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Key Words: Statins, geranylgeranyl transferase inhibitors, farnesy transferase inhibtors, Rho GTPase, asthma, chronic obstructive pulmonary disease, fibrosis, cancer,

Abbreviation List

15-epi-Lipoxin A_4: 15-epi-LXA_4
3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase: HMGCR
3-Hydroxy-3-Methylglutaryl-Coenzyme A: HMGCoA
Acute Lung Injury: ALI
Acute Lymphoblastic leukemia: ALL
Acute Respiratory Distress Syndrome: ARDS
Air Force Coronary Atherosclerosis Prevention Study: AFCAPS
Airway Hyperreactivity: AHR
Angiotensin Converting Enzyme: ACE
Angiotensin Receptor Blocker: ARB
Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lower Arm: ASCOT-LLA
N-acetylcysteine: NAC
Asthma Control Questionnaire: ACQ

Asymmetric Dimethylarginine: ADMA
Atrial Fibrillation: AF
Bronchial Epithelial: BE
Bronchoalveolar Lavage Fluid: BALF
Cardiovascular: CV
Cell Division Cycle-42: Cdc42
Chemokine (C-C motif) ligand: CCL
Cholesterol and Recurrent Events: CARE
Chronic Myeloid Leukemia: CML
Chronic Obstructive Pulmonary Disease: COPD
Cigarette Smoke: CS
C-jun NH2-Terminal Kinase: JNK
Collaborative Atorvastatin Diabetes Study: CARDS
Coronary Artery Disease: CAD
C- reactive protein: CRP
Cystic Fibrosis: CF
Emergency Department: ED
Endoplasmic Reticulum: ER
Epithelial Mesenchymal Transition: EMT
Extracellular Matrix: ECM
Farensyltrasferase: FT
Farnesyl Diphosphate: FDP
Farnesyl pyrophosphate: FPP
Farnesyltransferase: FTase
Farnesyltransferase Inhibitors: FTIs
Geranylgeranylpyrophosphate: GGPP
Geranyl Pyrophosphate: GPP
Gerenylgeranyltransferase: GGTase
Gerenylgeranyltransferase Inhibitors: GGTIs
GTPase Activating Proteins: GAPs
Guanine Dissociation Inhibitors: GDIs
Guanine Nucleotide Exchange Factors: GEFs
Guanosine Triphosphatase: GTPase
Hepatocellular Carcinoma Cells: HCC cells
Human Fetal Lung Fibroblast: HFL-1
Human Microvascular Endothelial Cells: HMEC-1
Hydroxycholesterol: HC
Hyperimmunoglobulinemia D Syndrome: HIDS
Inhaled Corticosteroid: ICS
Inter-Cellular Adhesion Molecule: ICAM
Interleukin: IL
Interstitial Lung Disease: ILD
Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin: JUPITER
Leukotriene B4: LTB4
Lipopolysaccharide: LPS
Long-Term Intervention with Pravastatin in Ischemic Disease: LIPID
Low-Density-Lipoprotein: LDL

Lymphocyte Function-Associated Antigen 1: LFA1
Matrix Metalloproteinase: MMP
Mevalonate: MVA
Mevalonate Kinase: MVAK
Multiple Myeloma: MM
Myocardial Infarction: MI
Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering: MIRACL
Nitric Oxide Synthase: NOS
Nitric Oxide: NO
Odds Ratio: OR
Ovalbumin: OVA
Pancreatic Carcinoma Cells: PCCs
Particulate Matter: PM
Peroxisome Proliferator Activated Receptor: PPAR
Pravastatin or Atorvastatin Evaluation and Infection Therapy: PROVE IT
Prenyltransferase: PTase
Prostaglandin E_2: PGE_2
Reversal of Atherosclerosis with Aggressive Lipid lowering: REVERSAL
Rho GTPase and Rho kinase: ROCK
Spontaneously Hypertensive: SH
Statin-Induced Lung Injury: SILI
T-acute Lymphoblastic Leukemia: T-ALL
Texas Coronary Atherosclerosis Prevention Study: TexCAPS
T-helper: Th
Thymic Stromal Lymphopoietin: TSLP
Toll-like Receptor: TLR
Transforming Growth Factor: TGF
Vascular Endothelia Growth Factor: VEGF
Vascular Endothelial: VE
West of Scotland Coronary Prevention Study: WOSCOPS

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Abstract

The cholesterol biosynthesis pathway, also known as the mevalonate (MVA) pathway, is an essential cellular pathway that is involved in diverse cell functions. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGCR) is the rate-limiting step in cholesterol biosynthesis and catalyzes the conversion of HMG-CoA to MVA.

Given its role in cholesterol and isoprenoid biosynthesis, the regulation of HMGCR has been intensely investigated. Because all cells require a steady supply of MVA, both the sterol (i.e. cholesterol) and non-sterol (i.e. isoprenoid) products of MVA metabolism exert coordinated feedback regulation on HMGCR through different mechanisms. The proper functioning of HMGCR as the proximal enzyme in the MVA pathway is essential under both normal physiologic conditions and in many diseases given its role in cell cycle pathways and cell proliferation, cholesterol biosynthesis and metabolism, cell cytoskeletal dynamics and stability, cell membrane structure and fluidity, mitochondrial function, proliferation, and cell fate.

The blockbuster statin drugs ('statins') directly bind to and inhibit HMGCR, and their use for the past thirty years has revolutionized the treatment of hypercholesterolemia and cardiovascular diseases, in particular coronary heart disease. Initially thought to exert their effects through cholesterol reduction, recent evidence indicates that statins also have pleiotropic immunomodulatory properties independent of cholesterol lowering.

In this review we will focus on the therapeutic applications and mechanisms involved in the MVA cascade including Rho GTPase and Rho kinase (ROCK) signaling, statin inhibition of HMGCR, geranylgeranyltransferase (GGTase) inhibition, and farnesyltransferase (FTase) inhibition in cardiovascular disease, pulmonary diseases (e.g. asthma and chronic obstructive pulmonary disease (COPD), and cancer.

Introduction

Triacylglycerols (16%), phospholipids (30%), cholesterol (14%), cholesteryl esters (36%) and unesterified long chain fatty acids (4%) form the major component of plasma lipids. Cholesterol was extracted from gallstones for the first time (cholestrine: solid bile) in ancient Greece (Endo, 2010), yet the molecular formula of cholesterol was first established only in 1888.

Cholesterol and cholesteryl esters are major constituents of plasma lipids which are also widely distributed in all cells of the body especially in nervous tissue (Vance, 2012). Cholesterol is a major component of the cell plasma membrane and plasma lipoprotein structure. Cholesterol has significant effects on membrane fluidity and membrane ultrastructure, and its unique structure is necessary for steroid biosynthesis (Simons & Vaz, 2004).

Cellular cholesterol content is tightly regulated despite wide fluctuations in extracellular serum concentrations (Simons & Ikonen, 2000). 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGCR) is the rate-limiting enzyme in cholesterol biosynthesis, it catalyzes the conversion of HMG-CoA to MVA, and is ubiquitously expressed in all cells (Goldstein & Brown, 1984, 1990).

The MVA pathway in humans is indispensable for *de novo* synthesis of cholesterol and other molecules essential for many cellular functions (Goldstein & Brown, 1990). The cholesterol molecule consists of 27 carbons, which is synthesized in 30 enzymatic reactions [with all of the carbon atoms originally derived from acetate] (Gaylor, 2002; Goldstein & Brown, 1990; Kovacs, Olivier, & Krisans, 2002). MVA itself is synthesized in an irreversible step from the HMG-CoA and is then further metabolized to the isoprenoids farnesyl diphosphate, a.k.a. farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), precursors for a number of important metabolites including the sterols, dolichols, ubiquinones (Coenzyme Q), isoprenoids, and carotenoids. These molecules are required for membrane formation (cholesterol), protein N-glycosylation (dolichols), mitochondrial

electron transport chain function (ubiquinone), protein-cell membrane anchoring (isoprenoids), and free radical scavengers (carotenoids) (Goldstein & Brown, 1990).

A schematic of the cholesterol biosynthesis pathway is shown in Figure 1. Upstream of cholesterol in the MVA pathway, FPP and GGPP are substrates for the post-translational modification (a.k.a. isoprenylation) of proteins including the Ras and Rho family GTPases (i.e. monomeric, small G proteins), which play a role in numerous cellular mechanisms (Goldstein & Brown, 1990; Swanson & Hohl, 2006).

The MVA pathway and in particular cholesterol biosynthesis have been extensively studied and found to be associated with several diseases such as hypercholesterolemia, coronary artery disease, and stroke. HMGCR is the most important and proximal enzyme in this pathway, and serves as the rate-limiting step in cholesterol biosynthesis (Goldstein & Brown, 1984, 1990). It is one of the most highly regulated enzymes known and is located in the endoplasmic reticulum (Goldstein & Brown, 1990).

The human HMGCR is composed of 888 amino acids (339 membrane-associated and 548 soluble catalytic residues) (Liscum, et al., 1985). Several studies have confirmed that both membrane and catalytic domains are highly conserved in different species (Luskey, 1988).

HMGCR plays a central role in cholesterol biosynthesis regulation and is regulated at different levels (Zammit & Easom, 1987) including HMGCR mRNA synthesis (Osborne, Goldstein, & Brown, 1985), mRNA translation (Panini, Schnitzer-Polokoff, Spencer, & Sinensky, 1989), HMGCR protein degradation (Gil, Faust, Chin, Goldstein, & Brown, 1985), and HMGCR enzyme activity (Alberts, et al., 1980b) via complex hormonal regulation (Simonet & Ness, 1988).

Cholesterol itself inhibits HMGCR gene expression via negative feedback mechanisms (Goldstein & Brown, 1990). Membrane fluidity of the endoplasmic reticulum also regulates HMGCR activity (Goldstein & Brown, 1990). HMGCR activity may also be regulated via phosphorylation

(inactive form) or dephosphorylation (active form) mechanisms which depend on the action of protein kinases (Goldstein & Brown, 1990).

A certain class of drugs, namely the statins, is capable of inhibiting the synthesis of endogenous cholesterol via competitive inhibition of HMGCR. Statins were originally discovered as *Penicillium citrinum*-derived metabolites with extremely potent inhibitory properties against HMGCR (Endo, Hasumi, & Negishi, 1985; Endo, Kuroda, & Tsujita, 1976). From this discovery, lovastatin was developed and used to reduce endogenous cholesterol synthesis serving as a valuable pharmacologic treatment for patients with hypercholesterolemia (Alberts, 1988b) (Montecucco, Quercioli, Mirabelli-Badenier, Viviani, & Mach, 2012; Raper, Kolansky, & Cuchel, 2012; Shepherd, et al., 1995a; Q. Zhou & Liao, 2009).

Over the past decade it has become evident that the statins also exhibit immunomodulatory,, anti-inflammatory, (Greenwood, Steinman, & Zamvil, 2006; Steffens & Mach, 2004) and neuroprotective (Greenwood, et al., 2006; Kivipelto, Solomon, & Winblad, 2005) effects.

Statins encompass a complex group of compounds, which differ from each other in their chemical structure, physiochemical and pharmacokinetic properties despite having similar biological activity. Statins can occur naturally as fermentation products of microorganisms (lovastatin, mevastatin, pravastatin, simvastatin) or obtained by chemical synthesis (atorvastatin, rosuvastatin, pitavastatin, cerivastatin) (Wierzbicki, 2001). Simvastatin and lovastatin active forms exist as the β-hydroxyacid open side chain, which is produced by liver carboxyestrases (CE) (Demierre, Higgins, Gruber, Hawk, & Lippman, 2005). Pravastatin does not require enzymatic conversion/activation because it exists in the active open ring form. Its structure is similar to lovastatin but with different side chain residues (Solomon & Freeman, 2008). Atorvastatin also exists in the active form and CE conversion is not required, however, due to liver extraction mechanisms its bioavailability is low in peripheral tissues (Goldstein & Brown, 1990).

In Figure 2 we have summarized the effects of the statins, GGTase inhibitors (GGTIs), and FTase inhibitors (FTIs) in the MVA cascade.

Pleiotropy of HMGCR Inhibitors (Statins)

The pleiotropy of statins occurs on several levels. First, by inhibiting the canonical target HMGCR, they deplete not only MVA but also the downstream isoprenoids FPP and GGPP, thereby reducing isoprenylation which affects their intracellular localization. Typically, non-isoprenylated GTPases remain cytosolic. Isoprenylated GTPases have the FPP or GGPP lipid attachment that then allows them to anchor in cell membranes. These anchored GTPases are then able to participate in signal transduction. Therefore, inhibiting isoprenylation results in the inactivation of the small GTPases (Rho, Ras, Rac and Cdc24) which are essential in many cellular events (e.g. intracellular signal transduction, and cellular proliferation, inflammation, and motility). Second, statins have off-target or non-canonical effects by directly inhibiting other enzymes such as leukocyte function antigen-1 (LFA-1) (Weitz-Schmidt, 2003; Zeki, Kenyon, & Goldkorn, 2011).

The GTPases play an important role in a variety of other cellular processes such as apoptosis, phagocytosis, vascular trafficking, cellular proliferation and transmigration, cytoskeleton dynamics, recruitment of inflammatory cells, and cell cycle regulation among other events(Zeki, et al., 2011)[Greenwood 2006]. Thus, by depleting the pool of available isoprenoids, statins indirectly alter GTPase function and thereby affect a multitude of cellular processes dependent on GTPase signaling.

Inhibitory effects of statins on cholesterol and isoprenoids affects endothelial nitric oxide synthase (NOS), a critical enzyme in the physiological and pathophysiological responses of the vascular endothelium (Mihos, Salas, & Santana, 2010) (Figure 2). Also, statins decrease the levels of inflammatory biomarkers such as C-reactive protein (CRP) and the transcription factor nuclear factor kappa-beta (NF-κB) indicating their role in inflammatory responses. Statins also exhibit pleiotropic

effects by inhibiting peroxisome proliferator activated receptor (PPAR) (i.e. functional inhibition) (Yano, et al., 2007; Q. Zhou & Liao, 2010).

In macrophages, statin treatment increases the transcriptional activity of PPARγ, inhibits lipopolysaccharide (LPS) induced tumor necrosis factor-alpha (*TNFα*) and monocyte chemoattractant protein-1 (*MCP-1*) mRNA expression, and represses the transcriptional activity of activated protein-1 (AP-1) through PPARα and PPARγ (Yano, et al., 2007; Q. Zhou & Liao, 2010). Statins also stabilize atherosclerotic plaques through activation of PPARγ (Yano, et al., 2007; Q. Zhou & Liao, 2010). Other pleiotropic effects include: inhibition of platelet aggregation; decrease in thromboxane A2 biosynthesis; reduction of migration and proliferation and increase of apoptosis in the vascular smooth muscle cells (Yano, et al., 2007; Q. Zhou & Liao, 2010).

Interestingly, statins also have effects on immunomodulation, the normalization of sympathetic neural outflow, and decrease the activation of the blood coagulating cascade (Mihos, et al., 2010). In macrophages, statins reduce cholesterol accumulation, MCP-1, and matrix metalloproteinase (MMP) secretion along with cell proliferation and activity (Q. Zhou & Liao, 2010).

The pleiotropic effects of statins may also be affected by their lipophilicity, half-life and potency (Q. Zhou & Liao, 2010). For example, the lipophilic statins such as simvastatin and fluvastatin can enter cells by passive diffusion, whereas the hydrophobic statins such as pravastatin and rosuvastatin cannot enter by passive diffusion, and instead require cell membrane protein transporters. Thus, the chemical structure of the different statins and their distinct physiochemical properties can influence their pleiotropic properties. Given these pleiotropic effects and the varied cellular mechanisms that are affected, statins have the potential to affect many disorders beyond just cardiovascular diseases.

CARDIOVASCULAR DISEASE

The Mevalonte Pathway, Statins, and Cardiovascular Disease

Dysregulated cholesterol metabolism is implicated as a significant risk factor for the development of atherosclerosis. Atherosclerotic narrowing of blood vessels leads to end-organ dysfunction, including the heart, kidneys, limbs, and brain. Furthermore, atherosclerotic plaque rupture leads to acute vessel thrombosis with end-organ mal-perfusion and infarction.

HMGCR inhibition using statins mitigates dyslipidemia and in turn improves cardiovascular (CV) outcomes in patients at risk not only through the prevention of atherosclerotic plaque formation, but also by stabilizing existing plaques. Many large scale multi-center trials have demonstrated the effectiveness of these compounds in reducing vascular event rates and in improving survival (Pedersen, et al., 2000; "Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)," 1994).

Statins were first discovered as secondary metabolites of yeast (Alberts, 1988a; Alberts, et al., 1980a). Subsequent clinical studies of these compounds for the treatment of dyslipidemia were quite encouraging with respect to improving human lipid profiles. Consequently, these compounds were then developed for the treatment of dyslipidemia as an important risk factor for the development of atherosclerosis and coronary artery disease (CAD) ("Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)," 1994; Shepherd, et al., 1995b).

Patients treated with statins were also found to have fewer non-fatal myocardial infarctions (MIs) and fewer strokes. This phenomenon was described in several large randomized control trials, such as the Cholesterol and Recurrent Events (CARE) trial (Sacks, et al., 1996), the Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) trial ("Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group," 1998), and

the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) (Downs, et al., 1998).

CRP is also a biomarker of vascular and systemic inflammation, and is an independent predictor of cardiovascular events (Ridker, et al., 2005). As demonstrated in the Pravastatin or Atorvastatin Evaluation and Infection Therapy (PROVE IT) study, patients who were taking statins and had a significant reduction in CRP had a significant improvement in event-free survival(Cannon, et al., 2004; Ridker, et al., 2005). The Reversal of Atherosclerosis with Aggressive Lipid lowering (REVERSAL) trial showed that aggressive treatment with atorvastatin improved lipid profiles and CRP levels compared to pravastatin, suggesting that this is not just a class effect, and may be dose related(Nissen, et al., 2004).

It is important to note that there is significant heterogeneity in the structure and function of the various compounds in the statin class, and this translates into non-uniform effects in cell signaling as well as clinical outcomes (Arnaboldi & Corsini, 2010; Hilgendorff, et al., 2003). Despite this apparent heterogeneity, further head-to-head comparisons in clinical studies are required to confirm these observations.

The dose effect of statins has also been extensively studied, and a meta-analysis of these major studies has confirmed that “intensive” statin therapy is more efficacious than “moderate” therapy (Cannon, Steinberg, Murphy, Mega, & Braunwald, 2006). Specifically in the context of acute coronary syndromes, the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) trial demonstrated a significant reduction in early recurrent ischemic events in patients receiving high dose atorvastatin (Schwartz, et al., 2001).

Due to the success of statins in secondary prevention, the use of statins for primary prevention in high-risk patients has also been studied. For example, The West of Scotland Coronary Prevention Study (WOSCOPS) study showed that men who had hyperlipidemia but no history of MI had a significant

reduction in cholesterol levels and less non-fatal MI or death from CAD with pravastatin administration (Shepherd, et al., 1995b). Further subgroup analysis of this study and the CARE study revealed that treated patients had a significant reduction in CV events compared to placebo despite having similar lipid profiles, suggesting a mechanism of action independent of cholesterol or low density lipoprotein (LDL) reduction(Sacks, et al., 1996; Shepherd, et al., 1995b).

Supporting this idea, the Anglo-Scandinavian Cardiac Outcomes Trial-lipid lower arm (ASCOT-LLA) demonstrated the protective effect of low dose atorvastatin in hypertensive, non-dyslipidemic patients (Sever, et al., 2003). The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial took it a step further, examining healthy individuals without hyperlipidemia but with elevated CRP levels. They found a significant reduction in the composite end-point of MI, stroke, arterial revascularization, hospitalization for unstable angina, or death from cardiovascular causes (Ridker, et al., 2008b).

These more recent studies, and especially the JUPITER trial, support the hypothesis that statins have lipid-independent effects and properties that mitigate systemic and endovascular inflammation.

Statin Pharmacotherapy in Cardiovascular Health and Diabetes

The beneficial effects of statins outlined above have been studied specifically in diabetics, and similar observations have been made in this patient population as well. For example, the Collaborative Atorvastatin Diabetes Study (CARDS) showed that low dose atorvastatin was safe and efficacious in reducing cardiovascular events in type 2 diabetics without high LDL cholesterol.

However, in the JUPITER trial, it was found that diabetes was diagnosed in 27% more patients receiving rosuvastatin than those receiving placebo. Despite this, patients in the JUPITER trial still experienced a significant reduction in cardiovascular events. A subsequent meta-analysis of WPSCOPS, HPS, LIPID, ASCOT, JUPITER and CORONA, totaling 57,593 patients suggested that

there was a small increase in diabetes risk with statin therapy (RR 1.13), but also that there was significant heterogeneity in these trials, making firm conclusions difficult (Rajpathak, et al., 2009).

Given these controversial results, dispensing warnings have been added to statin medications to inform patients of the potential risk. Most experts agree that at present, the health benefits of statins in patients with CV disease far outweigh these potential risks, and statins are still recommended for patients with CV risk factors and clear evidence of atherosclerosis.

Putative Mechanisms of Statin Pharmacotherapy in Cardiovascular Disease

The beneficial effects of statins beyond lipid-lowering are thought to occur through effects on the endothelium, vascular smooth muscle cells, and the myocardium itself. Atherosclerosis is a complex process that involves chronic low-grade vascular inflammation and lipid accumulation in the vessel wall. Therefore the effect of statins on lipid profile will positively affect lipid accumulation in the atherosclerotic plaque.

However, statins have been found to have anti-inflammatory properties through inhibition of the transcription factor NF-κB, a key mediator of inflammatory gene expression (Thurberg & Collins, 1998). NF-κB is also known as a key mediator of atherosclerosis (De Martin, Hoeth, Hofer-Warbinek, & Schmid, 2000). Beyond this mechanism, statins also affect other pathways such as nitric oxide metabolism, peroxisome proliferator-activated receptor (PPAR) and angiotensin signaling, small G-protein function, and adrenergic signaling in addition to direct immunomodulation, reviewed elsewhere (Jasinska, Owczarek, & Orszulak-Michalak, 2007)).

These factors culminate in the broad positive effects of statins on CV disorders where dyslipidemia *per se* may not be the only pathogenic event mediating disease outcomes.

Statins and Heart Failure

Heart failure patients who receive a statin have improved survival compared to those not on a statin (Horwich, MacLellan, & Fonarow, 2004). However, in a subsequent randomized controlled trial named CORONA (Controlled Rosuvastatin Multinational Study in Heart Failure) that investigated elderly patients with systolic heart failure taking rosuvastatin versus placebo, there was no difference in the composite end-point of death from cardiovascular causes, non-fatal MI or non-fatal stroke, despite improvements in CRP levels and LDL cholesterol compared to placebo. However, rosuvastatin reduced the number of cardiovascular-related hospitalizations.(Kjekshus, et al., 2007).

Therefore, despite the basic science data suggesting that statins provide a direct myocardial benefit (Dechend, et al., 2001; Y. Liao, et al., 2008; J. Liu, Shen, & Wu, 2008; Pliquett, Cornish, Peuler, & Zucker, 2003), these effects have not been realized clinically in patients with heart failure.

Statins and Atrial Fibrillation

There is substantial evidence supporting a link between inflammation and atrial fibrillation (AF) (Boos, Anderson, & Lip, 2006; Engelmann & Svendsen, 2005). A meta-analysis of randomized control trials on AF and statin therapy suggested a significant decline in the risk of AF in patients with sinus rhythm but with a history of previous AF, in patients undergoing heart surgery, or in patients after acute coronary syndrome (Fauchier, et al., 2008). However, this beneficial effect was not confirmed in a second contemporaneous meta-analysis of the PROVE IT-TIMI22 study and the A to Z trial (McLean, et al., 2008). On the other hand, and specifically in the cardiac surgical population, a very large meta-analysis of 54 reports encompassing over 90,000 patients demonstrated a clear reduction in peri-operative mortality, AF, stroke, ICU stay, and hospital stay (Kuhn, et al., 2013). It appears that statins may have a protective effect in some subpopulations and risk strata of AF, but not in all patients with AF.

New Developments in Mevalonate Pathway Pharmacotherapy in Heart Diseases

Given the success of statins in the treatment of cardiovascular diseases and their application to other disease, additional compounds that interfere with the MVA signaling pathway are being investigated. These include the GGTase inhibitors, FTIs, and squalene synthase inhibitors. These compounds have largely been studied in the context of cancer therapeutics with essentially only experimental studies on their cardiovascular effects.

For example, the FTI Manumycin A was found to prevent atherosclerosis development and reduce oxidative stress in apolipoprotein E-deficient mice (Sugita, Sugita, & Kaneki, 2007). This compound also inhibited the formation of cardiac allograft vasculopathy in a small animal model of cardiac transplantation (W. Stein, et al., 2011).

Individuals with the genetic disease Hutchinson-Gilford progeria syndrome have premature cardiovascular disease as a part of the illness. The FTI tipifarnib (R115777, Zarnestra) was shown to diminish both the onset and late progression of cardiovascular disease in a mouse model of this syndrome (Capell, et al., 2008) (Figure 2).

Whether these findings can be extrapolated to the general population at risk for cardiovascular disease remains to be determined. Finally, the squalene synthase inhibitor lapaquistat acetate has been studied in several trials. Despite reductions in LDL cholesterol and CRP, there were concerns over liver toxicity and therefore development and testing has been largely suspended (E. A. Stein, et al., 2011).

This remains an area open for further investigations, including potential combination therapies utilizing multiple pharmacologic inhibitors of the MVA pathway, perhaps in combination using lower doses.

CANCER

The Mevalonate Pathway and Cancer

The MVA pathway has been implicated in different aspects of tumorigenesis, as statins (being inhibitors of HMGCR) display novel anti-cancer capabilities (Thurnher, Nussbaumer, & Gruenbacher, 2012). Statins exert anti-proliferative, anti-angiogenic, pro-apoptotic, and anti-metastatic action on cancer cells via inhibition of both isoprenoid (e.g. FPP, GGPP) and cholesterol synthesis in the MVA pathway.

There is therefore considerable interest in modulating the MVA pathway in order to prevent and treat different types of cancers(Demierre, et al., 2005; Gazzerro, et al., 2011; Hindler, Cleeland, Rivera, & Collard, 2006; Slawinska & Kandefer-Szerszen, 2008; Swanson & Hohl, 2006). Some experts have even argued that HMGCR is a candidate 'metabolic oncogene' (Clendening, et al., 2010), given the role of HMGCR and MA pathway in cellular transformation (Clendening & Penn, 2012).

Various *in vitro* and *in vivo* studies have revealed the cytostatic and cytotoxic properties of statins on cancer cells. Cytostatic properties of statins are due to both impaired cholesterol synthesis and inhibition of isoprenoid production (Ghavami, Yeganeh, et al., 2012; Lewis, Holstein, & Hohl, 2005). The metabolites MVA and GGPP are able to fully overcome the anti-mitotic effect of statins (Seeger, Wallwiener, & Mueck, 2003; Soma, Corsini, & Paoletti, 1992). Moreover, lovastatin and simvastatin are able to alter the expression of various proteins regulating cell cycle (p21 and p27) and to arrest cancer cells in the G_1/S phases (Jakobisiak, Bruno, Skierski, & Darzynkiewicz, 1991; Koyuturk, Ersoz, & Altiok, 2004; Rao, Lowe, Herliczek, & Keyomarsi, 1998).

Statins (cerivastatin and lovastatin) also interfere with Ras- and Rho-dependent cell proliferation by inhibiting protein farnesylation (Bouterfa, et al., 2000; Denoyelle, et al., 2001). Immunohistochemical studies have revealed statin effects on karyokinesis and cytokinesis as well as chromosomal aberrations leading to the inhibition of cell growth and subsequent apoptosis (Hindler, et al., 2006; Lamprecht, et al., 1999).

Cholesterol, one of the key end-products of the MVA pathway is indispensable for the formation of blood vessels. Statins prevent angiogenesis by down-regulating pro-angiogenic vascular endothelial growth factor (VEGF), inhibiting endothelial cell proliferation, and blocking cell adhesion to extracellular matrix (ECM) (Feleszko, et al., 1999; Frick, et al., 2003; Nubel, Dippold, Kleinert, Kaina, & Fritz, 2004; Schaefer, et al., 2004; Weis, Heeschen, Glassford, & Cooke, 2002).

The ability of statins to induce apoptosis in cancer cells has been widely studied and several insights into the mechanism of action have been revealed. There are different models of promoting apoptosis in cancer cells depending on the cell type.

In multiple myeloma (MM) and acute lymphoblastic leukemia (ALL), statins (cerivastatin and lovastatin) have been shown to activate the mitochondrial pathway of apoptosis by activating caspase-3, caspase-8, and caspase-9 (Cafforio, Dammacco, Gernone, & Silvestris, 2005; I. K. Wang, Lin-Shiau, & Lin, 2000; W. W. Wong, Dimitroulakos, Minden, & Penn, 2002). The simvastatin-mediated induction of apoptosis in breast cancer cells correlates to the activation of pro-apoptotic Bax and the down-regulation of anti-apoptotic *Bcl-2* gene expression (Spampanato, et al., 2012).

In addition, suppression of NF-κB transcriptional activity is responsible for the pro-apoptotic effect of simvastatin in chronic myeloid leukemia (CML) (Ahn, Sethi, & Aggarwal, 2007). More detailed analyses have revealed that inactivation of NF-κB augments the PI3/Akt pathway, which results in enhanced sensitivity of lung and breast cancer cells to apoptosis (Denoyelle, et al., 2003; Hwang, et al., 2011).

Simvastatin can also induce apoptosis via involvement of c-jun NH_2-terminal kinase (JNK) in breast cancer cells offering a new approach of targeting the JNK signaling pathway for breast cancer treatment (Gopalan, Yu, Sanders, & Kline, 2013; Koyuturk, Ersoz, & Altiok, 2007). This raises the possibility that statins may serve as an important adjunctive treatment for certain types of cancers, including epithelial-based cancers such as breast cancer.

A fundamental feature of cancer cells is their ability to promote angiogenesis in order to increase their tumor size and achieve metastatic spread. As a result, malignant cancers are characterized by substantial secretion of MMPs. Because of their ECM-degrading activity, and the correlation between elevated levels of their activity and increased tumor metastasis, MMPs were primarily believed to facilitate tumor cell metastasis (Chang & Werb, 2001).

Of note, recent studies indicate that statins can prevent metastasis formation. Lovastatin, fluvastatin, and simvastatin can reduce the expression of MMPs (Lev, Gilburd, Lahat, & Shoenfeld, 2002; Luan, Chase, & Newby, 2003). Essential to the process of metastasis is the ability to alter vascular permeability and integrity. Statins take part in the induction of the vascular endothelial (VE)-cadherin expression in order to prevent intra- and extra-vasation of primary tumors (Duncan, El-Sohemy, & Archer, 2004; Hindler, et al., 2006; J. Zhang, et al., 2013). Additionally, statins alter cytoskeleton organization following modulation of adhesion, motility, and proteolysis in order to prevent metastasis (Collisson, et al., 2003; Farina, Bublik, Alonso, & Gomez, 2002).

Statins also exert anti-inflammatory action in the vicinity of the tumors in order to mitigate the host immune response. Lovastatin, simvastatin, and mevastatin are able to block lymphocyte function-associated antigen 1 (LFA-1), which activates migration of T-cells (Weitz-Schmidt, et al., 2001).

Another anti-inflammatory reaction of statins is the inhibition of NF-κB, which is essential for the synthesis of many cytokines and adhesion molecules necessary for the inflammatory response (Hilgendorff, et al., 2003).

An Overview of the Small Rho GTPase Proteins and Cancers

The Rho GTPase family belongs to the Ras super family of proteins that are conserved and widely expressed in different tissues and in mammalian cell lines (Foster, et al., 1996; Marks & Kwiatkowski, 1996). This family currently consists of three subfamilies, Rho (RhoA, RhoB, and

RhoC), Rac (Rac1, Rac2, and Rac3) and Cell Division Cycle-42(Cdc42) (CDC42Hs and G25K) (Boureux, Vignal, Faure, & Fort, 2007). However, the best-characterized family members are RhoA, Rac1, and Cdc42.

Activation of Rac has been shown to induce actin polymerization to form lamellipodia (broad web-like extensions), while Cdc42 activation stimulates the polymerization of actin to filopodia or micro-spikes (long and thin extensions) (Lamarche, et al., 1996). In contrast, Rho regulates bundling of actin filaments into stress fibers and the formation of focal adhesion complexes (Mackay, Esch, Furthmayr, & Hall, 1997). Furthermore, Rho appears to inhibit myosin phosphatase through the action of Rho kinase, activated by GTP (Kimura, et al., 1996).

Like the Ras oncoproteins, members of the small Rho GTPase family function as molecular switches, cycling between an active GTP-bound form and an inactive GDP-bound form (Oleksy, Opalinski, Derewenda, Derewenda, & Otlewski, 2006). The activity of small Rho GTPase is mainly regulated by Guanine nucleotide Exchange Factors (GEFs), which stimulate the exchange of GDP for GTP to generate the activated form of the enzyme. Rho GTPase activity is down-regulated by GTPase Activating Proteins (GAPs), which stimulate the hydrolysis of GTP to GDP (Oleksy, et al., 2006; Ridley, 2006). In addition, guanine nucleotide dissociation inhibitors (GDIs) block both nucleotide hydrolysis and exchange by interacting with the isoprenylated, GDP-bound form and thus control GDP/GTP cycling. This in turn affects the movement of Rho GTPase between cytosol and cell membranes (Figure 3).

Malignant cells are characterized by deregulated cell cycle control, reduced contact inhibition, loss of matrix-dependent growth regulation, increased cell survival, morphologic alteration, increased motility, and acquisition of invasive and metastatic properties.

Following activation, Rho GTPases bind different effector molecules (including enzymes, adaptor proteins, and actin nucleators) and trigger a signaling cascade to direct cellular responses linked

to cell proliferation, cell cycle, and survival. As they are the key regulators of all of these cellular processes, accumulating evidence from basic and clinical studies supports the concept that signaling pathways downstream of Rho GTPases play critical roles in tumor development and progression (Adnane, Muro-Cacho, Mathews, Sebti, & Munoz-Antonia, 2002; Burbelo, Wellstein, & Pestell, 2004; Kimmelman, et al., 2008; Wells, Ahmed, Masters, & Jones, 2005). Furthermore, Rho GTPases have been shown to regulate the release of pro-angiogenic factors to promote angiogenesis (Hoang, Whelan, & Senger, 2004; Uchida, et al., 2000)

Although activating mutations of the Ras isoform proteins are the most frequent oncogenic mutations in human cancer (Prior, Lewis, & Mattos, 2012), Rho proteins are rarely found mutated in tumors. In contrast, Rho proteins expression and/or activity are frequently altered in a variety of human cancers. For instance RhoA, RhoE, RhoC, RhoF Rac1, Rac2, Rac3, Cdc42 and Wrch2/RhoV are frequently overexpressed in many types of cancers (Faried, et al., 2005; Gomez del Pulgar, Benitah, Valeron, Espina, & Lacal, 2005; Gouw, Reading, Jenson, Lim, & Elenitoba-Johnson, 2005; Islam, et al., 2009; X. R. Li, et al., 2006; Ma, et al., 2010; Varker, Phelps, King, & Williams, 2003; C. Zhang, et al., 2007). Despite these observations, RhoA down-regulation in rare conditions such as human renal cell carcinoma has also been reported (Pu, et al., 2008).

While RhoA, RhoB, and RhoC all have the potential to interact with the same downstream effectors, their effects on cell shape and migratory properties are different. RhoC in particular, is involved in tumor growth and metastasis (Clark, Golub, Lander, & Hynes, 2000; Hakem, et al., 2005). In a colon cancer cell line, Bellovin and colleagues (Bellovin, et al., 2006) showed that *RhoC* expression is increased during epithelial-mesenchymal transition (EMT) and contributes to EMT-induced cell migration, while RhoA is decreased during EMT (Bellovin, et al., 2006).

Unlike RhoA and RhoC, RhoB is often down-regulated in human tumors and its expression significantly inhibits proliferation, migration, and invasion of gastric and lung cancer cells (Sato, et al.,

2007; J. Zhou, et al., 2011). In T-acute lymphoblastic leukemia (T-ALL), however, *RhoB* mRNA expression is up-regulated as compared to primary human T-cells (Bhavsar, Infante, Khwaja, & Ridley, 2013). This suggests that RhoB promotes T-ALL progression, in contrast to its inhibitory role in other cancers.

Genomic analysis of melanoma cells using DNA arrays revealed increased gene expression of *RhoC* in highly metastatic melanoma cells (Clark, et al., 2000). In invasive breast carcinoma cells, *RhoA* expression inhibits whereas *RhoC* enhances cancer cell invasion *in vitro* (Bellovin, et al., 2006; Simpson, Dugan, & Mercurio, 2004). Similarly, Dietrich and colleagues (Dietrich, et al., 2009) investigated the role of RhoA and RhoC in the tumorgenesis of pancreatic carcinoma cells (PCCs). They demonstrated that enhanced expression of *RhoC* results in a striking increase in the migration and invasion of PCCs, whereas over-expression of *RhoA* reduced their migration and invasion.

In human microvascular endothelial cells (HMEC-1), however, RhoA regulates the production of MMP-9, affecting matrix remodeling and enhancing migration of endothelial cells through a 3D-matrix protein gel (Abecassis, Olofsson, Schmid, Zalcman, & Karniguian, 2003). Further, *in vivo* studies using *RhoC*-deficient mice demonstrated that although loss of RhoC does not affect tumor initiation and development, it decreases tumor cell motility and metastasis (Hakem, et al., 2005).

Although it is not fully clear how RhoC is involved in cell invasion, increased *RhoC* expression triggered by the Twist-induced over-expression of microRNA-10b in breast cancer may be the possible cause for the induction of metastases (Ma, Teruya-Feldstein, & Weinberg, 2007). The microRNA miR-139 also interacts with ROCK-2 and reduces its expression in hepatocellular carcinoma cells (HCC cells) (C. C. Wong, et al., 2011). Down-regulation of miR-139 in HCC cells also increases invasiveness of these cells *in vitro* and HCC metastasis *in vivo*.

More recently, targeting syndecan-1 protein (an integral membrane protein) by microRNA miR-10b, can promote breast cancer cell invasiveness via a Rho-GTPase- and E-cadherin-dependent mechanisms (Ibrahim, et al., 2012).

The Rho subtypes play complex and different roles in any given cancer, and certainly have opposing roles depending on cancer and tissue type. This further highlights the interest that Rho GTPases play an important role in cancer and the need to continue our investigations in this realm.

Potential Role of Statins in Cancer Therapy

The statins may be promising preventative and therapeutic anti-cancer agents (Demierre, et al., 2005; S. Singh & P. P. Singh, 2013). As described above, existing experimental data provide compelling evidence that inhibition of the MVA pathway by statins leads to activation of still incompletely understood anti-cancer mechanisms. Specifically, there may be a role for statins in chemoprevention and the management of tumor development and progression (Mo & Elson, 2004). For example, Matzno and colleagues have shown that treatment of rat myoblasts with various statins (atorvastatin, cerivastatin, fluvastatin, simvastatin (at 3.0 μM) or pravastatin (at 3.0 μM) induced apoptotic cell death via depletion of farnesyl-anchored Ras protein from the cell membrane(Matzno, et al., 2005).

However, despite the significant anti-proliferative and tumoricidal effects of statins demonstrated *in vitro* (summarized in Table 1), their anti-tumor effects in animal models are modest. And their efficacy in clinical trials has been under debate and questioned by widely conflicting conclusions (Jukema, Cannon, de Craen, Westendorp, & Trompet, 2012). There could be many reasons for this.

As a general concept, any tissue-specific cancer is not simply a single disease but rather is a heterogeneous group of diseases with different underlying molecular mechanisms. This could

potentially explain why studies of cancer in statin-treated patients do not show consistent protective effects. If any, the beneficial effects of statins for cancer prevention and/or treatment will need to be targeted to the cancer molecular sub-type in question.

The statins may also interact in various ways with anti-tumor drugs, by either potentiating or diminishing their effectiveness. Elucidation of these interactions might change the choice of treatment in cancer patients as some combination therapies might be contraindicated, whereas others might elicit potentiated anti-tumor effects. A critical issue in chemoprevention is the weighing of risks versus benefits, where in one study statin-users had an increased risk for breast cancer (McDougall, et al., 2013), despite a larger body of evidence to the contrary. Thus, further studies are warranted to determine which patient groups or sub-groups could benefit from statin treatment.

Although current knowledge does not support the use of statins as anti-cancer monotherapy, supplementing current standard-of-care anti-cancer agents with statins might enhance the anti-tumor activity of different chemotherapeutic regimens. For example, statins exhibit synergistic anti-tumor effects with cisplatin (Kozar, et al., 2004), 5-fluorouracil (Agarwal, et al., 1999), doxorubicin (Feleszko, et al., 2002; Kozar, et al., 2004) and paclitaxel (Holstein & Hohl, 2001). Further research is needed in this avenue before such options can be recommended.

Supporting these observations, there is emerging evidence that statin use may lower the risk of developing various cancers (Bansal, Undela, D'Cruz, & Schifano, 2012; Bonovas, Filioussi, Flordellis, & Sitaras, 2007; P. P. Singh & S. Singh, 2013; Singh, Singh, Singh, Murad, & Iyer, 2013; Singh, Singh, Singh, Murad, & Sanchez, 2013). A recent large observational study of the Danish population showed that statin use prior to cancer diagnosis reduced cancer-related mortality up to 15% (Nielsen, Nordestgaard, & Bojesen, 2013). However, the role of statins in prolonging the survival of patients diagnosed with cancer still requires further study.

The goal of extending the scope of statins from cholesterol-lowering to cancer prevention and treatment might soon be within reach. In Table 1, some of anti-cancer effects of the various statins are summarized.

The Role of Prenyltransferases in Cancer Therapy

Difficulty in designing small GTPase inhibitors for cancer therapy (Bommi-Reddy & Kaelin, 2010), has prompted a global quest to develop FTIs and geranylgeranyltransferase (GGTase) inhibitors (GGTIs), together known as the prenyltransferase (PTase) inhibitors (PTIs), as potential anti-cancer drugs (Maynor, Scott, Rickert, & Gibbs, 2008).

The enzyme FTase is located in the cell cytosol, and it is one of the three enzymes in the PTase group that catalyzes most isoprenylation reactions. FTase adds a 15-carbon isoprenoid lipid (the farnesyl group) to proteins bearing a CAAX motif and its targets include members of the Ras superfamily of small GTP binding proteins critical to cell cycle progression. GGTase (types I and II) adds a 20-carbon isoprenoid lipid (the geranylgeranyl group) to proteins bearing a CAAX motif (for GGTase I) or a CXC motif (for GGTase II), and its targets include the Rho family GTPases.

Farnesyltransferase Inhibitors in Cancer Therapy

The FTIs comprise a novel class of anti-cancer agents recently developed to inhibit FTase with the downstream effect of preventing the proper functioning of the Ras protein, which is abnormally active in cancer (Heimbrook & Oliff, 1998; Niessner, et al., 2011). These ‘anti-Ras’ agents interrupt the crucial post-translational modification of Ras, reduce Ras function, and thus derive their potential therapeutic benefits in cancer (Niessner, et al., 2011).

Detailed information about the kinetics of the FTase reaction and the physicochemical nature of FTase substrates led to the rational design of FTIs for cancer therapy (Heimbrook & Oliff, 1998; Sebti & Hamilton, 1997). Based on their mechanism of action, existing FTIs can be divided into three categories: FPP competitive inhibitors, CAAX competitive inhibitors, and compounds that inhibit both FPP and CAAX (called "bisubstrate analogues") (Crul, de Klerk, Beijnen, & Schellens, 2001; Wasko, Dudakovic, & Hohl, 2011).

A number of selective inhibitors have been developed in each of these categories and have undergone rigorous *in vitro* and *in vivo* testing (Ohkanda, Blaskovich, Sebti, & Hamilton, 2003; Sebti & Hamilton, 2000); and their anti-tumor outcome has been linked with pleiotropic effects on apoptosis, angiogenesis, and the cell cycle (Appels, et al., 2011).

FPP (a.k.a. farnesyl diphosphoate (FDP)) analogues were the first reported active inhibitors of FTase and were designed based on the farnesyl moiety of the FPP substrate. Animal studies did not support the use of FPP analogues as anti-tumor agents (Rowinsky, Windle, & Von Hoff, 1999). Moreover, the use of FPP inhibitors in chemotherapy raises several concerns about toxic side effects, since FPP is involved in several biological pathways including cholesterol biosynthesis (Patel, et al., 1995).

The development of peptidomimetic inhibitors began upon discovering that FTase activity can be inhibited by a tetrapeptide having the CAAX motif (Goldstein, Brown, Stradley, Reiss, & Gierasch, 1991), which resulted in the development of low molecular-weight CAAX peptidomimetics as a principal strategy for FTase inhibition (Brown, Goldstein, Paris, Burnier, & Marsters, 1992; Duque, Vidal, & Rivas, 2011).

Non-peptidomimetic inhibitors constitute a heterogeneous group of FTIs with different action profiles for each target cell type (Manne, et al., 1995). R115777 and SCH66336 are orally active non-peptidomimetics and have been used in clinical trials (Castaneda, et al., 2011). *In vitro* tests of human

tumor cell lines show increased sensitivity to R115777 (End, et al., 2001; Epling-Burnette & Loughran, 2010). SCH66336 is a tricyclic halogenated compound and inhibits growth of several tumor cell lines as well as K-*ras*-transformed xenografts *in vivo* (Bishop, et al., 1995). BMS-214662 is a new class of non-peptide imidazole FTIs which exhibits complete tumor regression in various tumor xenograft models and has entered clinical trials.

The bisubstrate analogues combine features of FPP analogues and non-peptide CAAX peptidomimetics as FTIs and are highly potent *in vitro* (Manne, et al., 1995). BMS-186511 is a potent inhibitor of Ras signaling in transformed cells with minimal effects on normal cells (Manne, et al., 1995; Yan, et al., 1995).

Geranylgeranyltransferase Inhibitors in Cancer Therapy

Developing inhibitors of GGTase was not initially considered an attractive proposition because more cellular proteins undergo geranylgeranylation than farnesylation, and thus may have more toxic effects *in vivo* (Kazi, et al., 2009). Similar to FTIs, GGTIs have also shown encouraging results *in vitro* and in animal models, yet only one GGTI (GGTI-2418) is being used in a clinical trial (O'Dwyer, Gallagher, Nguyen, Waddell, & Chiorean, 2010). GGTI-298 inhibits N-Ras isoprenylation in various cancer cell lines *in vitro* (Lerner, et al., 1997). It reduces tumor invasiveness and decreases RhoA membrane association in variety of other cancer cell lines (Kusama, et al., 2003). By decreasing protein isoprenylation, GGTI-298 increases apoptosis in osteoclasts by affecting Rab-dependent functions such as intracellular membrane trafficking (Coxon, et al., 2001; Coxon, et al., 2000).

GGTI-298 also induces cell cycle arrest at G0/G1, which is p53-independent and is reproducible in several cancer cell lines (Adnane, Bizouarn, Qian, Hamilton, & Sebti, 1998; Vogt, Sun, Qian, Hamilton, & Sebti, 1997). Additionally, inhibition with a GGTI blocks cell cycle progression from G1

to S phase by increasing phosphorylation of the retinoblastoma protein in lung cancer cells (Sun, Qian, et al., 1999).

GGTI-2154 treatment for three days decreased breast tumor progression in MMTV-*v*-Ha-Ras transgenic mice (Sun, et al., 2003). In a mouse model of lung cancer, GGTI-297 or GGTI-2154 treatment reduced cancer development by 40 to 60%, respectively (Sun, Blaskovich, et al., 1999).

Taken together, these *in vitro* and animal studies demonstrate the efficacy of both GGTIs and GGTase conditional knockouts and thus support their clinical development as anti-cancer agents.

PULMONARY DISEASE

The Mevalonate Pathway and Statins in Pulmonary Disease

Pertubations in cholesterol and isoprenoid metabolism, i.e. changes to important MVA pathway metabolites affect cellular immune responses, tissue injury, chronic disease development and the manifestation of inflammatory systemic disorders (Greenwood, et al., 2006; Steinman, 2006).

A known deficiency in the human enzyme mevalonate kinase (MVAK), the first committed enzyme of cholesterol biosynthesis, produces two auto-inflammatory syndromes, mevalonate aciduria and hyperimmunoglobulinemia D syndrome (HIDS). Mevalonate aciduria manifests as failure to thrive, psychomotor retardation, cerebellar ataxia, visual impairment, dysmorphic features and recurrent febrile crises with organomegaly, arthralgias, and skin rashes. HIDS manifests as periodic fevers and some neurologic abnormalities across a spectrum, high immunoglobulin D and autoimmune-like phenomenon (Haas & Hoffmann, 2006).

Extreme depletion of the isoprenoids and cholesterol are thought to be the underlying cause, and recent evidence indicates upregulation of NACHT, LRR and PYD domains-containing protein 3 (NALP3) pointing to inflammasome-mediated mechanisms (Pontillo, Paoluzzi, & Crovella, 2010). A balance likely exists between extreme depletion and an overabundance of sterol and isoprenoid

metabolites, both of which may lead to disturbances in normal cellular function resulting in organ dysfunction, chronic illness, and inflammation.

Given the above 'natural' experiment of human MVAK deficiency, and given that many lung diseases are chronic inflammatory conditions, the following two questions arise: (1) *What role does MVA metabolism play in the pathogenesis of lung diseases?, and (2) How can MVA metabolism be modulated to mitigate these diseases?* The sections that follow will attempt to answer the above questions critically by reviewing and interconnecting what we have learned from the existing body of literature.

By conservative estimates from the Centers for Disease Control, 32 million Americans take statins, yet relatively little is known about their effects on lungs, pulmonary physiology health and disease. Due to the complexity of the MVA pathway and off-target effects of statins, investigators across disciplines have interrogated components of the MVA cascade in the laboratory, and examined the role of statins as a therapeutic agent. In pulmonary medicine, basic investigations, epidemiologic studies, and clinical trials have been conducted to address these questions.

In this part of the review we will summarize aspects of this literature and offer ideas for future directions. We will focus on the airway diseases asthma and COPD given that the majority of work has been done in these areas. However, a growing body of literature also exists for the following lung and related diseases which we will not review here: pneumonia, acute lung injury/acute respiratory distress syndrome (ALI/ARDS), pulmonary hypertension, pulmonary fibrosis, cystic fibrosis, lung transplant and rejection, and sepsis. Of note, lung cancer is discussed in previous parts of this review.

The Mevalonate Pathway in Pulmonary Health and Disease

The MVA cascade, its isoprenoid intermediates, and downstream cholesterol biosynthesis are essential for basic cell and healthy organ function. The isprenoids FPP and GGPP are critical

intermediates in the MVA cascade that modulate a diverse litany of cellular functions. They function as lipid adducts that covalently bind the small monomeric GTPases via the action of the prenyltransferases, allowing them to anchor in cell membranes for signal transduction (J. K. Liao, 2002; McTaggart, 2006; Perez-Sala, 2007).

These isoprenylated GTPases such as Ras, Rho, Rac, and Cdc42 participate in diverse immune functions (Greenwood, et al., 2006) (e.g. antigen presentation and processing, leukocyte migration, cytokine production, immune cell adhesion, etc.), the cell cycle, cytoskeletal dynamics, cell proliferation, endothelial and epithelial barrier integrity, redox balance, and inflammation (Table 2).

Lung resident cell function, including endothelial barrier integrity (W. Chen, Pendyala, Natarajan, Garcia, & Jacobson, 2008; Jacobson, et al., 2005), smooth muscle cell proliferation (Takeda, et al., 2006; Vigano, et al., 1995), extracellular matrix deposition (Schaafsma, et al., 2011), epithelial cell cytokine production (Iwata, et al., 2012; Murphy, et al., 2008; Sakoda, et al., 2006; W. Wang, et al., 2011; Zeki, Thai, Kenyon, & Wu, 2012), and cell fate phenomena such as endoplasmic reticulum (ER) stress, autophagy, and apoptosis (J. C. Chen, Wu, Huang, & Lin, 2008; Ghavami, et al., 2010; Ghavami, et al., 2011; Zeki, Franzi, Last, & Kenyon, 2009; J. Zhang, et al., 2013) depend on metabolites of the MVA pathway.

Cholesterol transport or efflux (rather than the absolute concentration of blood cholesterol) may be important for the proper development of lung alveoli, and abnormalities in cholesterol metabolism/transport along with Toll-like receptor (TLR)-4 activation can lead to emphysema even in the absence of cigarette smoke exposure (Goldklang, et al., 2012). Cholesterol metabolites also play a role in epithelial cell differentiation. Cholesterol sulfate accumulates in epithelial cells due to increased activity of cholesterol sulfotransferase during pathological epithelial cell squamous metaplasia (Rearick, Hesterberg, & Jetten, 1987). Human bronchial myocyte proliferation is mediated by the MVA pathway isoprenoids and the Rho GTPases in particular (Takeda, et al., 2006; Vigano, et al., 1995).

Inflammatory cells involved in lung and systemic inflammation including eosinophils (Adachi, et al., 2001), neutrophils (Dunzendorfer, et al., 1997), macrophages (W. Wang, Song, Wang, Chen, & Yan, 2013), mast cells (Kagami, et al., 2008), T-cells (Ghittoni, et al., 2005; Samson, et al., 2005; Shibata, et al., 2002; Shimada, Park, & Daida, 2006; Yamashita, et al., 1999) and dendritic cells (Yilmaz, et al., 2006) are all affected by HMGCR activity and MVA pathway metabolites and/or GTPases. Even given this limited sampling of a larger body of scientific work, this evidence collectively indicates a foundational role for the MVA pathway in respiratory health and disease.

Rho GTPase in Respiratory Disease

Because *Rho* is expressed in all cells and is involved in numerous cellular pathways, it is a mechanism that is highly relevant to lung health and disease. The Rho family GTPases are ubiquitous molecular switches mediating a wide array of biological events (Greenwood, et al., 2006; Ridley, 2001; Wettschureck & Offermanns, 2002). This family of monomeric GTPases (a.k.a. G-proteins) has at least 20 members with many regulators and effector molecules, the most heavily studied being RhoA, Cdc42, and Rac1.

The small GTPase Rho has three isoforms, RhoA, RhoB, and RhoC (Ridley, 2001; Wheeler & Ridley, 2004); these are hydrolases that bind GTP/GDP and hydrolyze GTP to GDP to carry out their signal transduction functions within cells (Figure 3). The Rho GTPases transduce their signal via the downstream ROCK enzymes which mediate outside-to-inside cell signals. Therefore, pharmacological or biologic interference with this signaling pathway often involves Rho GTPase or ROCK inhibition or both.

Therefore, the Rho GTPase family in all, and RhoA in particular, are involved in many diverse cellular processes relevant to respiratory health and are thus deserving of further study (Etienne-Manneville & Hall, 2002) (Table 2).

Asthma

Rho GTPase has been implicated in a variety of lung specific phenomena that contribute to different pulmonary diseases. The ROCK pathway plays a critical role in asthma and in particular related to airway remodeling and hyperresponsiveness (Schaafsma, Gosens, Zaagsma, Halayko, & Meurs, 2008; Witzenrath, et al., 2008).

In animal models, RhoA modulates bronchial smooth muscle cell contraction under (Interleukin)-13 (IL13) and IL4 T-helper 2 (Th2) stimulation, a mechanism highly relevant to the pathogenesis of allergic asthma (Chiba, Nakazawa, et al., 2009). Rho GTPase mediates actin polymerization in acetylcholine-induced airway smooth muscle contraction (W. Zhang, Du, & Gunst, 2010). By a related mechanism, bronchodilators can suppress Rho activation *ex vivo* and thereby reduce airway muscle contraction (C. Liu, Zuo, & Janssen, 2006), while prednisolone inhibits TNFα- and IL13-induced *RhoA* expression in bronchial smooth muscle cells (Goto, Chiba, Sakai, & Misawa, 2010). Inactivation of RhoA by simvastatin or a geranylgeranyltransferase inhibitor (GGTI) also reduces human airway smooth muscle cell proliferation (Takeda, et al., 2006).

Geranylgeranyltransferase I (GGTase I), which geranylgeranylates Rho family GTPases, also modulates autophagy and apoptosis in human airway smooth muscle cells (Ghavami, Mutawe, et al., 2012). Pharmacological inhibition of GGTase I induces apoptosis and autophagy in these smooth muscle cells. In animal models of allergic asthma, GGTase inhibitors reduce eosinophilia and airway smooth muscle hyperresponsiveness (Chiba, Sato, Hanazaki, Sakai, & Misawa, 2009; Chiba, Sato, & Misawa, 2009a). Because smooth muscle cell hypertrophy and eosinophilic inflammation are central features of asthma, GGTIs may be an important drug to develop for the treatment of asthma.

In rodent models of allergic asthma, specific inhibition of the Rho downstream effector Rho kinase also attenuates eosinophilic inflammation, goblet cell hyperplasia, and airway hyperreactivity (AHR) (Taki, et al., 2007). Interestingly, in obese mice (leptin-deficient (ob/ob) mice), *RhoA*

expression is increased in the airway nasal epithelium and tracheal smooth muscle compared to wild type mice (Ross, Darrah, Hodges, Lang, & Kelley, 2013). Given the role of RhoA in established models of allergic asthma, we predict that Rho mechanisms might mediate increased AHR not only in all asthma, but in particular in the human obese asthmatic.

Eosinophils are the main effector leukocyte in allergic asthma. The eosinophil chemokine eotaxin (of which three types exist: eotaxin-1,-2, and -3) stimulates ROCK activation which is necessary for eosinophil chemotaxis *in vitro* (Adachi, et al., 2001). Human eosinophil motility in a 3-dimentional microenvironment is also regulated by Rho GTPase (Muessel, Scott, Friedl, Bradding, & Wardlaw, 2008). Therefore, Rho signaling has implications for the persistent eosinophilia seen in more severe forms of human asthma (Coleman, et al., 2012) and for the development of new therapies that could target Rho-mediated eosinophilic inflammation.

Such insight from *in vivo*, *ex vivo*, and *in vitro* models highlights a major role of ROCK in asthma and Th2-polarized allergic inflammation (Schaafsma, Bos, Zuidhof, Zaagsma, & Meurs, 2008). Inhibition of this pathway in animal models of asthma mitigates allergic inflammation and AHR, and is thus a promising area for further research in novel therapeutics (Schaafsma, Bos, Zuidhof, Zaagsma, & Meurs, 2006; Schaafsma, Bos, et al., 2008; Taki, et al., 2007; Witzenrath, et al., 2008).

Cigarette Smoke-Induced Lung Injury and COPD

In a mouse model of cigarette smoke (CS)-induced inflammation and apoptosis, CS impairs apoptotic cell clearance via oxidant-mediated activation of the ROCK pathway (Richens, et al., 2009). Rho GTPases are also essential molecules that differentially regulate efferocytosis (the clearance of apoptotic cells), where RhoA and Rho kinase inhibit efferocytosis, and Rac1 and Cdc42 stimulate it (Moon, Lee, Park, Chong, & Kang, 2010; Morimoto, Janssen, Fessler, McPhillips, et al., 2006; Richens, et al., 2009). Rho family GTPases such as Rac1 and Cdc42 also differentially modulate CS-

induced human airway epithelial cell migration relevant to healing and carcinogenesis (L. Zhang, Gallup, Zlock, Finkbeiner, & McNamara, 2013). Thus, varied roles exist for the different Rho family GTPases warranting additional studies into the mechanisms involved in smoking-related diseases such as COPD.

Pulmonary endothelial dysfunction, based on pulmonary arterial relaxation response and vasodilatory eNOS expression, is present in smokers with normal lung function (Duong-Quy, et al., 2011). The mechanism may occur via ROCK inhibition of NO synthesis. Such a mechanism has relevance for the pathogenesis of human COPD and may be a therapeutic opportunity for drugs that inhibit the ROCK pathway in lungs (Fernandes, Henry, & Goldie, 2007).

Future research should focus on delineating the roles of RhoA, Rac1/2, and Cdc42 and Rho kinases in smoke-induced injury before specific therapies can be successfully developed.

The Airway Epithelium and Lung Inflammation

In a model of acute lung inflammation using lipopolysaccharide (LPS), the antioxidant N-acetylcysteine (NAC) inhibited RhoA activity in alveolar macrophages and promoted neutrophil apoptotic cell clearance. A Rho kinase inhibitor mimicked the effects of NAC suggesting that Rho inhibition has a role to play in mitigating acute pulmonary inflammation (Moon, et al., 2010). However, Rho kinase inhibition with Y27632 at high doses (i.e. 100 uM) induces loss of actin stress fibers in human airway epithelial cells leading to epithelial apoptosis (Moore, Marroquin, Gugliotta, Tse, & White, 2004). Rho kinase and possibly Rho GTPase may be regulators of epithelial apoptosis, and whether this has harmful or beneficial effects on disease pathogenesis remains unknown.

Linking apoptosis to inflammation, and apoptotic epithelial cell clearance, the Rho family member protein Rac1 GTPase is necessary for normal apoptotic epithelial cell engulfment by neighboring airway epithelial cells. This is a recently described novel airway epithelial mechanism to clear dead cells and thus mitigate Th2 inflammation in asthma (Juncadella, et al., 2013; Lambrecht &

Hammad, 2013). This suggests that at least in the case of Rac1, its inhibition could have potential adverse consequences in allergic asthma, further adding to the complex and interconnected role that Rho family GTPases play in lung health and disease (Henson & Bratton, 2013).

Also relevant to asthma, rhinovirus infection activates p38-MAPK via membrane lipid rafts and RhoA suggesting that RhoA inhibition could mitigate epithelial cell viral infection (Dumitru, Dreschers, & Gulbins, 2006). Relevant to both innate and adaptive immune responses in primary human small airway epithelial cells, RhoA GTPase is activated by Toll-like receptors (TLR)-2 and -3 leading to Src and NF-kB signaling, where RhoA is required for NF-kB activation (Manukyan, Nalbant, Luxen, Hahn, & Knaus, 2009).

Conversely, in macrophages, inhibition of the Rho family GTPases (RhoA, Cdc42, Rac1) increases TNFα production after LPS exposure indicating a potentially negative role for Rho inhibition (Monick, Powers, Butler, & Hunninghake, 2003). It is probable that Rho GTPase has dual and paradoxical effects depending on the inhibitor dose used and cell type in question, or inhibitor effects on other Rho GTPases such as Rac (Boulter, Estrach, Garcia-Mata, & Feral, 2012; Boulter, et al., 2010).

The Rho GTPase/Rho kinase pathway also modulates *NOS2* expression in human alveolar epithelial cells (A549). In A549 cells, mevastatin increases cytokine-induced activation of the NOS2 promoter in a GGPP-dependent fashion, thereby highlighting the involvement of Rho in NO lung biology (Kraynack, Corey, Elmer, & Kelley, 2002). In *in vitro* models of cystic fibrosis (CF) epithelium, Rho GTPase inhibition with mevastatin restores *NOS2* expression, which is thought to be lacking in human CF and a contributor to severe inflammation (Kreiselmeier, Kraynack, Corey, & Kelley, 2003).

Epithelial Barrier and Healing

The various Rho family GTPases interact in complex ways to maintain barrier function via epithelial tight junctions (Braga & Yap, 2005; Harhaj & Antonetti, 2004; Ivanov, Parkos, & Nusrat, 2010). Maintenance or increase in epithelial cell-cell contact, induced by microtubule depolymerization, depends on ROCK pathway signaling. However, Rac1 GTPase opposes this pathway, where inhibition of Rac1 signaling promotes epithelial barrier function as measured by transepithelial resistance (Lorenowicz, et al., 2007). Conversely, in human airway epithelial cells, the EGF receptor (EGFR) promotes permeability barrier development and function through Rac1 GTPase (Terakado, et al., 2011).

RhoA GTPase and its downstream effector molecule Rho kinase both suppress airway epithelial wound healing via a mechanism involving microtubule depolymerization (Desai, Aryal, Ceacareanu, Hassid, & Waters, 2004), suggesting that Rho inhibition or modulation of some kind could benefit epithelial healing and wound closure relevant to asthma and COPD. Given that the airway epithelium holds center stage in current thinking, this becomes a very important question for the development of novel and airway-targeted therapies.

We speculate that the dose of ROCK inhibitor(s) has opposite effects depending on drug concentration and possibly the cell type studied. In the case of Rho kinase inhibitor Y27632, lower doses (≤ 0.5 uM) promote airway epithelial wound healing, whereas higher doses (≥ 10 uM) do not have this benefit and may achieve the opposite (Desai, et al., 2004). Such contradictory results may be due to differences in inhibitor dose and type, experimental design and/or epithelial cell type, however, it may also be due to the complex crosstalk between all the Rho GTPases and the effect of RhoGDI1 protein, a dissociation inhibitor that keeps Rho in the cytosol and in the inactive state(Boulter, et al., 2010).

Pulmonary Hypertension

Downstream of RhoA GTPase is the Rho kinase signaling pathway, and these work in concert to execute cell signal transduction. In pulmonary hypertension, this signaling cascade plays an important role in disease pathogenesis. Thus, the ROCK inhibitors have been proposed as a potential treatment for pulmonary hypertension (Duong-Quy, Bei, Liu, & Dinh-Xuan, 2013; Oka, Fagan, Jones, & McMurtry, 2008).

Several different mechanisms in pulmonary hypertension are interconnected to Rho signaling, of which we highlight only a few. In mice, protein kinase G (PKG-I) deficiency causes pulmonary hypertension through activation of the ROCK pathway to induce vasoconstriction and pulmonary vascular remodeling (Zhao, et al., 2012). In primary cultured pulmonary artery smooth muscle cells as a model of pulmonary vascular remodeling, ROCK signaling mediates endothelin-1-induced *MMP-2* expression (M. Li, Li, & Sun, 2008). In murine pulmonary artery smooth muscle cells, endothelin-1 also acts via Rho kinase to alter pH homeostasis leading to pulmonary arterial hypertension (Undem, Rios, Maylor, & Shimoda, 2012).

In pulmonary artery fibroblasts, hypoxia induces Rac1-p38 MAPK-dependent proliferation and mitogen release. Low-dose fluvastatin (1 uM) inhibits hypoxia-induced adventitial fibroblast proliferation and mitogen release. The Rac1 guanine exchange factor inhibitor NSC-23766 mimicked the observed beneficial statin effect (Carlin, et al., 2012; Carlin, Peacock, & Welsh, 2007). Given this statin-sensitive mechanism, fluvastatin or Rac1 inhibitors may have therapeutic potential in pulmonary hypertension.

In human idiopathic pulmonary hypertension, ROCK activities are increased along with increased RhoA serotonylation, and this pathway is involved in pulmonary artery smooth muscle cell contraction and proliferation (Guilluy, et al., 2009). Both inhibitors of ROCK attenuate the prolonged vasoconstriction and vascular remodeling seen in pulmonary hypertension.

Pulmonary Fibrosis

In pulmonary fibrosis, fibroblast proliferation/turn-over involves cell cycle protein cyclin D1 and is over-expressed in the lungs of patients with idiopathic pulmonary fibrosis (IPF). This dysregulation in IPF occurs by a RhoA-dependent mechanism that mediates lung fibroblast proliferation (Watts, Cottrell, Hoban, & Spiteri, 2006).

Hyperoxia also induces RhoA activation in lung fibroblasts and mediates collagen synthesis/deposition. In oxygen-induced lung fibrosis, the ROCK pathway mediates myofibroblast transformation and collagen synthesis (Ni, Dong, Han, Kondrikov, & Su, 2013). In a mouse model of bleomycin-induced lung fibrosis, Rho kinase inhibition with fasudil attenuates these fibrotic changes (Jiang, et al., 2012).

Rho signaling also mediates profibrotic pathways involving connective tissue growth factor (CTGF) and transforming growth factor-beta (TGFβ) in human lung fibroblasts, a process inhibited by simvastatin (Watts & Spiteri, 2004). This in turn has therapeutic implications not only for Rho inhibition but also for statins in the treatment of fibrotic lung diseases.

Endothelial Barrier Integrity

Human pulmonary artery endothelial cell barrier integrity is modulated by Rho family GTPases RhoA and Rac1 (Birukova, et al., 2004). Specifically, inhibition of RhoA and Rac1 membrane localization by simvastatin enhances endothelial barrier integrity in the lung (W. Chen, et al., 2008; Jacobson, 2009). In an earlier study by the same group, simvastatin improved barrier protection but increased the amount of GTP-bound Rac (Jacobson, et al., 2004). In neither study did the authors evaluate both GTP-binding status and membrane/cytosol location, or GTPase activity.

These findings may not be contradictory if under certain circumstances statin can increase the cytosolic fraction of GTP-bound Rac via effects on guanine dissociation inhibitors (GDIs), guanine

nucleotide exchange factors (GEFs), and GTPase activating proteins (GAPs), the proteins that co-regulate GTPase function and intracellular location (Boulter, et al., 2010; Turner, Zhuang, Zhang, Boss, & Pilz, 2008).

Thus, it may be that both over-activation and inhibition (Lu, et al., 2011) of these GTPases play important roles in the pathogenesis of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and/or severe pneumonia (Jacobson, et al., 2005). The challenge is the proper accounting of GTPase location, activity, and induction of downstream signals that confirms true Rho activation.

The Statins as Therapeutic Agents in Pulmonary Diseases

Asthma – the basic science

The first study to show that statins have an anti-inflammatory effect in allergic asthma was by McKay *et al.* These authors showed that systemic treatment with simvastatin in ovalbumin (OVA)-allergic BALB/c mice reduced total and eosinophil cell counts, reduced IL4 and IL5 levels in bronchoalveolar lavage fluid (BALF), and attenuated histologic evidence of airway/lung inflammation (McKay, Leung, McInnes, Thomson, & Liew, 2004).

Since then multiple animal studies have confirmed and further expanded these findings using different statins besides simvastatin including pravastatin, atorvastatin, lovastatin, and rosuvastatin (Chiba, Sato, & Misawa, 2009b; C. F. Huang, et al., 2013; Imamura, et al., 2009; D. Y. Kim, Ryu, Lim, Lee, & Ro, 2007; Zeki, et al., 2009; Zhu, et al., 2012).

Statins also inhibit airway smooth muscle cell proliferation (Takeda, et al., 2006; Vigano, et al., 1995) and inducible mitogenic responses to contractile agents (Capra & Rovati, 2013) relevant to airway remodeling. Adverse airway remodeling leads to fixed airflow obstruction in asthma and has no effective current treatment. Therefore, the statins and other inhibitors of the MVA pathway, including

isoprenylation inhibitors (e.g. FTIs and GGTIs (Figure 2)), may be promising agents for the treatment of severe asthma or prevention of irreversible airway remodeling.

Ex vivo studies also indicate the key role that protein isoprenylation plays in mediating LPS-induced contractile responses in human airways (Cazzola, et al., 2011). Simvastatin and the Rho kinase inhibitor Y27632 both abolish airway bronchoconstriction further supporting their potential therapeutic role in treating AHR.

In cultured primary human airway smooth muscle cells and human airway fibroblasts, simvastatin has pro-apoptotic properties. Statins could potentially abolish the smooth muscle cell hypertrophy and hyperplasia that leads to airway remodeling and subsequent chronic airflow obstruction (Ghavami, et al., 2010; Ghavami, et al., 2011). Long term human clinical trials are needed to test this hypothesis in order to assess the clinical impact of these agents.

Of interest, dietary cholesterol enhances OVA-induced eosinophilic inflammation, and independently in the same model pravastatin treatment markedly attenuates OVA-induced allergic inflammation (Yeh & Huang, 2004). The mechanism of this anti-eosinophilic effect is thought to be mediated by the MVA pathway, where MVA+simvastatin co-treatment abolishes the anti-inflammatory effect of simvastatin suggesting that HMGCR, at least partially, mediates allergic inflammation (Zeki, et al., 2009). Statins also inhibit the growth and IgE-dependent histamine release of human lung mast cells in a MVA-dependent fashion (Krauth, et al., 2006).. Given that HMGCR is the rate-limiting step for cholesterol biosynthesis in the MVA pathway, these studies highlight the importance of metabolic pathways in allergic lung disease.

Also, statins inhibit the growth and IgE-dependent histamine release of human lung mast cells in a MVA-dependent fashion (Krauth, et al., 2006). The role of cholesterol and lipoproteins in asthma and other lung diseases, and their effects on pulmonary immune responses is an active area of current

research (Gowdy & Fessler, 2013; Zeki, et al., 2011). Cholesterol and the non-sterol isoprenoids of the MVA pathway also likely alter the function of lung resident cells.

The L-arginine/arginase/nitric oxide synthase (NOS) pathway plays a critical role in both human and murine asthma (Bratt, Zeki, Last, & Kenyon, 2011; Holguin, et al., 2013; Morris, et al., 2004). Increased arginase activity contributes to airway remodeling, depletes L-arginine which reduces local lung levels of nitric oxide (NO), and thereby contributes to AHR and asthma symptoms.

In an acute model of OVA-induced allergic asthma, systemic treatment with simvastatin decreases lung arginase-1 protein expression and enzyme activity, while also mitigating markers of airway remodeling such as goblet cell hyperplasia/metaplasia (Zeki, Bratt, Rabowsky, Last, & Kenyon, 2010). In acute and chronic mouse models of allergic asthma, systemic treatment with simvastatin improves dysfunctional nitric oxide metabolism in bronchial epithelial cells and mouse lungs. It also mitigates allergic inflammation, AHR, and airway remodeling; and reduces asymmetric dimethylarginine (ADMA), oxo-nitrative stress, apoptosis, and epithelial injury (Ahmad, et al., 2011).

Thus, while it is important to develop novel inhibitors of the arginase enzyme, statins may serve as a readily available, but safe alternative to be used in sub-phenotypes of asthma where arginase may be playing a major role in pathogenesis (Holguin, et al., 2013).

Although much work has been done to show statins' anti-Th2 effects in allergic mouse models of asthma, these drugs can also modulate Th1 (Samson, et al., 2006), Th17 (Imamura, et al., 2009; Maneechotesuwan, Ekjiratrakul, Kasetsinsombat, Wongkajornsilp, & Barnes, 2010), and T regulatory cell (Maneechotesuwan, et al., 2010) responses in animal models and cell culture systems relevant to asthma. This immunomodulatory repertoire (Greenwood, et al., 2006) becomes increasingly important in sub-phenotypes of asthma where current therapies are lacking, in particular in severe asthma which is typically corticosteroid-resistant, manifesting neutrophillic- rather than eosinophilic-predominant airway inflammation. Lovastatin in particular has anti-inflammatory and pro-resolving effects in

acutely inflamed lungs and airways. By increasing the generation of the pro-resolving mediator 15-epi-lipoxin A_4 (15-epi-LXA_4), lovastatin decreases human leukocyte-airway mucosal injury (Planaguma, et al., 2010).

Because statins appear to have both systemic and local tissue effects, there may be benefits to administering statins via the inhaled route instead of *or* in addition to the standard oral route. Direct application of simvastatin onto Calu-3 cells (grown under air-liquid interface conditions to induce mucus production) significantly reduces mucus production (Marin, et al., 2013). However, there is also evidence of cytotoxicity with reductions in epithelial barrier integrity and loss of viability(Marin, et al., 2013), indicating that optimal dosing studies are needed before statins can be safely given to humans via the inhaled route.

However, simvastatin given via inhalation and intratracheal (i.t.) instillation using OVA-allergic mice, significantly reduces airway inflammation and eosinophilia, goblet cell hyperplasia, submucosal collagen deposition, while simultaneously reducing airway resistance and improving dynamic lung compliance (L. Xu, et al., 2012). Interestingly, low-dose inhaled (5 mg/mL) and i.t. (2 mg/kg) simvastatin are as potent as dexamethasone (1 mg/kg) given intraperitoneally (i.p.) on reducing BALF total cell numbers and eosinophils.

This raises the possibility that lung- or airway-targeted approaches for statin delivery might be an important area for innovation in the treatment of asthma. Inhaled statins, therefore, have the potential to become a new class of inhaler drugs for the treatment of asthma.

Asthma – the clinical science

Several investigators have called attention to the importance of studying statins in human respiratory diseases (E. Hothersall, McSharry, & Thomson, 2006) and in particular the most common airway diseases asthma (Camoretti-Mercado, 2009; Yuan, et al., 2012; Zeki, et al., 2011) and COPD

(Janda, Park, FitzGerald, Etminan, & Swiston, 2009; Mancini, 2007; Young, Hopkins, & Eaton, 2009b). Several large epidemiological studies have revealed a protective statin effect on exacerbations and lung function (Alexeeff, Litonjua, Sparrow, Vokonas, & Schwartz, 2007; Huang, et al., 2011; Lokhandwala, West-Strum, Banahan, Bentley, & Yang, 2012).

Most recently, two large epidemiologic observational studies suggest that long-term use of statins has benefits in asthma. In the first study by Tse *et al.*, statin use and exacerbations were assessed over a 24-month period in 14,566 statin users. They reported that statin exposure was associated with decreased odds of having asthma-related emergency department (ED) visits (OR 0.64, 95% CI 0.53-0.77, $p<0.0001$), and two or more dispensing of oral corticosteroids (OR 0.90, 95% CI 0.81-0.99, $p=0.04$) (Tse, Li, et al., 2013).

In the second study by Tse *et al.*, statin use and asthma-related ED visits and/or hospitalizations were assessed over a 12-month period and stratified by ICS use (3,747 ICS users and 2,905 non-ICS users). They found similar results to their first study. Among ICS users, statin use was significantly associated with decreased odds of asthma-related ED visits (OR=0.77, 95% CI 0.64-0.94, $p=0.008$), but not with asthma-related hospitalizations (OR=1.09, 95% CI 0.92-1.30, $p=0.31$). No significant associations were found among non-ICS users (Tse, Charland, et al., 2013).

These studies indicate that long-term statin use may have benefit even in ICS users, and could reduce acute exacerbations of asthma. Despite a number of large studies with positive outcomes, most of these have been observational and a call for additional prospective studies remains (Silva, Couto, Delgado, & Moreira, 2012).

To date, several randomized clinical trials in patients with mild or moderate allergic asthma have failed to show a consistent and significant clinical benefit to statins (Braganza, et al., 2011; Cowan, Cowan, Palmay, Williamson, & Taylor, 2010; E. J. Hothersall, et al., 2008; Maneechotesuwan, et al., 2010; Menzies, et al., 2007). However, these studies had several limitations including short

duration (≤ 4 to 8 weeks maximum), varied statin drug choice and doses, and lack of robust hard clinical endpoints such as acute exacerbations, emergency department (ED) visits and hospitalizations. .

Interestingly, some of the trials did consistently show that statins (atorvastatin, simvastatin) exert anti-inflammatory effects by reducing sputum inflammatory cell counts (eosinophils, macrophages) and other markers of inflammation (e.g. leukotriene B_4 (LTB_4)). In two studies, adding simvastatin to an inhaled corticosteroid (ICS) further reduces sputum %eosinophil counts beyond the ICS alone (sputum % eosinophils: 9.9% vs. 22.7% (p=0.047) (Cowan, et al., 2010), and for Δ sputum eosinophils (%): ~ -5 vs. -10% (p=0.02) (Maneechotesuwan, et al., 2010).These statin effects on human airway eosinophilia were also observed in several murine models of asthma (Imamura, et al., 2009; D. Y. Kim, et al., 2007; McKay, et al., 2004; Zeki, et al., 2009).

One possible mechanism for this anti-eosinophilic statin effect is the inhibition of eosinophil adhesion to inter-cellular adhesion molecule (ICAM)-1 under conditions of physiologic shear stress (Robinson, et al., 2009). This in turn would reduce bronchial influx of eosinophils during acute allergic airway inflammation and provides a unique mechanism different from that of ICS.

In two of these clinical trials, statin use resulted in some mild clinical improvements, but the short duration of these studies (≥ 4 and/or 8 weeks) could not answer the question of whether statins could have greater clinical benefits over a longer period of use. Cowan *et al.* showed that simvastatin in atopic asthmatics with sputum eosinophilia had no corticosteroid-sparing effects. However, in asthmatics tapered off of their inhaled fluticasone, simvastatin use improved symptom scores (Asthma Control Questionnaire (ACQ)), increased FEV1, and reduced sputum eosinophils compared to placebo (Cowan, et al., 2010). Braganza *et al.* showed that atorvastatin treatment over 4 and 8 weeks in smokers with mild-to-moderate asthma did not alter lung function (i.e. morning peak expiratory flow), but did improve asthma quality of life (Braganza, et al., 2011).

These studies suggest that there may be a role for statins as an adjunctive therapy in asthma; however, it remains unclear for which sub-phenotype of asthma statins would work best. Severe asthma has some features similar to COPD including neutrophilic inflammation, fixed airflow obstruction, and steroid resistance. Given what we know about statins and COPD, patients with severe asthma may be a population worthy of further study. Although no clinical trials have been reported in this population, a recent observational study indicates that obese severe asthmatics using statins for a median of one year have better control of asthma symptoms (Zeki, et al., 2013).

COPD – the basic science

As the interest in statins and COPD increased in the clinical arena, a multitude of basic science work emerged to explore mechanisms of action. Both animal models and *in vitro* cell culture work have demonstrated the anti-inflammatory, anti-proliferative, and anti-fibrotic effects of statins and other modulators of the MVA cascade such as the prenyltransferase inhibitors.

Animal models of COPD and emphysema have demonstrated at least a partial benefit to treatment with statins. In a guinea pig model of cigarette smoke-induced COPD, simvastatin (50 mg/kg) was started 3 months after smoke exposure, and continued for another 3 months to complete a 6 month experiment modeling chronic disease. Early (within 4 weeks) effects of simvastatin include reversal of pulmonary arterial hypertension. Simvastatin also ameliorated pulmonary arterial remodeling and emphysema at 6 months, and partially reversed smoke-induced loss of vascular nitric oxide generation. However, statin treatment did not prevent small airway remodeling as measured by airway wall area, collagen deposition, and elastin content (Wright, et al., 2011).

In a rat model of cigarette smoke-induced emphysema and pulmonary hypertension, simvastatin administered orally (5 mg/kg) for 16 weeks ameliorates the development of emphysema, pulmonary hypertension, pulmonary vascular remodeling, and reduces peribronchial and perivascular

inflammation and the induction of MMP-9 activity in lungs (J. H. Lee, et al., 2005). Furthermore in another study using rat alveolar macrophages, simvastatin inhibits cigarette smoke extract-mediated MMP-9 induction by blocking Ras isoprenylation and downstream NF-kB activation (S. E. Kim, et al., 2009).

In mice, systemic treatment with simvastatin (20 ug/200 ul i.p.) for 3 weeks, prevents the development of elastase-induced lung emphysema, reduces neutrophilic inflammation, and promotes alveolar cell proliferation and regeneration (Takahashi, et al., 2008).

Using spontaneously hypertensive (SH) rats, simvastatin (20 mg/kg i.p.) given 1 week pre-treatment before and during 3 days of cigarette smoke exposure prevents smoke-induced leukocyte bronchial influx and bronchial epithelial sloughing (Davis, et al., 2012). This study suggests that statins may have a preventative effect or immune priming effect that pre-conditions a less injurious host response to cigarette smoke, in addition to a possible direct epithelial cytoprotective effect. The clinical implications of this observation is the public health impact statins could have, especially given that COPD is decades in the making.

The statins not only have systemic immune effects, but cell specific phenomena appear to occur as well. One important pathologic process in COPD is the accumulation of apoptotic cells and ineffective efferocytosis, i.e. the phagocytosis of dead cells and debris, or clearance of apoptotic cells that is carried out by macrophages (Krysko, Vandenabeele, Krysko, & Bachert, 2010; Morimoto, Janssen, Fessler, McPhillips, et al., 2006; Morimoto, Janssen, Fessler, Xiao, et al., 2006). As discussed above, Rho GTPases are essential regulators of this response, where statins directly enhance efferocytosis. Lovastatin for example enhances efferocytosis in a MVA- or HMGCR-dependent manner in human primary and alveolar macrophages taken from patients with COPD, and in mouse lungs (Morimoto, Janssen, Fessler, McPhillips, et al., 2006).

Excess mucus production is a key feature of both asthma and COPD. There are many factors that trigger and regulate mucin production in airway epithelial cells ranging from environmental aeroallergens to smoke to infectious agents.

In a MVA-dependent manner, simvastatin attenuates acrolein-induced goblet cell hyperplasia and metaplasia in airways and inhibits the expression of *Muc5AC* at both the mRNA and protein levels (Y. J. Chen, et al., 2010). In an *in vitro* model using Calu-3 epithelial cells grown under air liquid interface conditions, simvastatin treatment for 14 days causes a significant inhibition in mucus production (Marin, et al., 2013). Although these are not primary bronchial epithelial cells, it does confirm prior *in vivo* data from murine models (using various noxious stimuli) which show a reduction in goblet cell metaplasia.

In a related fashion, small airway remodeling is a key hallmark of COPD (Hogg, et al., 2004). In a rat model of cigarette smoke-induced small airway remodeling, simvastatin treatment for 16 weeks attenuates small airway wall thickening, and prevents markers of airway fibrosis (hydroxyproline and collagen deposition). Statin treatment also down-regulates TGFβ1 and CTGF and the SMAD2/3 signaling molecules, while also reducing TNFα levels in BALF (Ou, et al., 2009).

As adverse structural remodeling contributes to chronic airflow obstruction, we speculate that long-term statin treatment could improve lung function and functional capacity in patients with COPD.

Statin effects on the airway epithelium have also been reported. The airway epithelium (or mucosa) is the initial site of contact with environmental insults, and thus is a key modulator of inflammation and remodeling signals in asthma and COPD. The epithelial mucosal immune response and its barrier functions are central to allergic, infectious, and smoke-induced lung diseases. Th2-mediated bronchial inflammation is also important in COPD with features of atopy.

Thymic stromal lymphopoietin (TSLP), a hub cytokine that stimulates Th2 inflammation, is over-produced in viral stimulated bronchial epithelial cells procured from patients with COPD.

Simvastatin can selectively inhibit dsRNA-induced IRF3 activation and production of TSLP and IFNβ in these epithelial cells (Brandelius, et al., 2013). Because viral infections are thought to be one mechanism that induces acute exacerbations in asthma and COPD, statins may serve to mitigate exacerbations by this mechanism.

Several different authors using a mixture of primary epithelial cells and cell lines have shown a beneficial statin effect by reducing the induction of pro-inflammatory cytokines. Although this can apply to many diseases beyond COPD, it remains highly pertinent to the current discussion. The underlying presumption based on clinical studies is that prolonged expression of pro-inflammatory and pro-fibrotic signals mediates chronic airway injury and therefore disease (Barnes, 2006, 2008).

In one of the earliest studies using the KB human epithelial cell line, simvastatin mitigated IL1α-induced *IL6* and *IL8* expression by a MVA- and GGPP-dependent mechanism (Sakoda, et al., 2006). Sakoda *et al.* further showed that simvastatin reduces NF-κB and AP-1 promoter activity and dominant-negative Rac1 also inhibits IL1α-induced NF-κB and AP-1 promoter activity (Sakoda, et al., 2006). These data indicate that Rac1 GTPase mediates inflammatory signals in human epithelial cells.

Using LPS-stimulated human bronchial epithelial cells (BEAS-2B), pitavastatin and pravastatin inhibit *IL6, IL8* and *GM-CSF* mRNA expression and their protein translation in a MVA-dependent manner (Iwata, et al., 2012). Atorvastatin inhibits *CRP* expression (Xing, et al., 2011) and LPS-induced *COX-2* expression and subsequent prostaglandin E_2 (PGE_2) production (Wu, et al., 2005) in A549 human alveolar epithelial cells.

Using primary bronchial epithelial cells derived from stable lung allografts, simvastatin inhibits pro-inflammatory cytokines important in neutrophilic inflammation and remodeling, namely basal and IL17- and/or TGFβ-induced IL6, IL8, GM-CSF, MMP-2 and MMP-9 (Murphy, et al., 2008). However, while atorvastatin inhibits particulate matter $(PM)_{10}$-induced cytokine production (e.g. IL1β, IL8, GM-

CSF, IL6, TNFα) in human alveolar macrophages, it does not do the same in human bronchial epithelial cells (Sakamoto, et al., 2009).

We speculate that this lack of effect in epithelial cells could be due to the relatively lower statin dose (nanomolar range rather than typical micromolar range used in other studies), different cell type (normal donors rather than donors with COPD), or different mechanisms involving PM_{10}.

However, in another study, statin-users were reported to have reduced expression of Chemokine (C-C motif) ligand *(CCL)* 5 *(CCL5)*, *CCL11*, *IL5*, *IL13* and *IL13RA1* in their nasal mucosal tissue (W. Wang, et al., 2011). Furthermore, PM induces *CCL5*, *CCL11* and *IL13RA1 in vitro* and treatment with a statin reduces their mRNA expression in human primary nasal epithelial cells.

Oxidized-LDL is known to play an important role in impaired surfactant protein metabolism. In a MVA-dependent manner, simvastatin inhibits oxidized-LDL-induced TGFβ1 production in A549 cells by a mechanism that involves the Ras/ERK pathway (Guo, et al., 2012). This suggests a possible link between airway cholesterol metabolism/transport and inflammation.

Interestingly, 25- and 27-hydroxycholesterol (HC) are increased in the airways/lungs of COPD patients and may have a pro-inflammatory role in airway epithelial cells mediating innate immune responses and neutrophilic inflammation (Kikuchi, et al., 2012; Koarai, et al., 2012; Sugiura, et al., 2012). *In vitro* studies using human fetal lung fibroblasts (HFL-1) suggest that 25-HC also promotes fibroblast mediated tissue remodeling, by increasing α-smooth muscle actin, collagen I, MMP-2, MMP-9, and TGFβ1, via a NF-κB signaling mechanism (Ichikawa, et al., 2013). In primary human airway epithelial cells, 25-HC enhances IL6 and IL8 release after stimulation of TLR3 and may therefore potentiate the innate immune response in chronic airway diseases (Koarai, et al., 2012).

Pertinent to both pneumonia and COPD, persistent neutrophilic inflammation incurs severe injury to lungs and airways. In both primary human bronchial epithelial cells and mouse lungs, simvastatin (30 ug/30 uL) suppresses polyinosinic–polycytidylic acid (poly I:C)-induced RANTES

(regulated on activation, normal T cell expressed and secreted) production and neutrophilia (C. S. Lee, et al., 2013). One mechanism of the protective statin effect at the mucosal level involves the pro-resolving mediator 15-epi-lipoxin A_4 (15-epi-LXA_4). Lovastatin decreases acute airway mucosal total and neutrophilic inflammation by increasing the production of 15-epi-LXA_4 *in vivo* (Planaguma, et al., 2010). This suggests that at least one mechanism whereby statins afford mucosal protection is by inducing pro-resolving endogenous mediators during human leukocyte-airway epithelial interactions.

Despite these promising results, statins have the potential to be cytotoxic at higher doses (i.e. > 10 to 20 uM range). Thus, given that the majority of these studies did not account or control for this factor, much of the *in vitro* work mentioned above may be open to criticism. *In vitro* studies in particular should report cell viability assays to determine whether statin treatment is cytotoxic at the pharmacologic dose and treatment duration used to evaluate their outcome of interest.

Therefore, we urge investigators and readers to consider the understudied and potential harmful effects of statins on airway epithelial and other lung resident cells. Detailed studies are therefore needed to hone the optimal experimental conditions using appropriate animal and cell culture models.

COPD – the clinical science

Much interest has been focused recently on COPD with respect to cardiovascular drug effects (Marin, Colombo, Bebawy, Young, & Traini, 2011; Mortensen, et al., 2009; Sheng, Murphy, MacDonald, Schembri, et al., 2012) including statins, because it has long been appreciated that patients with COPD have a higher incidence of cardiovascular disease and atherosclerosis (Rennard, 2005; Sin & Man, 2003). Coronary artery disease, in particular, is prevalent and under-diagnosed in patients with advanced lung diseases including COPD (Reed, et al., 2012).

Risk stratification including odds of death in patients with COPD is improved by the addition of cardiovascular event risk scores to lung function data. This allows for more accurate predictions of

long-term survival in COPD (H. M. Lee, et al., 2012). In addition, elevated plasma CRP, fibrinogen, and leukocyte count is associated with a 2- to 4-fold increased risk of major co-morbidities in COPD including myocardial infarction, heart failure, diabetes, pneumonia, and lung cancer (Thomsen, Dahl, Lange, Vestbo, & Nordestgaard, 2012).

As in asthma, statin use in patients with COPD has been associated with improved clinical outcomes in several epidemiologic studies (Dobler, Wong, & Marks, 2009; Janda, et al., 2009; Young, et al., 2009b). The statins have also garnered special attention in COPD because of their pleiotropic pharmacological effects and potential as anti-inflammatory, anti-remodeling, and anti-cancer agents in COPD (Young, Hopkins, & Eaton, 2009a).

Thus, statins have been associated with three main improvements with respect to COPD: reduction in acute exacerbations, improvement in lung function, and decrease in mortality.

In smokers, ex-smokers and in those with obstructive and restrictive pulmonary deficits who use a statin have a significantly lower decline in lung function as measured by FEV1 (-0.005 ± 0.20 L/yr vs. 0.085 ± 0.17 L/yr, $p < 0.0001$) and FVC (-0.046 ± 0.45 L/yr vs. 0.135 ± 0.32 L/yr, $p < 0.0001$) , compared to their respective controls (Keddissi, et al., 2007). Interestingly, patients with obstructive physiology had a lower incidence of respiratory related urgent care visits favoring statin users (0.12 ± 0.29/patient-years vs. 0.19 ± 0.32/patient-years; $p=0.02$).

In the VA Normative Study, Alexeef *et al.* studied 803 elderly men with chronic bronchitis, asthma or emphysema and also reported a marked reduction in lung function (FEV1, FVC) decline over 10 years even after controlling for known potential confounders and the healthy user effect (Alexeeff, et al., 2007).

In large cohorts of patients with COPD, statin use is associated with less acute exacerbations and hospitalizations (Huang, et al., 2011). The use of statins in patients hospitalized for an acute COPD exacerbation is associated with a lower number of and risk of acute COPD exacerbations after 1 year

follow-up ((HR: 0.656 (95% CI: 0.454-0.946); p=0.024)) (Bartziokas, et al., 2011). In 1,085 subjects (292 on statins and 793 not on statins) as part of the COPDGene Study, statin use is associated with improved lower airway luminal area and with reduced exacerbations over 12 months (0.40±0.94 vs. 0.56±1.14, p=0.03) (Bartziokas, et al., 2011). Similarly, in a retrospective cohort study in patients with COPD exacerbations with 1 year follow-up, statin use is associated with a lower incidence of both acute exacerbations and endotracheal intubations (Blamoun, et al., 2008).

Statin and angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) use is associated with significant reductions in COPD hospitalizations (Mancini, et al., 2006) and mortality in subjects hospitalized for COPD exacerbation (Frost, Petersen, Tollestrup, & Skipper, 2007; Mancini, et al., 2006; Mortensen, et al., 2009). In COPD, statins also protect against cardiovascular events and mortality when done as secondary prevention of known cardiovascular disease (Sheng, Murphy, MacDonald, Schembri, et al., 2012). Statin consumption is also associated with reductions in all-cause mortality in COPD in both primary and secondary prevention of cardiovascular disease (Sheng, Murphy, MacDonald, & Wei, 2012).

In a large retrospective study of 854 patients with COPD exacerbation, statin administration was associated with improved survival, where concomitant use of ICS increased the survival benefit associated with statins (with the following hazard ratios: 0.75 (0.58–0.98) for ICS only; 0.69 (0.36–1.3) for statins only; and 0.39 (0.22–0.67) for combined ICS and statin treatment compared to no such treatment) (Soyseth, Brekke, Smith, & Omland, 2007).

A recent study of 1,687 patients with COPD showed that statin use is associated with a 30% reduction in all-cause mortality over 4 years, independent of prior history of cardiovascular disease or diabetes mellitus (Lawes, et al., 2012). Long-term statin use (>2 years) is associated with a 39% decrease of death in patients with COPD. Impressively, if stratified by the level of systemic inflammation (according to serum C-reactive protein (CRP) levels), statin use was associated a 78%

reduction in mortality if the high sensitivity (hs)-CRP level is >3 mg/L, versus a non-significant 21% reduction in mortality if hsCRP ≤ 3 mg/L (Lahousse, et al., 2013).

Fibrin clots in COPD are denser, more resistant to lysis, and contribute to overall vascular dysfunction, but statins mitigate this COPD pathology (Undas, et al., 2009). This suggests that IL6-mediated systemic inflammation and endothelial dysfunction likely play a role via a confluence of factors involving decrement in lung function, acute exacerbations, and cardiovascular dysfunction all leading to poor outcomes in COPD.

Because statins are known to mitigate cardiovascular events and particularly in patients with high CRP (Ridker, et al., 2008a), they could have benefits in COPD by decreasing inflammation and endovascular dysfunction. However, growing evidence from animal and *in vitro* studies indicate that statins may also have direct effects on lung resident cells (W. Chen, et al., 2008; Davis, et al., 2012; Ghavami, et al., 2010; Ghavami, et al., 2011; Iwata, et al., 2012; M. Li, et al., 2008; Marin, et al., 2013; Murphy, et al., 2008; Schaafsma, et al., 2011; Zeki, et al., 2012).

In another study evaluating cancer in patients with COPD, statin use is associated with a reduced risk of extrapulmonary cancer mortality (HR 0.49; 95% CI 0.24 to 0.99) (van Gestel, et al., 2009). Mortality from COPD and mortality from influenza/pneumonia was reduced in patients who used ≥ 4mg/day of a statin, where the odds ratio (OR) of death was much lower in the COPD group (OR, 0.17; 95% CI, 0.07 to 0.42) than in influenza/pneumonia group (OR, 0.60; 95% CI, 0.44 to 0.81) (Frost, et al., 2007).

The statin benefit also crosses ethnic differences, where a large population-based study in Japan found results similar to the aforementioned studies. Statin consumption was associated with a significant reduction in COPD-related mortality, pneumonia, and all-cause mortality, but no differences in the number of malignancies (Ishida, et al., 2007).

In another Japanese cross-sectional study (853 patients; over age 40 including never smokers, current smokers, and past smokers), statin administration was associated with a 5-fold lower risk of having airflow obstruction (FEV1/FVC <70%) compared to non-users (Bando, et al., 2012). There were no statistically significant changes in smoking status and statin use. This is an important study because it was not done in established COPD. Rather, it was part of a COPD screening study for patients who visited their primary care provider. This suggests that statin use may have a protective mechanism in *preventing* the development of obstructive lung diseases such as COPD.

Similarly, a Taiwanese nationwide retrospective nested case-control study of 14,316 COPD patients showed that statin use was associated with at least a 30% reduced risk of acute exacerbations, and this risk reduction was dose-dependent achieving greater benefit with higher statin doses (M. T. Wang, et al., 2013). Clinical trials evaluating both the preventative and treatment potential of statins in COPD are therefore warranted.

Systemic inflammation is a major contributing factor in COPD (Agusti & Faner, 2012; Walter, et al., 2008). Emerging evidence indicates that systemic inflammation in COPD, and higher levels of serum CRP in particular, predict worse lung function and higher mortality in COPD providing incremental prognostic information (Mancini, et al., 2006). Systemic inflammation is particularly important given the known interface between cardiovascular disease, endothelial dysfunction, and airway disease. In essence, therapies that mitigate systemic inflammation may in turn help treat COPD. In the case of statins, they are known inhibitors of serum CRP and IL6, are protective of the endothelial system in cardiovascular health, and have extra-hepatic effects including in the lung. However, it remains unknown whether these statin effects on lung health are in sum helpful or harmful. Despite the overwhelming epidemiologic data indicating benefit in COPD, we still require multiple RCTs before we can know with certainty whether statins will be beneficial in COPD.

In the Copenhagen City Heart Study, 1,302 patients with COPD were followed over 8 years, and their CRP levels and COPD outcomes were measured. The authors reported that CRP is a strong and independent predictor of future COPD hospitalizations and death (Dahl, et al., 2007). More recently, systemic inflammation in COPD (as assessed by IL6 measurements over 3 years) is seen as progressive and associated with increased mortality (Hazard Ratio 2.68, 95% CI 0.13-1.84, p = 0.02) and decreased exercise tolerance (Ferrari, et al., 2013).

Although large-scale multi-center RCT data are not yet available, in a small study using a case management approach, COPD patients treated with a statin after 3 months had an approximately 50% reduction in serum CRP (p=0.02) (McDonald, Higgins, Wood, & Gibson, 2013). While statins reduce CRP levels, inhaled corticosteroids (ICS) do not affect CRP in elderly patients with bronchial airflow obstruction (Melbye, et al., 2007). This suggests that an important element in the pathogenesis of obstructive airway diseases is unaffected by ICS, a cornerstone of current standard-of-care therapy.

Thus, understanding the pathogenic role of systemic inflammation in COPD, which is at least partially represented by elevated CRP blood levels, is important given these therapeutic implications. Based on what we have learned about CRP and associated outcomes based on CRP levels (the higher the CRP, the worse the COPD outcome), future studies using statins should stratify treatment based on whether CRP is > or < 3 mg/L.

Because most of the studies evaluating the effects of statins in COPD are observational, the effect is at best correlative, not causal. Even such consistent associations have been challenged by some authors due to inherent biases to observational study designs (Suissa, 2010). In truth, the best answers are derived from multiple different RCTs and ideally from different centers. Although a few RCTs have been published, several ongoing RCTs (www.clinicaltrials.gov) will evaluate important clinical outcomes in COPD and help answer the question: Is there a statin indication in COPD?

Two RCTs in COPD demonstrate a statin advantage with respect to functional capacity and systemic anti-inflammatory effect (T. M. Lee, Chen, Shen, & Chang, 2009; T. M. Lee, Lin, & Chang, 2008). Lee *et al.* showed that pravastatin (40 mg daily) for 6 months not only reduced serum CRP (by 70%), but significantly increased exercise time by 54% ($p<0.0001$) as compared to placebo. In addition, pravastatin-treated patients with a greater decrease in CRP had a significant improvement in exercise time compared to those without a decrease in CRP (T. M. Lee, et al., 2008). In a subsequent trial also using pravastatin (40 mg daily) for 6 months, Lee *et al.* showed that pravastatin significantly improved exercise tolerance, decreased dyspnea, and reduced pulmonary artery pressures in patients with COPD and pulmonary hypertension (T. M. Lee, et al., 2009).

In another short (3 month) RCT using simvastatin (40 mg/day) in patients with COPD, there was no reduction in circulating inflammatory biomarkers (fibrinogen, CRP, TNFα, IL6, MMP-9) and no change in FEV1 or FVC despite a decrease in total cholesterol and LDL (Kaczmarek, et al., 2010). No hard clinical outcomes such as exacerbation or survival were determined in this clinical trial.

Given the different sub-phenotypes of COPD being reported (Agusti & Faner, 2012; Beghe, Verduri, Roca, & Fabbri, 2013; Hurst, et al., 2010), the design of future clinical trials should take this into consideration in order to enroll patients most likely to benefit from a statin.

Potential Pulmonary Adverse Reactions Due to Statins

Despite the diverse and extensive body of literature indicating a benefit to statin use in lung diseases, there is some evidence to suggest potential harm. Statin-induced lung injury (SILI) is a rare but serious condition associated with statin use (L. K. Huang, et al., 2013) as is statin-induced fibrotic non-specific interstitial pneumonia, an interstitial lung disease (ILD) (Lantuejoul, Brambilla, Brambilla, & Devouassoux, 2002).

In the COPDGene cohort, statin use is associated with interstitial lung abnormalities (ILA) in older smokers (age >65) and enhances bleomycin-induced lung inflammation and fibrosis in mice by a mechanism involving NLRP3-inflammasome activation (J. F. Xu, et al., 2012). However, in younger patients (age 45-55) there is a trend towards reduced risk of statin-associated ILA, raising the question of whether age and smoking status interacts with statin use in a manner that gives these paradoxical results (Thannickal & Hagood, 2012; J. F. Xu, et al., 2012).

Also, in at least three prior animal studies, statins prevented or attenuated bleomycin-induced lung fibrosis (J. W. Kim, et al., 2010; Ou, et al., 2008; Schroll, et al., 2013), contrary to the findings by Xu *et al* (J. F. Xu, et al., 2012). Furthermore, subsequent to the study by Xu *et al.*, a large study of 6,665 patients with ILD was conducted and found no association between statin use and the incidence of ILD (Saad, Camus, Suissa, & Ernst, 2013).

These studies indicate that in some rare cases, statins could pose a potential harm to patients. However, for the vast majority of patients, statins given for approved indications are probably safe; where the benefits likely outweigh the potential pulmonary risks.

Summary and Future Directions

In this focused review we have discussed the critical role the MVA cascade and its intermediates play in cardiovascular diseases, cancer and pulmonary diseases. Cholesterol, the isoprenoids and their isoprenylation targets the Ras and Rho family GTPases, all have diverse and complex roles in basic cellular biology and physiology.

In cardiovascular diseases, asthma and COPD alone there is a large body of evidence supporting this, but an emerging literature also indicates the involvement of these pathways in cancers and other lung diseases such as ALI/ARDS, pneumonia, IPF, pulmonary hypertension, etc.

Statins have emerged as pleiotropic drugs with wide-ranging effects beyond cholesterol lowering. In both large epidemiologic studies and basic science experiments, statins have demonstrated a consistent beneficial effect on heart and lung physiology and pathological mechanisms.

We see statins as having not a singular role in a therapeutic armamentarium, but rather as an important adjunctive therapy to current standard-of-care regimens. Statins therefore hold a unique place in the world of emerging and innovative new therapies because of their overwhelming safety profile and potential to treat comorbid conditions, beyond atherosclerosis.

We believe that clinical trials targeting subpopulations of cancer patients, asthma and COPD with the 'right' sub-phenotype will yield fruitful results in the years to come. Ongoing studies should evaluate not only statins and their optimal route of administration, but also combination therapies with Rho and Ras inhibitors, Rho kinase inhibitors (i.e. Y27632) and prenyltransferase inhibitors.

Conflict of Interest

All authors confirm that there is not any conflict of interest with individuals or organizations within three years of initiating the work that could inappropriately influence, or be perceived to influence, the study design or data interpretation.

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Table 1: Potential Anti-Cancer Effects of Statins

Tumor type	Statin	Anti-cancer effect
Anti-proliferative capability		
Breast cancer Bladder cancer	Lovastatin	Inhibition of cell proliferation by arresting cells in the G_1 phase of the cell cycle (Rao, et al., 1998) Downregulation of proliferation-associated proteins (Jakobisiak, et al., 1991)
Breast cancer	Cerivastatin	Inhibition of cell proliferation by arresting cells in the G_1 phase of the cell cycle (Denoyelle, et al., 2001)
Breast cancer Glioma	Simvastatin	Inhibition of cell proliferation by activation of JNK and c-Jun phosphorylation (Koyuturk, et al., 2004)
Glioblastoma multiforme	Lovastatin	Inhibition of cell proliferation by inhibition of Ras farnesylation and interference with actin cytoskeleton (Bouterfa, et al., 2000)
Pro-apoptotic capability		
Multiple myeloma (MM) Lymphoblastic leukemia (LL)	Lovastatin Cerivastatin	Induction of apoptosis by activation of mitochondrial pathway of apoptosis (Cafforio, et al., 2005) (I. K. Wang, et al., 2000)
Breast cancer Chronic myeloid leukemia (CML) Lung cancer	Simvastatin	Induction of apoptosis by activation of proapoptotic Bax and decrease in anti-apoptotic Bcl-2 (W. W. Wong, et al., 2002) (Spampanato, et al., 2012) Induction of apoptosis by activation of c-Jun (Hwang, et al., 2011) (Koyuturk, et al., 2007)
Anti-angiogenic capability		
Murine Lewis lung cancer model Ras-3T3 tumors	Cerivastatin Atorvastatin Lovastatin	Decrease of tumor vascularization by down-regulation of VEGF and inhibition of endothelial cells proliferation (Weis, et al., 2002) (Feleszko, et al., 2002)
Anti-metastatic capability		
Ras-3T3 tumors Melanoma	Lovastatin Fluvastatin Simvastatin Atorvastatin	Reduction of MMPs expression by inhibition of Ras isoprenylation (Lev, et al., 2002) (Luan, et al., 2003) (Collisson, et al., 2003)
Colon cancer	Lovastatin	Inhibition of intra- and extravasation of the primary tumors by downregulation of the endothelial leukocyte adhesion molecule E-selectin (Nubel, et al., 2004)
Breast cancer	Lovastatin	Preventing metastasis by alterations in cytoskeleton organization (Farina, et al., 2002)
Pancreatic cancer Colon cancer	Lovastatin Fluvastatin	Reduction of EGF-mediated liver metastases (Kusama, et al., 2002)
Renal cancer Breast cancer	Lovastatin Fluvastatin	Reduction of lung metastases by decreased phosphorylation of Rac-1 and cytoskeleton reorganization (Farina, et al., 2002; Horiguchi, Sumitomo, Asakuma, Asano, & Hayakawa, 2004)

Table 2: Varied Roles of Rho Signaling in Lung Biology[∞]

Cellular or Tissue Effect[∞]

Cell Growth, Death and Mobility

Cell motility/migration (Birukova, et al., 2012; Muessel, et al., 2008; Xiao, Li, & Liu, 2012).

Cell proliferation (Guilluy, et al., 2009; Takeda, et al., 2006; Vigano, et al., 1995; Watts, et al., 2006).

Cytoskeleton dynamics (Citi, Spadaro, Schneider, Stutz, & Pulimeno, 2011; Fukata, Amano, & Kaibuchi, 2001; Ivanov, et al., 2010; Jacobson, et al., 2004; Kato, Hashikabe, Iwata, Akimoto, & Hattori, 2004; Koch, Benz, Schmidt, Olenik, & Aktories, 1997).

Apoptosis (Ghavami, Mutawe, et al., 2012; Moore, et al., 2004; Shi & Wei, 2007).

Efferocytosis (Moon, et al., 2010; Morimoto, Janssen, Fessler, McPhillips, et al., 2006; Morimoto, Janssen, Fessler, Xiao, et al., 2006; Richens, et al., 2009).

Tumor cell growth and invasion (Agarwal, et al., 1999; Ahn, et al., 2007; J. Chen, et al., 2012; Koyuturk, et al., 2007; J. Zhang, et al., 2013).

Immunity

Antigen presenting/processing (Greenwood, et al., 2006).

Leukocyte transendothelial migration (Muller, 2011).

Inflammation (Chiba, Arima, Sakai, & Misawa, 2008; Greenwood, et al., 2006; Silveira, Dominical, Lazarini, Costa, & Conran, 2013; Zeki, et al., 2009).

Epithelial Biology

Epithelial-neutrophil adhesion (Yagi, et al., 2006)

Ciliogenesis (Pan, You, Huang, & Brody, 2007)

Maintenance of cell-cell contact and tight junctions (Braga & Yap, 2005; Harhaj & Antonetti, 2004; Popoff & Geny, 2009; Xiao, Qin, Ping, & Zuo, 2013).

Epithelial barrier integrity (Braga & Yap, 2005; Citi, et al., 2011).

Epithelial wound closure (Desai, et al., 2004).

Epithelial mucociliary clearance (Seminario-Vidal, et al., 2011).

Alveolar barrier function (DiPaolo & Margulies, 2012; Sawafuji, et al., 2005; Takahashi, et al., 2008).

Fibrosis

Extracellular matrix (Adiguzel, Hou, Sabatini, & Bendeck, 2013; M. Li, et al., 2008; Schaafsma, et al., 2011).

Fibrosis/collagen deposition (Jiang, et al., 2012; Ni, et al., 2013; Watts, et al., 2006; Watts & Spiteri, 2004).

Endothelial Biology

Endothelial barrier function and integrity (Aslam, et al., 2013; Birukov, 2009; Birukova, et al., 2004; Birukova, et al., 2012; Xiao, et al., 2013).

Angiogenesis (Park, et al., 2002)

Eosinophil adhesion to pulmonary endothelium (Sashio, et al., 2012)

Mesenchymal Biology

Smooth muscle cell contraction (Chiba, Nakazawa, et al., 2009; Fukata, et al., 2001; Guilluy, et al., 2009; C. Liu, et al., 2006; W. Zhang, et al., 2010).

Smooth muscle cell proliferation (Takeda, et al., 2006; Vigano, et al., 1995).

Airway smooth muscle cell contraction & bronchial hyperresponsiveness (Chiba, et al., 2008; Chiba, Nakazawa, et al., 2009; Schaafsma, Bos, et al., 2008; Schaafsma, Roscioni, Meurs, & Schmidt, 2008)

Infectious Diseases

Bacterial virulence (Boquet & Lemichez, 2003)

Viral Virulence (Dumitru, et al., 2006).

Respiratory viral infection (Dumitru, et al., 2006; Pastey, Crowe, & Graham, 1999).

Metabolic Signals

Nitric oxide synthase (NOS) activity (Kato, et al., 2004; Kraynack, et al., 2002; Muniyappa, Xu, Ram, & Sowers, 2000).

Redox balance (Antonopoulos, Margaritis, Shirodaria, & Antoniades, 2012; Melo, et al., 2013).

∞ ***This table is not all inclusive, but it does cite the key citable references related to lung pathophysiology.***

Figure Legends

Figure 1. Overview of the cholesterol biosynthesis pathway. (A) Farnesol or related isoperinoids regulate Ras farnesylation and other GTPases like Rho and Ras, resulting in GTPase activation and p53-mediated induction of apoptosis and cell growth regulation. (B) Inhibition of squalene synthase (SQS) decreases raft-associated cholesterol levels, thus attenuates cancer cell proliferation and also induces death of cancer cell. (C) Suppression of cholesterol biosynthesis from lanosterol leads to inhibition of cell cycle progression and also cell differentiation. (D) AEBS ligands are associated with zymosterol and 7-dehydrocholesterol, which can induce cancer cell differentiation and death through the production of reactive oxygen species (ROS) and oxysterols. Suppression of ROS production by antioxidants leads to cell survival through an autophagic process by induction of AEBS ligands. (E) Cholesteryl esters of fatty acids (CEFA) is the product of intracellular cholesterol esterification that is catalyzed by the Acyl-coA:Cholesterol Acyl Transferase (ACAT) using cholesterol and fatty acyl-coenzyme A esters (RCoA). CEFA is the major lipid found in foam cells which plays important role in atherosclerosis. CEFA is also implicated in the stimulation of cancer cell proliferation, invasiveness and mitogenesis.

Figure 2. Effect of different inhibitors on the mevalonate pathway. Statins, bisphosphonates, FTIs and GGTIs are different classes of drugs which have various inhibitory effects on the MVA pathway. Statins block the conversion of HMG-CoA to MVA by suppressing the HMGCR and thereby inhibits Rac geranylgeranylation and Ras farnesylation. Statins also attenuate reactive oxygen species (ROS) derived from NADPH oxidase. Bisphosphonates (BPs) inhibit the IPP isomerase and isoprenoid biosynthesis downstream by targeting FPP synthase and indirectly interfering with protein isoprenylation. FTIs and GGTIs are prenyltransferase inhibitors. The formation of a covalent bond between the isoprenoids FPP and GGPP and the GTPases (e.g. Ras, Rho and Rac) by prenyl

transferases is targeted by FTIs and GGTIs. Other inhibitors such as squalene synthase inhibitors (SQSIs) and oxidosqualene cyclase inhibitors (OSCIs) target the synthesis of the cholesterol precursor squalene and lanosterol synthesis, respectively.

Figure 3. The Rho GTPase molecular switch. When cell is in resting state, Rho GTPases exist mostly in the cytosol, in inactive (GDP-bound) form, in complexes with Rho GDI. Following an activation signal, Rho GTPases are targeted to the membrane by post-translational modification of their COOH termini with lipid moieties (i.e. farnesyl, geranyl-geranyl, palmitoyl and methyl) by geranyl-geranyltransferases (GGTases). This reaction allows the activated small GTPases (in the GTP-bound form) to interact with the cell membranes, where they exert their function.

Cycling between an inactive GDP-bound and an active GTP-bound is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs release guanosine diphosphate (GDP) from Rho GTPases promoting the binding of guanosine triphosphate (GTP) and activation of Rho GTPases. In the GTP-bound form, Rho proteins undergo a conformational change which allows them to interact with effector proteins and initiate a downstream cellular response. GTPase activating protein (GAP) converts GTP-bound form of Rho GTPases to inactive GDP-GTPases by hydrolyzing GTP into GDP and terminate the signal transduction.

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Figure 1

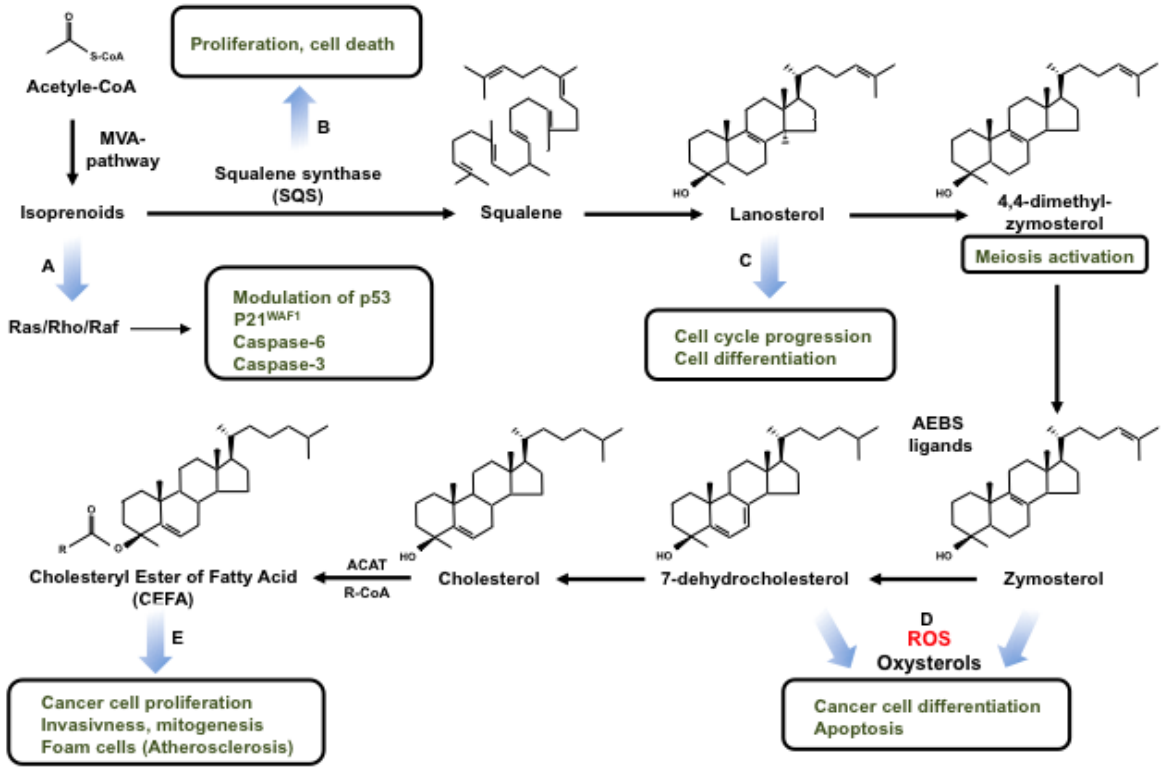

Figure 2

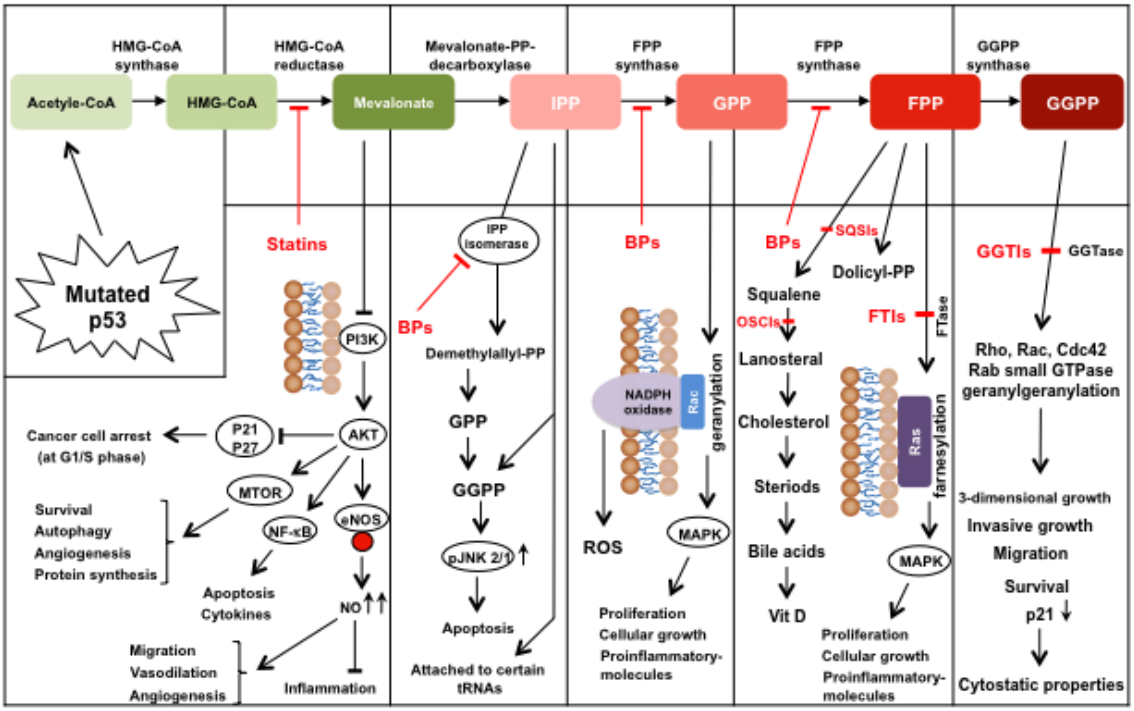

Figure 3