins on cholesterol biosynthesis pathway, which also generates isoprenoid intermediates, farnesylpyrophosphate (FTT) and geranylgeranylpyrophosphate (GGPP). Both these products are used in post-translational modification of proteins via prenylation.*

Figure 2: *Summary of the mechanisms of statin transport into effector cells and the downstream effects of indirect inhibition of GTPase signalling that contributes to the pleiotropic effects of statins. The statin mediated depletion of intracellular isoprenoid intermediates causes a reduction in prenylation anchorage of GTPases (including Ras, Rac, Rho, and Cdc42) on the inner cell membrane. Subsequently, the Rho protein can assume two conformations: inactive GDP-bound date or active GTP-bound state. Hence, statins impair the activation of the GTPase by guanine nucleotide exchange factor (GEF). This leads to inactivation of the several kinases that functions to regulate the downstream mitogen activated protein kinase (MAPK) p38 and the nuclear factor (NF)-κB pathways that are associated with inflammation, oxidative stress, mucus production and tissues remodelling. CES: carboxylesterases, CYP: Cytochrome P450, CR: cellular receptor, FPP: Farnesyl pyrophosphate, GDP: guanosines diphosphate, GAP: GTPase-activating protein, GGP: geranylgeranyl pyrophosphate, GTP: guanosine triphosphate, NOS: notric oxide synthase, Rho: Ras homologous protein, ROS: reactive oxygen species, OAT: Organic anion transporter, OATP: Organic anion transporting polypeptide*

Figure 3: *Summary of the proposed mechanisms by which statins act to regulate cellular processes. Statins inhibit synthesis of prenyl groups (Figure 1) that are required for GTPase (Rho/Rac/Cdc42/Rap) signaling. Thus, statin-mediated inhibition of this pathway, results in the decreased downstream transcription of pro-inflammatory cytokines (TNF-α, IFN-Y, IL-6, and the increased transcription of anti-oxidative proteins. Further to this, Cdc-42 has been shown to regulate that transcription of the cholesterol transporters, ABC transporters, via the ERK1/2 - Cox-2 signaling axis, signaling pathway. As a result of statin treatment, ABC transporters are down regulated resulting in decreased reverse cholesterol transport (RCT) and increasing intracellular levels of cholesterol (shown in grey). The increased intracellular cholesterol is transported to the endoplasmic reticulum (via an unknown mechanism, indicating a possible role for Rab proteins) causing a 'fatty acid overload', that in turn results in activation of the ERK1/2-Cox-2 signaling pathway to increase the transcription and translation of ABC transporters. ERK1/2 (extracellular-signal regulated kinase 1/2); Cox-2 (cyclooxygenase-2); PPAR (peroxisome proliferator-activated receptors); LXRs (liver X receptors); ABC transporters (ATP-binding cassette transporters); MAPK p38 (mitogen activated protein kinase p38); NF-κB (nuclear factor-κB); TNF-α (tumour necrosis factor -α); IL (interleukin-6); IFN-Y (interferon-Y); G-CSF (granulocyte–colony stimulating factor); VCAM (vascular cell adhesion molecule); ICAM (intracellular adhesion molecule); NAPDH (nicotinamide adenine dinucleotide phosphate); ROS (reactive oxygen species) MEK5 (mitogen-activated protein kinase kinase-5); (ERK)-5 (extracellular signal-regulated kinase-5); MEF2 (monocyte enhancing factor 2); KLF2 (krupple-like factor2); eNOS (endothelial nitric oxide synthase).*

	Pravastatin	Rosuvastatin	Pitavastatin	Fluvastatin	Atorvastatin	Lovastatin	Simvastatin
Chemical structure							
Prodrug	No	No	No	No	No	Yes	Yes
Lipophilicity (log P)	-0.23 (Hydrophilic)	0.13 (Hydrophilic)	1.49 (Lipophilic)	3.24 (Lipophilic)	Acid form: 1.53 Lactone form: 4.2 (Lipophilic)	4.3 (Lipophilic)	4.68 (Lipophilic)
Synthesis	Fungal mevinic acid derived	Fully synthetic	Synthetic heptonoic acid derived	Synthetic heptonoic acid derived	Synthetic heptonoic acid derived	Fungal mevinic acid derived	Fungal mevinic acid derived
~~**Lipophilicity (log P)**~~	~~-0.23 (Hydrophilic)~~	~~0.13 (Hydrophilic)~~	~~1.49 (Lipophilic)~~	~~3.24 (Lipophilic)~~	~~Acid form: 1.53 Lactone form: 4.2 (Lipophilic)~~	~~4.3 (Lipophilic)~~	~~4.68 (Lipophilic)~~
Statin generation	1st	3rd	3rd	1st	2nd	1st	2nd
GIT Absorption (%)	3.4	40-60	80	98	30	30	60-80
Systemic Bioavailability (%)	18	20	60	24	14	<5	<5
Protein binding (%)	50	88	96	98	>98	>95	95
Major P450 metabolic enzyme	None	CYP2C9 (minor)	CYP2C9	CYP2C9 (minor)	CYP3A4	CYP3A4	CYP3A4
Active metabolites	No	Minimal	No	No	Yes (2)	Yes (4)	Yes (3)
Elimination half-life (h)	2	19	11	<3	14	2	1.4-3
Transporter	OATP1B1	OATP1B1	OATP1B1, OATP1B3, OAT3, NTCP	Passive diffusion, OATP 1B1, OATP2B1, OATP1B3	Passive diffusion, P-gp, OATP C	Passive diffusion	Passive diffusion
Efficacy (%)*							

• *Serum LDL reduction*	34	63	48	24	50	34	41
• *Serum HDL reduction*	12	10	-[#]	8	6	9	12
• *Serum triglyceride reduction*	24	28	23	10	29	16	18

Table 1: *Chemical properties of statins affecting their pharmacokinetics profile and efficacies on various lipid factors after oral administration [137, 156]. *Efficacy was elicited in hypercholesterolemia patients taking a daily oral dose of 40mg for atorvastatin, fluvastatin, lovastatin, pravastatin, simvastatin and rosuvastatin and 4 mg for pitavastation; [#]no significant effect reported; LCL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol; GIT: gastrointestinal track, OATP: Organic anion transporting polypeptide; OAT: Organic anion transporter, NTPC: sodium-taurocholate co-transporting polypeptide; P-gp: P-glycoprotein*

Disease	Study	Study type	Statin used	Sample size	Duration	Main outcomes (statin group as compared to control group)
Asthma	Zeki et al., 2013 [157]	Retrospective study	Simvastatin, atorvastatin, lovastation, pravastatin	165	1-5 years	• No significant difference in pulmonary function results (FEV_1, FVC, $FEF_{25-75\%}$) • No significant difference in serum inflammation (total white blood cell count, eosinophils) • Significantly higher asthma control (ACT) score
	Tse et al., 2013 [158]	Retrospective study	Simvastatin, lovastatin, atorvastatin, pravastatin, rosuvastatin, fluvastatin, cerivastatin, and pitavastatin	3747	12 months	• Significantly lower emergency department visits in asthma patients who are using inhaled corticosteroid concomitantly
	Braganza et al., 2011 [159]	RCT	Atorvastatin (40mg/day)	71	4 weeks	• No significant difference in morning peak expiratory flow • No significant difference in inflammatory biomarkers in sputum and serum • Improvement in asthma control (ACQ and AQLQ) *All asthmatic patients are smokers
	Ostroukhova et al., 2009 [160]	Retrospective study	Any statin	80	2 years	• 3-5% median worsening of FEV_1 in the statin group • Increased used of maintenance medication, nocturnal awakenings and albuterol in statin group
	Fahimi et al., 2009 [161]	RCT	Atorvastatin (10mg/day)	17	4 weeks	• No significant improvement in pulmonary function tests (PEF, FVC and FEV_1/FVC)
	Maneechotesuwan et al., 2010 [162]	RCT	Simvastatin (10mg/day)	50	8 weeks	• Significant reduction in sputum eosinophils when combined with inhaled budesonide • No change in lung function (FEV_1)
	Horthersall et al., 2008 [163]	RCT	Atorvastatin (40mg/day)	54	8 weeks	• Significant reduction in sputum macrophage counts when combined with inhaled corticosteroid • No change in lung function (PEF, FEV_1) • No improvement in ACQ score • No significant change in sputum inflammatory biomarkers (IL-8, TNF_α, IFN_γ, LT B4, MPO)
	Huang et al., 2011	Retrospective	Any statins	3965	Not	• Statin use was associated with lower incidence of

	[164]	study			reported	hospitalisations due to asthma attack
	Badar et al., 2014 [165]	RCT	Atorvastatin (40mg/day)	60	12 weeks	• Improvement in asthma control ACQ score • Decrease in sputum cell counts • Significant reduction in asthma exacerbation risk • No significant improvement in lung function (FEV_1, PEF)
	Lakhandwala et al., 2012 [166]	Retrospective study	Any statins	1437	1 year	• Significantly lower asthma-related hospitalisation and/or emergency room visit for patients on statins
	Pagovich et al., 2010 [167]	Retrospective study	Atorvastatin, simvastatin	70	1 month	• Improvement in lung function measurement (peak flows) • Decrease in albuterol use
	Cowan et al., 2010 [168]	RCT	Simvastatin (40mg/day)	43	4 weeks	• Improvement in lung function (PEF, FEV_1) • Significant reduction in sputum eosinophils and higher lymphocytes • Reduced asthma control with decrease in ACQ score
	Moini et al., 2012 [169]	RCT	Atorvastatin (40mg/day)	62	8 weeks	• No difference in lung function measurements (FEV_1, FVC) • No difference in blood eosinophil counts • No difference observed with ACT score
	Olgun Tildizeli et al., 2017 [170]	RCT	Rosuvastatin (40mg/day)	51	8 weeks	• Improvement in lung functions measurements (FEV_1/FVC, $FEF_{25-75\%}$) • Decreased in sputum eosinophilia • Significant reduction sputum in inflammatory markers (IL-6, TNF_α) • No change in ACT and AQLQ scores
COPD	Criner et al., 2014 [171]	RCT	Simvastatin (40mg/day)	885	641 days	• No significant difference in exacerbation rates and time to first exacerbation.
	Lee et al., 2009 [172]	RCT	Pravastatin (40mg/day)	53	6 months	• Improvement in exercise capacity • Decreased pulmonary hypertension demonstrated by significant reduction in echocardiographically derived systolic PAP (pulmonary artery pressure) and dyspnea during exercise

						• No difference in pulmonary function parameters (FEV_1, FEV_1/FVC, total lung capacity or inspiratory capacity)
	Blamoun et al., 2008 [173]	Retrospective study	Simvastatin, lovastatin, atorvastatin, pravastatin, fluvastatin	185	1 year	• Fewer episodes of exacerbation • Lower number of required intubations secondary to COPD exacerbations
	Lahousse et al., 2013 [174]	Retrospective study	Any statins	7983	>2 years	• Long terms statin use associated with decreased risk of death • Long-term statin use associated with a significant 78% reduced mortality if hsCRP level > 3 mg/L (a marker of systemic inflammation)
	Van Gestel et al., 2008 [175]	Retrospective study	Fluvastatine, simvastatin, pravastatin, atorvastatin, and rosuvastatin	3371	Not reported	• Statin use associated with improved short- (30 days follow-up) and long- (10 years follow-up) term survival in patients with peripheral arterial diseases with and without COPD • COPD patients should be treated with intensified doses of statins to achieve improved short and long term survival
	Rezk and Elewa, 2013 [176]	RCT	Simvastatin and atorvastatin	28	10 months	• Decrease in sputum inflammatory markers (total inflammatory cell counts, neutrophils, leptins) • Lower rates of exacerbations • No difference in pulmonary function test parameters (FEV_1, FEF) * All COPD patients in the study were ex-smokers
	Bartziokas et al., 2011 [177]	Retrospective study	Atorvastatin, simvastatin, fluvastatin, lovastatin, rosuvastatin	245	1 year	• No significant different in short and long-term survival after initial hospitalization with COPD exacerbation • Patients with statins had a significantly lower number of exacerbation, less severe exacerbation and prolonged time to next exacerbation leading to better quality of life compared to control
	Neukamm et al., 2015 [178]	RCT	Rosuvastatin (10mg/day)	99	12 weeks	• No significant difference in endothelium-dependent vascular or pulmonary function • Reduction in systemic inflammation (IL-6, hsCRP)

	Mroz et al., 2015 [179]	RCT	Atorvastatin (40mg/day)	18	12 weeks	• Significant reduction in sputum neutrophil counts, $CD45^+$ cell expression in lung biopsies, health related quality of life questionnaire scores, serum hs-CRP levels and key genes involved in inflammatory processes, immune response and leukocyte activation in lung tissues. • No difference in six-minute walk distance
	Maneechotesuwan et al., 2015 [180]	RCT	Simvastatin (20mg/day)	26	4 weeks	• Simvastatin reverses the Th-17/IL-10 (anti-inflammatory and pro-inflammatory) imbalance in the airways • Reduced sputum macrophage counts but not neutrophils • Improved CAT symptom scores • No difference in lung function (FEV_1, FVC or lung resistance)
Acute Respiratory Distress Syndrome/ Acute Lung Injury	McAuley et al., 2014 [181]	RCT	Simvastatin (80mg/day)	540	28 days	• No significant difference in number of ventilator-free days, days free of non-pulmonary organ failure or mortality at 28 days.
	Truwit et al., 2014 [182] and Dinglas et al., 2016 [183]	RCT	Rosuvastatin (10 or 20mg/day)	745	Varies	• No improvement in clinical outcomes in terms of 60-day in-hospital mortality and mean ventilator free days • Rosuvastatin contributed to hepatic and renal organ dysfunction
	Kor et al., 2009 [184]	Retrospective study	Any statins	178	Not reported	• No evidence for protection against pulmonary or non-pulmonary organ dysfunction • No improvement in clinical outcomes with respect to ventilator free days, ICU mortality and hospital mortality
	O'Neal et al., 2011 [185]	Prospective cohort study	Statins and aspirin	575	Not reported	• Prehospital use of statin found to be protective against sepsis or acute lung injury/ acute respiratory distress syndrome which may be potentiated by aspirin in use • No difference found with in-hospital mortalities

	Shyamsundar et al., 2009 [186]	RCT	Simvastatin (40 or 80 mg/day)	30	4 days	• Study performed in human model of inflammatory lung injury with healthy subjects inhaling a dose of lipopolysaccharide (50μg) • Pretreatment with simvastatin reduces inflammation by decreasing neutrophilia, myeloperoxidase, matrix metalloproteinases 7,8 and 9, TNFα, CRP in bronchoalveolar lavage.
	Craig et al., 2011 [187]	RCT	Simvastatin (80mg/day)	60	14 days	• Simvastatin decrease IL-8 in bronchoalveolar lavage by 2.5 folds and showed improvement in non-pulmonary organ dysfunction • No significant differences in oxygenation, respiratory mechanics and mortality
Pulmonary arterial hypertension (PAH)	Wilkins et al., 2008 [188]	RCT	Simvastatin (80mg/day)	42	6 months	• Transient and significant reduction in right ventricular hypertrophy and N-terminal pro-B-type natriuretic peptide but not sustained over 12 months
	Zeng et al., 2012 [189]	RCT	Atorvastatin (10mg/day)	220	24 weeks	• No beneficial effect was observed • No significant difference observed between groups in 6-minute walk distance, pulmonary vascular resistance, cardiac output and proportion of patients who improved, remained stable or showed deterioration.
	Kawut et al., 2011 [190]	RCT	Simvastatin (40mg/day)	65	6 months	• No significant different in 6-minute walk distance or biomarkers of endothelial dysfunction or injury
	Barreto et al., 2008 [191]	RCT	Rosuvastatin (10mg/day)	60	6 months	• Lower P-selectin levels, which is crucial in inflammation and thrombosis in PAH • No significant changes in other markers (tissue-plasminogen activator and von Willebrand factor antigen
	Liu et al., 2013 [192]	RCT	Atorvastatin (20mg/day)	68	6 months	• Increase in migration and adhesion activity of endothelial progenitor cells • Reduction in pulmonary artery pressure
	Moosavi et al., 2013 [193]	RCT	Atorvastatin (40mg/day)	45	6 months	• No significant difference in PAH patients with COPD (systolic pulmonary arterial hypertension,

						cardiac output, right ventricular size, CRP, 6-minute walk distance test and spirometry)
	Reed et al., 2011 [194]	Retrospective study	Atorvastatin, simvastatin	112	Not reported	• Study performed in patients with secondary PAH to COPD • In patients with COPD, significant reduction in pulmonary arterial pressure and pulmonary artery pressure was observed • No difference in pulmonary vascular resistance

Table 2: *Clinical studies performed in the past 10 years using oral statins to treat chronic inflammatory lung disease. ACT: asthma control test, ACQ: Asthma Control Questionnaire, AQLQ: Asthma Quality of Life Questionnaire, CAT: COPD Assessment Test, COPD: Chronic Obstructive Pulmonary Disease, FEV1: forced expiratory volume in the first second, FVC: forced vital capacity, $FEF_{25-75\%}$: forced expiratory flow at 25-75% of the pulmonary volume, hsCRP: high-sensitivity C-reactive protein, IFNγ: interferon γ, IL: interleukin, LT: leukotriene, MPO: myeloperoxidase, PEF: peak expiratory flow rate (FVC), RCT: randomised controlled trial, TNFα: tumour necrosis factor α*