Transversal Document:
Lipid Biology

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1 BACKGROUND

1.1 Smoking-related disease mechanisms

Smoking cigarettes strongly contributes to the development and progression of a number of serious diseases, including cardiovascular disease (CVD) (Office of the Surgeon General 2014, Price 1999, Siasos 2014) and chronic obstructive pulmonary disease (COPD) (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2014, Office of the Surgeon General 2014, Pauwels 2004, Postma 2015).

CVD describes a group of diseases affecting the heart and blood vessels that can be broadly classified into coronary heart disease, cerebrovascular disease, and peripheral vascular disease. Buildup of fatty deposits called atherosclerotic plaques on the inner walls of blood vessels supplying the heart or brain is the common feature shared by all of these diseases, and the resulting vessel blockage with narrowing of the vascular lumen and induction of a hypercoagulable state leads to serious, and at times fatal, events such as myocardial infarction or stroke (Office of the Surgeon General 2010b).

Cigarette smoking is one of the major risk factors for CVD, and is causally linked to the development of CVD contributing to endothelial injury and dysfunction, a pro-atherogenic lipid profile, chronic inflammation, and an abnormally increased tendency toward coagulation. Evidence gathered from *in vitro* and *in vivo* studies, as well as population-based observational studies, has led to the recognition of vascular inflammatory processes as being central to all stages of atherogenesis, from local disturbance of low-level physiological platelet–endothelial interactions to increased pathophysiological interactions with dysfunctional endothelial cells to plaque development and rupture (Libby 2002, Luscher 1993, Ross 1999). In addition, multiple lines of evidence suggest that reactive oxygen species (ROS) are key contributors to molecular signaling pathways underlying the development of atherosclerosis (Harrison 2003, Madamanchi 2005), and play a central role in modifying most of the known main risk factors for CVD, including hypertension, hypercholesterolemia, obesity, and diabetes (Asmat 2015, Ceriello 2004, Keaney 2003, Rodriguez-Porcel 2001).

Healthy endothelium produces a range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation in response to various inflammatory and oxidative stimuli (Münzel 2008). Endothelium is maintained in a quiescent state through the inhibitory signaling of nitric oxide (NO), an important vasodilator (Deanfield 2007).

When endothelium is injured, a host response is evoked that involves a switch from NO-mediated silencing of cellular processes to redox signaling, ultimately resulting in activation of transcription factors such as nuclear factor kappa B (NFκB) and subsequent production of inflammatory mediators including interleukin (IL)-1, IL-8, and tumor necrosis factor alpha (TNF-α) (Mudau 2012, Yang 2006). Endothelial nitric oxide synthase (eNOS), the source of NO under physiological conditions, becomes uncoupled and produces superoxide O_2^-, which can interact with NO, thus reducing its bioavailability, while forming the highly reactive peroxynitrite $ONOO^-$ and adding to the overall oxidant burden (Nedeljkovic 2003, Versari 2009). Activated endothelium expresses surface molecules (e.g., P-selectin and integrins) that attract platelets and

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mediate platelet rolling and adhesion. By releasing vasoactive substances such as adenosine tri- and diphosphate as well as serotonin, platelets can stimulate the formation of NO and prostacyclin (also called prostaglandin I2/PGI2) within endothelial cells, a possible protective mechanism providing platelet inhibition and increased local blood flow at sites of platelet activation. In addition, platelets also can stimulate the formation of the vasoconstrictor peptide endothelin-1 within endothelial cells, and platelet-derived substances, such as thromboxane and serotonin, can activate vascular smooth muscle cells and cause profound vasoconstriction. Under normal physiological conditions, platelet–vessel wall interaction is very low because of an overabundance of protective mechanisms (Luscher 1993).

In disease states, however, endothelial dysfunction increases platelet–vessel wall interaction, leading to platelet activation and the subsequent release of growth factors (e.g., PDGF, TGF-β, EGF, bFGF), chemokines (e.g., CCL5, CXCL4, CXCL5), cytokines (e.g., IL-1β), chemoattractants (e.g., MIP-1α) and coagulation factors (e.g., factor V, factor IX, plasminogen, plasminogen activator inhibitor-1) that in turn promote the recruitment of monocytes to the site of inflammation, platelet aggregation, coagulation, cell survival, and proliferation (Gawaz 2005).

Increased levels of circulating high-density lipoprotein (HDL) are vasculoprotective, not only because of the ability of HDL to remove cholesterol from cells via reverse cholesterol transport, but also by its anti-inflammatory, antithrombotic, and anti-oxidative properties, which include the release of NO (Andrews 2010). Low-density lipoprotein (LDL) markedly reduces endothelium-dependent relaxations to aggregating platelets and serotonin. Even more marked changes in endothelial function occur in atherosclerosis. These functional alterations of platelet–vessel wall interaction may play an important role in the pathogenesis of CVD (Luscher 1993).

Smoking increases the concentrations of oxidized low-density lipoprotein (oxLDL) and other modified blood lipids, and oxidative stress exerted directly or indirectly by cigarette smoke constituents can disturb the physiological low-level platelet–endothelial interaction leading to increased pathophysiological interactions with dysfunctional endothelial cells. There is increasing evidence that HDL may become potentially pro-inflammatory through oxidative modifications of HDL-associated lipids and proteins, such as apolipoprotein (Apo) A1 and paraoxonase-1, and its endothelial-protective properties are markedly altered (Kratzer 2014).

Monocytes that have been attracted to the site of endothelial damage attach to the endothelial surface, transmigrate to the subendothelial space, and differentiate into macrophages, which then begin to secrete pro-inflammatory mediators supporting the establishment of chronic inflammation (Facchinetti 2007, Libby 2012). These macrophages also phagocytose oxidized lipids, which are elevated in smokers (Bloomer 2007, Yamaguchi 2005), and develop into lipid-laden foam cells, eventually forming the fatty streaks that are the first visible signs of atherosclerosis (Fearon 2011). Attracted T cells release cytokines that attract, in turn, vascular smooth muscle cells, which start migrating from their location in the media into the intima layer of the vessel just beneath

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the endothelial cell layer where they proliferate and contribute to the growth of the developing plaques. This can eventually give rise to complex atheromas.

In humans, the final stage of atheroma formation is reached after years of continuous exposure to environmental noxious stimuli, such as cigarette smoke and a "Western" diet rich in saturated fats and poor in antioxidants (Celermajer 1997). The natural disease progression results in plaque growth and positive vessel remodeling to maintain a functional lumen size. Although atherosclerotic plaques remain clinically silent for decades, they may evolve to become advanced lesions that are prone to calcification, cap thinning, hemorrhage, and rupture. It should be noted, however, that an apparently increasing proportion of fatal arterial thromboses arises from superficial erosion sites in vascular walls that are characterized by endothelial cell apoptosis on an underlying lipid-poor but proteoglycan- and glycosaminoglycan-rich plaque structure. However, statin treatment, changing lifestyle, and other factors may favor the stabilization of the classical lipid-rich plaques (Libby 2015).

COPD is a respiratory disease characterized by progressive airflow limitation and is associated with an abnormal inflammatory response of the lungs to noxious particles and gases (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2014). The development of COPD in smokers has been linked to a number of sustained cellular stress responses, including endoplasmic reticulum (ER), hypoxic, and autophagic stresses (Chen 2008, Hengstermann 2008, Hwang 2010, Malhotra 2009, Vasilios 2008). Most if not all of these stress responses are executed as an answer to oxidative insults that arise from direct exposure to cigarette smoke and cause tissue injury through oxidative stress.

While most data addressing the response of the lungs to cigarette smoke at the molecular level have focused on genes and/or proteins, a few studies have demonstrated that specific lipid classes play key roles in lung pathobiology (Telenga 2014, Yang 2011). There is evidence that cigarette smoke exposure can modulate lung lipid metabolism, e.g., lungs chronically exposed to cigarette smoke show signs of altered ceramide and eicosanoid metabolism (Cantin 2010, Lundstrom 2011). Cigarette smoke-related stress responses also lead to the attraction, activation, and release of pro-inflammatory mediators from immune cells, which in turn produce a number of (endogenous) oxidative stressors capable of perpetuating an inflammatory response and eventually leads to irreversible tissue damage in the small airways and alveolar tissue. These processes cause a self-perpetuating chronic bronchitis with mucus hypersecretion, remodeling of the small airways characterized by fibrosis and mucous plugging (small airway disease), and a proteolytic and apoptotic breakdown of the alveolar walls (emphysema), which are the typical components of COPD, as well as a systemic inflammatory condition and comorbidities, particularly in the advanced stages (Barnes 2009, Martin 2013).

Exposure to cigarette smoke has been reported to induce or potentiate lung inflammation through a variety of mechanisms involving multiple cell types (Barnes 2003, Stampfli 2009, Thorley 2007). Airway epithelial cells, resident lung macrophages, and dendritic cells are the initial cellular targets of cigarette smoke exposure, and in response, these cells produce potent chemoattractants that recruit inflammatory cells from the systemic circulation; notably, neutrophils, macrophages, and T cells, into the lung

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microenvironment (Barnes 2003, Stampfli 2009, Yoshida 2007). Chronic exposure can lead to increased airway cell damage/death, which further exacerbates pulmonary inflammation through the release of damage-associated molecular pattern molecules (Chen 2010, Morbini 2006, Reynolds 2011). Self-perpetuation of inflammatory processes is mainly accomplished by an increase in T cells (more CD8+ or increased CD8+/CD4+ ratio) (Tetley 2005), but adaptive immunity with B cell responses, lymphoid follicle formation, and autoimmune mechanisms may also contribute to persisting inflammation even after smoking cessation (Brusselle 2011, Seys 2015, Taraseviciene-Stewart 2006).

1.2 Lipid changes as causes and consequences of inflammatory processes and oxidative stress

The 2010 U.S. Surgeon General's Report "How Tobacco Smoke Causes Diseases" identifies inflammation and oxidative stress, among others, as key mechanisms underlying all major smoking-related diseases (Office of the Surgeon General 2010b). Cigarette smoke is a complex and dynamic mixture consisting of more than 8,000 unique chemical compounds (Perfetti 2011). Both the gas and particulate (tar) phases of cigarette smoke contain high levels of oxidants, with an estimated 10^{15} radicals per puff and 10^{17} radicals per gram of tar (Pryor 1992, Pryor 1997). Many of these free radicals, but also endogenously generated ROS and reactive nitrogen species are the prime mediators of endothelial dysfunction in smokers, a key event in the development of atherosclerosis (Salahuddin 2012).

It is very likely that the biological effects exerted by cigarette smoking are related to the complex interaction of these compounds and their metabolites with each other and with various cells, including those of the respiratory tract and the cardiovascular system, where smoke exposure elicits a series of adaptive cellular stress responses including oxidative stress responses (Müller 2006).

Oxidative stress results from an imbalance between the levels of ROS and antioxidants. ROS and other reactive species act directly on cellular macromolecules, damaging lipids, proteins, and DNA. In general, cells respond to oxidative stress by activation of the transcription factor Nrf-2 (encoded by the nuclear factor erythroid-derived 2-like 2 gene), a key regulator of detoxification and antioxidant genes (Ishii 2002). As recently reviewed by Muller & Hengstermann, cigarette smoke-dependent Nrf-2 activation is assumed to be based on a strong electrophile-driven sulfhydryl (-SH) reactivity inherent to cigarette smoke, exerted by numerous cigarette smoke constituents, including aldehydes, metal ions, and polyphenols. This may lead to intracellular generation of oxidants such as reactive intermediates and ROS. These electrophiles can be sensed by their interaction with specific cysteine residues provided by Nrf-2's negative cytosolic regulator Kelch-like ECH-associated protein 1, which appears to be the major route leading to Nrf-2 activation (Muller 2012).

The products of the antioxidant and detoxification genes that can be activated by Nrf-2 either act as quenchers to eliminate the oxidative insult or contribute to the regulation of downstream effectors, which determine cell fate based on the extent of damage. For example, oxidative damage is known to elicit a number of other cellular responses triggering various protein kinase signaling cascades, most notably those of the mitogen-

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activated protein kinase (MAPK) family, and resulting in proliferation, growth arrest, senescence, or cell death, typically depending on the type of stressor and the duration and extent of stress (Martindale 2002).

In addition to the Nrf-2-mediated antioxidant response, cigarette smoke-related intracellular ROS, often generated by plasma membrane-bound NADPH oxidase upon interaction with cigarette smoke constituents, can trigger another redox-sensitive transcription factor, the pro-inflammatory NFκB. In a multistep kinase reaction involving MEKK, NIK, protein kinase C, and IKK alpha and beta, and also including histone acetylation and phosphorylation, the transcription of a number of inflammatory mediator genes such as cytokines can be enhanced, and there is cross-talk with Nrf-2 signaling. Post-translational modification of HDAC2 by cigarette smoke-induced oxidative stress inactivates its anti-inflammatory activity, which would prevent histone acetylation and thereby attenuate NFκB-induced cytokine transcription (Yao 2012, Yao 2007).

Not surprisingly, most if not all of these stress responses are executed as an answer to oxidative insults that arise from direct exposure to cigarette smoke and cause tissue injury through oxidative stress. Cigarette smoke-related oxidative stress also leads to the attraction, activation, and release of pro-inflammatory mediators from immune cells, which in turn produce a number of (endogenous) oxidative stressors capable of perpetuating an inflammatory response. Inflammatory processes triggered not only by oxidative stress-induced cytokines, but also by various other insults, such as infection, tissue injury, and cellular stress, are a first-line defense mechanism that rapidly triggers the release of more pro-inflammatory substances, including cytokines, free radicals, hormones, and other small molecules. These molecules combat the stressor and also initiate distinct inflammation-resolving processes in parallel, such as the adaptive immune response (Berridge 2014) or the production of so-called resolvins, local-acting anti-inflammatory mediators that are biosynthesized from the essential fatty acids eicosapentaenoic acid and docosahexaenoic acid (Ariel 2007). While this acute inflammatory response can lead to pathological consequences, it is in itself not a disease but a means to maintain homeostasis, and how the response is eventually executed typically depends on the original trigger. However, if resolution of acute inflammation is not achieved, the inflammatory mediators elicit additional oxidative and other cellular stresses, and chronic cigarette smoke exposure or ongoing stress may lead to the development of smoking-related diseases (Demirovic 2013, Glover-Cutter 2013).

While sustained unfolded protein response and ER stress and hypoxic stress have been reported to contribute to all of these disease-related processes, most of the evidence to date focuses on the roles of oxidative stress and inflammation in the development and progression of CVD (Austin 2009, Kaufman 2002, Minamino 2010a, Minamino 2010b, Tabas 2010, Wang 2012). Excessive superoxide anion production, e.g., during the respiratory burst response of activated macrophages and neutrophils, can lead to peroxynitrite formation with the physiologically generated NO, and the highly reactive peroxynitrite can increase the vasoconstrictive and thrombogenic effects of thromboxane A2 (TXA2) and prostaglandin H2, while inhibiting the production of vasorelaxant and anti-thrombogenic prostacyclin (Zou 2007). As reasoned in a review by Gryglewski, there appears to be a synergistic interaction between various endogenous antioxidant

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defense mechanisms such as the prostacyclin synthase–prostacyclin–adenylate cyclase–cAMP axis, the eNOS–NO–guanylate cyclase–cGMP axis, and the heme oxygenase (HO-1)–CO–biliverdin–biliverdin reductase–bilirubin axis that can potently inactivate reactive endogenous or exogenous molecules. Their pharmacological steering could provide targets for the therapy of systemic diseases including atherosclerosis (Gryglewski 2008).

In addition to these enzymatically derived prostanoids that are generated within a framework of biologically regulated or controllable processes, there is a complex family of isomers of these prostaglandins/prostanoids, called isoprostanes, which are produced *in vivo* predominantly by free radical-catalyzed peroxidation of polyunsaturated fatty acids (PUFA), such as arachidonic acid or linoleic acid (lipid peroxidation). Their origin from direct chemical reactions of free radicals makes them interesting as quantitative oxidative stress markers (Nam 2011).

Most work has focused on a group of isomers of the enzyme-derived PGF(2α), called F(2)-isoprostanes, which have been shown to be associated with oxidative stress in the pathogenesis of various diseases including atherosclerosis, COPD, and Alzheimer's disease, although their exact contributions may need further exploration. In addition to the passive role as end products of lipid peroxidation, F2-isoprostanes have been demonstrated to actively contribute to the mediation of oxidative stress effects and in the self-propagation of oxidative stress (Milne 2005, Milne 2008, Nam 2011, Pratico 1999, Rahman 2002). Isoprostanes can give rise to various reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), with the capability to form various adducts, e.g., with proteins or DNA (Nam 2011). However, some isoprostanes have been demonstrated to reduce the inflammatory response in macrophages by inhibiting lipopolysaccharide-stimulated IκBα degradation and subsequent NFκB nuclear translocation and transcriptional activity (Musiek 2005).

Taken together, these relationships illustrate the mutual interdependence of oxidative stress and inflammatory processes and the important roles of pro- and anti-inflammatory lipid mediators and oxidized lipids in the vicious cycle that may result in progressive tissue destruction if inflammation does not resolve. In the following chapters, examples of lipid mediators and oxidized lipids and their roles in CVD and COPD pathomechanisms will be described in greater detail.

2 LIPIDS IN THE PATHOGENESIS AND DIAGNOSIS OF CVD

Aside from cigarette smoking, CVD risk factors that contribute to the development of atherosclerosis are typically associated with a Western lifestyle. These include obesity, high blood pressure, high total cholesterol (TC) levels, and diabetes. TC levels do not accurately reflect CVD risk, as this measurement represents the sum of cholesterol carried not only by atherogenic lipoproteins, such as LDL, intermediate-density lipoprotein (IDL), and very-low-density lipoprotein (VLDL), but also by anti-atherogenic lipoproteins (HDL) (Carmena 2004). Of these, the particle heterogeneity and oxidation status of LDL and HDL have emerged as principal factors affecting particle atherogenic potential (Krauss 2005). In addition to triacylglycerols (TAG) and cholesterol levels,

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advanced lipid testing methods providing particle size and number, and apolipoprotein concentrations, are now available to clinicians (De Leon 2015).

During the pathogenesis of CVD, atherogenic, oxidized, or otherwise modified lipids mostly circulating in the bloodstream in the form of lipoprotein complexes (LDL, HDL, VLDL) are internalized and retained in the arterial intima layer by an inflammatory mechanism inducing endothelial secretion of chemotactic substances and the expression of adhesion receptors that favor monocyte adhesion and transmigration into the arterial wall. In the subendothelial space, monocytes differentiate into macrophages, which start to accumulate modified lipids (e.g., oxLDL) leading to foam cell formation (Figure 1). Foam cells aggravate the atherogenic process by releasing growth factors, cytokines, metalloproteinases, and ROS, all of which lead to a vascular remodeling process that culminates in destabilization and rupture of the atherosclerotic plaque (Libby 2010).

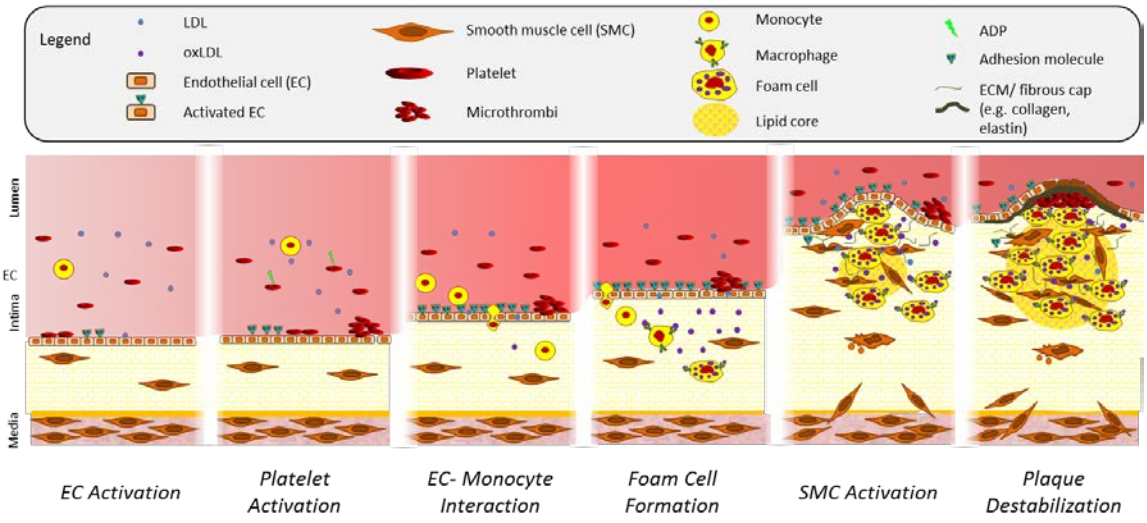


Figure 1. Cellular and molecular interactions between inflammatory cells, mediators, and lipids underlying atherogenesis in the vessel wall

Schematic of atherogenesis in the vessel wall. Endothelial dysfunction leads to the recruitment of inflammatory cells and platelet activation. These events promote transendothelial migration of monocytes from the circulation, macrophage and foam cell differentiation, and smooth muscle cell activation within the atherosclerotic plaque. Complex plaque lesions are prone to destabilization and rupture (De Leon 2015).

2.1 Blood lipid profiles and oxLDL in smokers

Lipids are essential for a number of cellular processes including synthesis of cellular membranes, formation of lipoprotein particles, signal transduction, and energy storage, among others; tight regulation of their metabolism contributes to the maintenance of cellular homeostasis (Liu 2011). Circulating lipoproteins such as VLDL and LDL are particles that transport TAG and cholesterol in the bloodstream between the liver and peripheral tissues and cells, and HDL manages reverse cholesterol transport. These

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processes are controlled mainly by interactions between the protein components of lipoproteins, called apolipoproteins, and their receptors located on target cells (for details, see next chapter).

Various factors have been shown to interfere with regular lipid shuttling and metabolism leading to an accumulation of lipids in cells, and an increase or modification in circulating lipoproteins. Not surprisingly, cigarette smoking has been linked to high levels of LDL-cholesterol, VLDL-cholesterol, and TAG, and decreased HDL-cholesterol levels; furthermore, abnormal lipid profiles have a strong positive correlation with endothelial dysfunction and CVD risk (Craig 1989, Khurana 2000, Mammas 2003, Rao 2013, Tang 1997b, Willett 1983). Studies conducted in diverse populations have shown that cigarette smoke is a risk factor for the development of coronary artery disease and peripheral artery disease (Office of the Surgeon General 2010a, Price 1999), whereas smoking cessation results in a decline in CVD risk within the first 5 years, as estimated by inflammatory markers and traditional risk factors (e.g., family history and low HDL levels) (Bakhru 2005). Of interest, smoking cessation was shown to have a positive effect on the lipid profile, with HDL-cholesterol increasing and TAG and LDL-cholesterol levels tending to decrease in as little as 2–8 weeks since quitting (Allen 1994, Eliasson 2001, Komiyama 2013, Moffatt 1988, Noh 2012, Tang 1997a).

The initiating event of endothelial injury lies in the accumulation of oxidized or otherwise modified (e.g., acetylated (Goldstein 1979), glycated (Horiuchi 2003) or enzymatically modified (Torzewski 2006)) lipids (Siegel-Axel 2008), although unmodified LDL may also markedly reduce endothelium-dependent relaxations to aggregating platelets and serotonin (Luscher 1993) and thereby enhance the severity of endothelial dysfunction, eventually leading to monocyte adhesion and transmigration that initiate plaque formation (Figure 1).

One of the prime examples of oxidized lipids whose physiological roles have been studied extensively over the last three decades in the context of CVD is oxLDL. Although its biological effects are well understood, it is still unclear where oxidation of LDL occurs and which enzymes contribute to LDL oxidation *in vivo* and to what extent (Steinberg 1997). Some *in vitro* evidence supports the involvement of lipoxygenases (Folcik 1995) and of myeloperoxidase (Clark 2010, Leeuwenburgh 1997).

Higher levels of circulating oxLDL have been reported in smokers relative to nonsmokers (Bloomer 2007, Linna 2008, Yamaguchi 2005). Using human umbilical vein endothelial cells, we have demonstrated that combining non-toxic concentrations of cigarette smoke bubbled through PBS (smoke-bubbled PBS (sbPBS)) with native LDL (nLDL) significantly reduces the amount of bioavailable NO. The effect is comparable with that seen with oxLDL, but has not been seen with sbPBS or nLDL alone (Steffen 2012). Mechanistic investigations showed that the combination of sbPBS+nLDL did not reduce the amount of eNOS, but did inhibit its enzymatic activity. Concomitantly, both sbPBS+nLDL and oxLDL significantly increased the production of ROS in the form of superoxide anions (O_2^-) and peroxynitrite ($ONOO^-$) in human umbilical vein endothelial cells. Selective inhibition of NADPH oxidase prevented this response. Incubation of sbPBS+nLDL revealed the formation of 7-ketocholesterol and 7-hydroxycholesterol,

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which are indicators for oxidative modification of LDL. This could explain the reported increase in circulatory levels of oxLDL in smokers. Our results suggest that reduction of functional NO in response to a combination of sbPBS+nLDL is secondary to both reduction of eNOS activity and stimulation of NADPH oxidase activity (Steffen 2012). Thus, we believe measurements of lipids and oxLDL in the circulation might provide a useful insight into the extent of cigarette smoking-induced oxidative alterations to circulating lipids.

2.2 Lipid-lowering interventions have limited success in disease prevention

Over the past 40 years, technological advancements in the determination of plasma lipoproteins and their associated Apo have delivered a wealth of data documenting the association between certain lipid particles (LDL and HDL) and CVD risk (De Leon 2015). Accordingly, pharmacological research has provided a range of drugs to normalize lipid profiles, particularly the statins. Statins are widely used in the treatment of dyslipidemia and associated cardiovascular abnormalities including atherosclerosis, hypertension, and coronary heart disease. Their cholesterol-lowering effects are based on inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase, a rate-limiting enzyme of cholesterol biosynthesis. Besides cholesterol-lowering effects, statins possess pleiotropic anti-inflammatory, antioxidant, antiplatelet, and antifibrotic properties, which may additionally play imperative roles in statin-mediated cardiovascular protection. The potential modulatory role of statins on eNOS, a key enzyme involved in the regulation of cardiovascular function by generating endothelium-derived relaxing factor (often represented as “nitric oxide”), appears to be quite important (Balakumar 2012).

In spite of the well-recognized cardiovascular defensive potential of statins, a large residual risk is still observed for patients optimally treated with current lipid-lowering drugs, as has been shown in a recent review of a large number of clinical trials (De Leon 2015). This may indicate that additional factors are involved. Several studies have probed deeper and shown that other lipid species within tissues, or circulating in plasma associated with lipid particles, also play a role in atherogenesis. The authors concluded that further reductions in clinical event rates require identification of different targets, better biomarkers of disease progression, and development of novel therapeutic strategies. Lipidomics approaches represent a sound strategy to characterize the molecular lipid species and pathways implicated in CVD onset and progression (De Leon 2015).

2.3 Apolipoproteins and cholesterol transport

Apolipoproteins are lipid-binding proteins that form lipoproteins, a heterogeneous population of soluble macromolecular aggregates responsible for the transport of water-insoluble lipid molecules (mainly TAG, cholesterol, fatty acids, and phospholipids) from their site of synthesis (e.g., liver) or absorption from food (e.g., gut), to sites of use (e.g., muscle and adipose tissue). A schematic overview is provided in Figure 2.

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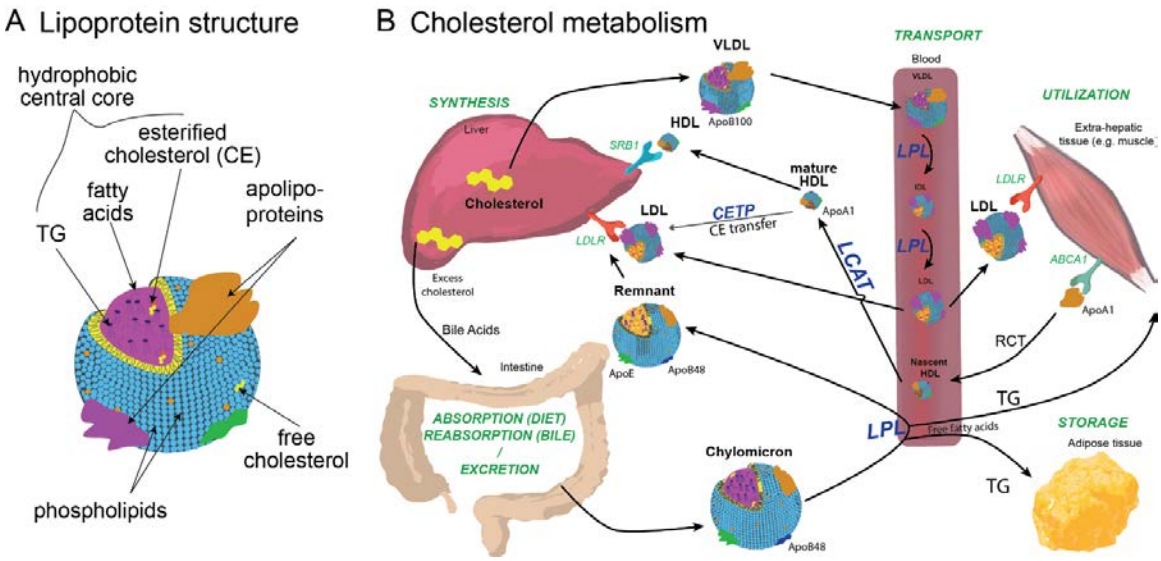


Figure 2. Lipoprotein structure and cholesterol metabolism and transport

(A) Schematic of a lipoprotein particle. Lipoproteins have a spherical geometry with a monolayer of amphipathic lipids and proteins encircling a core of neutral lipids. Apolipoproteins (orange and purple) embedded in the phospholipid layer confer structural and functional properties to the molecule (specific transport of VLDL, LDL, or HDL). Lipoprotein contains TAG, free fatty acids, free cholesterol, and phospholipids. **(B)** Cholesterol synthesized in hepatocytes is transported as VLDL to the blood circulation. VLDL contain TAG, fatty acids, free cholesterol, and phospholipids. VLDL particles are transported by ApoB100. VLDL is then remodeled and transformed into IDL and LDL by the action of LPL in the blood circulation. Cholesterol could also be transported by chylomicrons. Chylomicrons are synthesized by enterocyte cells in intestinal tissue after dietary absorption or bile acid reabsorption. Chylomicrons transport cholesterol from the intestines to the circulation via ApoB48. LPL triggers transformation of chylomicrons into remnants with the release of free fatty acids into the circulation. These free fatty acids are stored in adipose tissue in the form of TAG or oxidized by non-hepatic tissues such as muscles. LDL particles and remnants are removed from the circulation through interactions with LDL receptors. Excess cholesterol can be cleared from tissue via reverse cholesterol transport via ApoA1 lipoprotein. ApoA1 is synthesized in hepatocytes and released from the liver as lipid-poor ApoA1. These particles bind to ATP-binding cassette A1 transport proteins located on the surface of peripheral cells, leading to the transfer of unesterified free cholesterol. ApoA1 accumulates free cholesterol and free fatty acids, and the particles become mature HDL by the action of lecithin-cholesterol acyltransferase and cholesteryl ester transfer protein. HDL particles are then cleared from the bloodstream by scavenger receptor B1 expressed by hepatocytes. ApoA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; ApoB48: Apolipoprotein B48; VLDL: very-low-density lipoprotein; IDL: intermediate-low density lipoprotein; LDL: low-density lipoprotein; LPL: lipoprotein lipase; ABCA1: ATP-binding cassette A1; FC: free cholesterol; CETP: cholesteryl ester transfer protein; LCAT: lecithin-cholesterol acyltransferase; SRB1: scavenger receptor B1; TG: triglycerides/triacylglycerols; FC: free cholesterol; PL: phospholipids. From (De Leon 2015).

The main apolipoprotein of LDL, IDL and VLDL particles is ApoB, which mediates the interaction of non-HDL-cholesterol with vascular endothelium via engagement of the LDL receptor (Olofsson 2007). ApoB is also the ligand of the LDL receptor (Boren 1998a), and elevated plasma levels of ApoB are strongly associated with increased risk for CVD (Gigante 2012). Two molecular species of ApoB exist: ApoB100 is the full-length molecule that is synthesized in the liver where VLDL particles are assembled for

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release into the circulation (Olofsson 2012), while ApoB48 is the N-terminal fragment of ApoB100 that is synthesized in the intestines, where it participates in the assembly of chylomicrons. ApoB100 is thought to play a key and direct role in the retention of LDL upon binding to negatively charged proteoglycans in the arterial wall (Boren 1998b).

ApoA1, a major protein component of HDL-cholesterol, plays a crucial role in cellular cholesterol efflux and transport, a capability that has been characterized as a main contributor to the anti-atherogenic effects of HDL-cholesterol (Ansell 2005). It activates lecithin-cholesterol acyltransferase, resulting in esterification of cholesterol, a key step in reverse cholesterol transport (Andrikoula 2008). Because ApoB associates with LDL-cholesterol at a ratio of 1:1, its measurement can provide insight into the number of circulating LDL particles and hence the level of pro-atherogenic cholesterol (Andrikoula 2008). This was also, at least in part, the basis for proposing the ApoB/ApoA1 ratio as an alternative to LDL/HDL indices (Walldius 2006). ApoB/ApoA1 ratios calculated from non-fasting lipid levels in over 20,000 individuals in the INTERHEART study were a better predictor of myocardial infarction than any other cholesterol measure (Raitakari 2013). Multiple studies have shown strong associations between the ApoB/ApoA1 ratio and risk for coronary artery/heart disease (Boekholdt 2006, Lu 2011, Simons 2009), myocardial infarction (Dunder 2004, Yusuf 2004), heart failure (Ingelsson 2005), and stroke (Kostapanos 2010, O'Donnell 2010). It was also shown to correlate well with early atherosclerosis, as indicated by intima-media thickness (Panayiotou 2008), and to perform better than the traditional lipid profile in predicting the risk for CVD and major cardiovascular events (Boekholdt 2006, Ingelsson 2005, Pan 2014, Yusuf 2004). In addition, cigarette smoking was linked to increased ApoB and decreased ApoA1 levels (Jensen 1995, Meenakshisundaram 2010, Slagter 2013), and successful quitters exhibited higher ApoA1 serum levels than current smokers in as little as 12 weeks (Iwaoka 2014, Richard 1997).

The terminal step of reverse cholesterol transport to the liver requires apolipoprotein E (ApoE) protein, which forms complexes with lipoprotein remnants and HDL and helps target them either to the LRP1 receptor or the LDLR receptor, respectively, in hepatocytes for final uptake and lysosomal processing/recycling (Heeren 2006). In addition to its long-recognized function in reverse cholesterol transport, recent experimental evidence suggests that HDL may also exert antiplatelet effects and thereby prevent platelet aggregation, fibrinogen binding, granule secretion, and liberation of the vasoconstrictive and thrombogenic lipid mediator, TXA2. These antiplatelet activities are mediated, in part, by scavenger receptor B1 and/or the ApoE receptor, LRP8, and leading, among others, to protein kinase C stimulation and generation of NO (Nofer 2010).

Molecular defects in ApoE structure, e.g., mutated ApoE2, can result in pathogenic hypercholesterolemia and increased atherosclerosis, such as type III hyperlipoproteinemia and dysbetalipoproteinemia, especially when additional risk factors are present (Mahley 1985). Knockout of the ApoE gene in mice has been leveraged as a widespread atherogenesis model in pharmacological and toxicological investigations (Meir 2004).

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2.4 Thromboxanes and metabolites

Thromboxanes are members of the eicosanoid family of molecules and belong to the labile prostanoids resulting from COX1 (PTGS1) or COX2 (PTGS2)-mediated cyclooxygenation of PUFA and the subsequent action of thromboxane A synthase (Gryglewski 2008). They are of interest in the context of cigarette smoke-induced alterations of platelet biology and vascular function. Upon activation, platelets release arachidonic acid from the plasma membrane, which is then enzymatically converted into TXA2. Its production increases under conditions of oxidative stress, which leads to peroxynitrite formation, which in turn activates TXA2 synthase (Gryglewski 2008). TXA2 promotes platelet aggregation, vasoconstriction, and smooth muscle cell proliferation (Needleman 1976), even though it is highly unstable and rapidly converted into the more stable thromboxane B2 (TXB2). Levels of the major TXB2 metabolites 11-dehydro- and 2, 3-dinor-thromboxane B2 in plasma or urine are thought to be reflective of systemic TXA2 production and can be easily assessed by mass spectrometry analysis (Catella 1986, Lawson 1986).

As mentioned by the 2001 Institute of Medicine report "Clearing the Smoke", these TXB2 metabolites can serve, together with flow-mediated dilation and other hemodynamic parameters, as useful indicators of altered endothelial and platelet function (Stratton 2001a). Using this method, significantly higher urinary levels of TXB2 metabolites were measured in smokers than in nonsmokers (Calapai 2009, Frost-Pineda 2011, Lowe 2009, Rångemark 1992, Uedelhoven 1991, Wennmalm 1991), and 11-dehydro- and 2, 3-dinor-thromboxane B2 levels rapidly declined in smokers who quit (Rångemark 1993, Saareks 2001, Wennmalm 1993). This responsiveness to changes in cigarette smoke exposure together with the ease of their detection and measurement makes thromboxane metabolites suitable biomarker candidates for monitoring oxidative stress-induced platelet activation. However, it has been reported that although urinary excretion of TXB2 metabolites was elevated in smokers, the capacity of their platelets to generate TXA2 *in vitro* was significantly reduced, pointing to a possible chronic smoking-induced alteration of the platelet TXA2 system (Weber 2000).

2.5 Prostacyclin

Like thromboxanes, prostacyclin (also called prostaglandin I2 or PGI2) is an unstable prostanoid derived from the linear PUFA arachidonic acid by enzymatic cyclooxygenation, but then followed by the action of prostacyclin synthase. Prostacyclin is produced by the vascular wall (predominantly by endothelium) and it acts as a physiological antagonist of TXA2, i.e., as a vasorelaxant and antithrombotic mediator. Prostacyclin *per se* is a powerful cytoprotective agent that exerts its action through activation of adenylate cyclase, followed by intracellular accumulation of cyclic-AMP in various types of cells (Gryglewski 2008). As already mentioned in chapter 1.2, peroxynitrite (formed by the reaction of NO with superoxide anion) can inhibit the function of prostacyclin synthase and thereby abolish the beneficial vasodilatory and antithrombotic effects of both NO (which is bound as peroxynitrite) and prostacyclin (which is not replenished and rapidly disappears, with a half-life of approximately 4 min) (Gryglewski 2008, Zou 2007). More stable prostacyclin analogs, such as iloprost and

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epoprostenol have been synthesized for the treatment of vascular diseases including peripheral artery disease and pulmonary hypertension (Bozkurt 2013, Gryglewski 2008).

The relationship between prostacyclin levels in smokers *vs.* nonsmokers appears to be controversial. While some authors report decreased prostacyclin production in smoke-exposed endothelial cells *in vitro* and in cardiovascular tissues of cigarette smoke-exposed Apoe$^{-/-}$ mice (Barbieri 2011, Mahfouz 2006, Sotoda 2015), approximately 70% higher prostacyclin levels (measured as the stable urinary prostacyclin metabolite, 2, 3-dinor-6-keto-PGF1α) were observed in smokers compared with nonsmokers, and they were dependent on COX2 activity (McAdam 2005). Three-times higher urinary prostacyclin metabolite excretion was reported for smokers *vs*. nonsmokers, as well as a 40% decrease following 2 weeks cessation in a study by Saareks *et al.* (Saareks 2001); whereas only a marginally higher prostacyclin metabolite level was observed in urine of smokers in a study by Smith *et al.* (Smith 2001). One possible explanation of the heterogeneous findings on smoking-related changes in absolute prostacyclin metabolite levels could be an imbalance in prostacyclin and TXA2 production in favor of the latter (Huang 2011).

2.6 Other arachidonic acid metabolites

In addition to TXA2 and TGI2, several other biologically active prostanoids are derived from arachidonic acid, including stable prostaglandins, e.g., PGE(2), PGF(2α), PGD(2), PGJ(2), as well as labile prostanoids, e.g., PG endoperoxides (PGG(2), PGH(2)) (Gryglewski 2008). Prostaglandin E2 (PGE2) is of particular interest because it exerts vasodilatory effects in the respiratory tree but can also increase vascular permeability, control vascular smooth muscle cell tone, and contribute to atherogenic processes (Gomez 2013). Plasma from current smokers showed higher concentrations of PGE2 and tissue factor activity compared with never smokers. Similar results were obtained in mice and in mouse cardiac endothelial cells after treatment with aqueous cigarette smoke extracts, and inhibitor experiments demonstrated that PGE2 modulated tissue factor expression and activity through the EP1/SIRT1 pathway (Amadio 2015).

Another group of biologically active arachidonic acid metabolites originates from the enzymatic activity of cytochrome P450 (CYP) isoenzymes: 20-hydroxyeicosatetraenoic acid (20-HETE) is a metabolite generated by CYP4A isoforms, and epoxyeicosatrienoic acids (EET) are generated by CYP2C and CYP2J activity. Both contribute to the regulation of renal tubular and vascular function. They were reported to enhance vascular inflammation and injury in models of diabetes, ischemia/reperfusion, and cerebrovascular oxidative stress (Christmas 2015, Fan 2015, Hoopes 2015). 20-HETE, as well as the endoperoxides PGG2 and PGH2, and isoprostanes (see next chapter), have been shown to activate the thromboxane receptor as autocrine or paracrine ligands, promoting angiogenesis and vascular constriction (Chamorro 2009). From a drug perspective, these oxidative stress-derived thromboxane receptor agonists can bypass a pharmaceutical blockade of TXA2 generation, e.g., by aspirin, and abolish the success of the therapy (Capra 2014a). For the EET, anti-inflammatory properties were also described in the context of CVD and metabolic syndrome (Christmas 2015).

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An additional anti-inflammatory arachidonic acid metabolite, leukotriene, 15-epi-LXA4, can be generated by CYP enzymes (including CYP2C9) through hydroxylation of arachidonic acid to form 15R-HETE, which subsequently is converted by 5-LOX and LXA4 synthase. 15-epi-LXA4 can inhibit a range of inflammatory mediators and pro-inflammatory cytokines, as well as NFκB activation (Jozsef 2002, Oliw 1993, Yuan 2015).

2.7 Isoprostanes and related lipid peroxidation products

Lipid peroxidation is a free radical-driven, non-enzymatic oxidation of PUFA such as linoleic acid or arachidonic acid. Oxidation products can either be isomers of enzymatically generated prostanes, called isoprostanes, or reactive aldehydes like MDA or 4-HNE (for an overview, see (Nam 2011, Roberts 2009)).

Peroxidation of arachidonic acid gives rise to isoprostanes, prostaglandin-like compounds produced in tissues by non-enzymatic reactions that are catalyzed by free radicals. A variety of isoprostane molecules exhibiting widely differing biological activities can be generated by oxidative damage *in vivo* (Janssen 2001). The most widely studied group, F2-isoprostanes, have been established as a reliable indicator of global oxidative injury at the organism or tissue level, and their accuracy and stability in body fluids makes them a "gold standard for the assessment of oxidative damage" (Nam 2011). Numerous studies have demonstrated that quantification of F2-isoprostanes using mass spectrometry is the most accurate measure of oxidative stress *in vitro* and in animal models of oxidant injury (Basu 2008, Milne 2005, Roberts 2000, Visioli 2000). In smokers, plasma and urinary free and esterified F2-isoprostanes were found to be elevated relative to nonsmokers (Helmersson 2005, Morrow 1995, Reilly 1996) and rapidly declined upon smoking cessation (Chehne 2001, Pilz 2000, Reilly 1996). More recently, induced sputum and exhaled breath condensate (EBC) have been investigated for markers of inflammation and oxidative stress. Significantly higher levels of 8-isoprostane have been observed in sputum and/or EBC of patients with inflammatory lung diseases and in smokers *vs.* nonsmokers (Borrill 2008, Inonu 2012, Ko 2006, Mazur 2011a, Montuschi 2005, Nussbaumer-Ochsner 2015), and acute increases in 8-isoprostane in EBC were observed in asthmatics but not in healthy controls within 90 min after smoking two cigarettes (Papaioannou 2010).

Many attempts have been undertaken to investigate the suitability of F2-isoprostanes not only as biomarkers of exposure and of biological effects, but also as a disease biomarker in diseases that are associated with pronounced oxidative stress, including atherosclerosis, COPD, and Alzheimer's disease (Pratico 1999). With regard to atherosclerosis, the search for clear implications of F2-isoprostanes as causal mediators in typical pathomechanisms is still ongoing. 8-isoprostane F2 alpha (8-iso-PGF2α) is a potent pulmonary and renal vasoconstrictor (Jourdan 1997, Takahashi 1992) and mediator of platelet aggregation (Minuz 1998, Morrow 1992). There are indications that F2-isoprostanes mediate neutrophil adhesion induced by minimally modified LDL (Davi 2004), and they can, like TXA2, 20-HETE, PGG2 and PGH2, activate the pleiotropic thromboxane prostanoid receptor involved in platelet aggregation, thrombosis, and vascular tone. Unlike the other thromboxane prostanoid ligands, radical-driven

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generation of F2-isoprostanes is insensitive to conventional drug treatment (aspirin) (Capra 2014a). Despite their correlation with oxidative stress levels and atherosclerosis, data from many clinical studies have not yet provided clear evidence that oxidative biomarkers including the F2-isoprostanes are useful as biomarkers of disease risk, and it was concluded that high levels of F2-isoprostanes in urine or blood may be a non-specific indicator of CVD (Pastori 2014, Zhang 2013). Nevertheless, we consider F2-isoprostanes to be a useful indicator of smoking-related oxidative stress (both local and systemic being relevant for a variety of diseases including CVD and COPD) in future clinical studies aimed at the assessment of oxidative stress responses in smokers switching to potential modified risk tobacco products (MRTP).

Cyclopentenone isoprostanes are highly reactive isomers of PGA2 and PGJ2, and are also formed non-enzymatically from arachidonic acid. In an *in vitro* study on murine macrophages, these isoprostanes inhibited IκBα degradation and subsequent NFκB nuclear translocation as well as transcriptional activity, and expression of iNOS and COX2 via a PPARγ-independent, partially redox-dependent mechanism (Musiek 2005). Whether such an anti-inflammatory, possibly negative feedback mechanism plays a distinct role in the pathogenesis of CVD may require further investigation. There were also attempts to link the proven anti-atherosclerotic activity of fish-oil-derived docosahexaenoic acid to its oxidatively derived cyclopentenone isoprostanes (A4/J4 neuroprostanes), which were also effective at suppressing NFκB-mediated inflammatory responses and at increasing Nrf-2 to DNA binding and downstream NAD(P)H quinone dehydrogenase 1 (NQO1) expression in vascular endothelial cells *in vitro* (Majkova 2011).

Lipid peroxidation of PUFA such as linoleic acid or arachidonic acid can also lead to the formation of reactive aldehydes, the best known of which are MDA and 4-HNE. The latter is also an end product of cardiolipin peroxidation, occurring in damaged mitochondria (Zhong 2014). These aldehydes form adducts with cellular nucleophiles such as proteins, nucleic acids, and glutathione, which have been related to various diseases. Examples are the highly mutagenic MDA-dG adducts that have a considerable baseline also in hepatocytes of healthy individuals, possibly reflecting the physiological level of endogenous lipid peroxidation. Likewise, DNA adducts with 4-HNE have been observed to induce p53 gene mutations (Nam 2011). With regard to atherosclerosis, foam cell-derived 4-HNE was shown to activate PPARδ in endothelial cells, and subsequently, the pro-oxidant thioredoxin-interacting protein was expressed, which induced cellular senescence in an *in vitro* system; hints of this mechanism were also observed in atherosclerotic plaques (Riahi 2015). 4-HNE can also form highly concentrated adducts in LDL particles (Annangudi 2008). An interesting mechanism by which oxLDL increases oxidative stress and ER stress involves inactivation of the ER-resident chaperone, protein disulfide isomerase, by 4-HNE adduct formation through 4-HNE formed from oxLDL or from other endogenous origins, leading eventually to increased protein misfolding, ER-stress, apoptosis and plaque progression (Muller 2013). Further possible atherosclerotic implications of 4-HNE have recently been reviewed (Mali 2014).

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3 LIPIDS IN THE PATHOGENESIS AND DIAGNOSIS OF COPD

COPD is a respiratory disease characterized by progressive airflow limitation and is associated with an abnormal inflammatory response of the lungs to noxious particles and gases. Pathological changes characteristic of COPD are found in the proximal airway, peripheral airway, lung parenchyma, and pulmonary vasculature, and include chronic inflammation, with increased numbers of specific inflammatory cell types in different parts of the lung, and structural changes resulting from repeated injury and repair. The development of COPD in smokers has been linked to a number of sustained cellular stress responses. Pulmonary cells are exceptionally susceptible to the harmful effects of environmental insults including cigarette smoke, and chronic exposure to cigarette smoke has been linked to the development and progression of a variety of lung diseases including COPD (Barnes 2008, Tuder 2006, Vestbo 2013). Although the specific sequence of events leading to the development of these lung diseases is still under active investigation, recent studies have highlighted the central contribution of inflammatory responses to their pathogenesis (Chung 2008, Crotty Alexander 2015, Sopori 2002, Tetley 2005).

Upon injury, e.g., following infection, tissue injury, or exposure to noxious agents, the healthy lung can mount an effective acute inflammatory response aimed at eliminating the insult, limiting the extent of tissue damage and protecting the host (Levy 2014). Depending on the trigger of inflammation, inflammatory molecules are released, signaling to circulating leukocytes and attracting them to the site of injury. Well-known key players in this process are neutrophils, which are rapidly recruited following the release of IL-1 and TNF-α from resident macrophages executing an acute phase response, including a massive burst of ROS generation, to the inflammatory trigger. Inflammatory cells release not only ROS, thereby increasing oxidative stress that further enhances inflammation, but also many inflammatory mediators from different chemical classes, among which lipid mediators play an important role, e.g., the prostanoids and leukotrienes in COPD (Barnes 2004). There is evidence that cigarette smoke exposure can modulate lung lipid metabolism, e.g., lungs chronically exposed to cigarette smoke show signs of altered ceramide and eicosanoid metabolism (Cantin 2010, Lundstrom 2011). Recently, Morissette *et al.* presented an intriguing model for how cigarette smoke-induced damage of surfactant lipids, with the involvement of foam cell-like macrophages, can elicit lung inflammation (Morissette 2015b). To limit inflammation and prevent collateral injury of healthy, uninvolved tissue, the lung orchestrates the formation of specialized pro-resolving (lipid) mediators, specifically lipoxins, resolvins, protectins, and maresins. These immunoresolvents are agonists for resolution and interact with specific receptors on leukocytes and structural cells to inhibit further inflammation, e.g., neutrophilic resolvins and protectins, and are potent anti-inflammatory lipid mediators that exert their effects by reducing neutrophil infiltration and enhancing macrophage phagocytic capabilities thus limiting and resolving inflammation. These anti-inflammatory mechanisms appear to be defective in several common lung diseases that are characterized by excess or chronic inflammation (Kohli 2009, Levy 2014).

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Although numerous mechanistic data on changes of a multitude of lipids in various lipid classes being either causes or consequences of inflammatory and degenerative changes in the lungs of COPD patients have been generated, the overall correlation between COPD pathogenesis and specific lipid metabolic changes appears to be less well understood than the role of lipids in CVD (Azimzadeh Jamalkandi 2015). This is now being addressed in untargeted lipidomics studies (mass spectrometry-based analyses of lipids), which revealed key roles in lung pathobiology for specific lipid classes (Telenga 2014, Yang 2011). It is anticipated that the recent progress in lipidomics will facilitate further insights into the complex compositions and responses of lung lipidomes (Ejsing 2009, t'Kindt 2015).

A comparably low number of epidemiological investigations has been reported on this topic. One aspect was an apparent protective aspect of high dietary intake of anti-inflammatory omega-3 PUFA (principally eicosapentaenoic acid and docosahexaenoic acid from fish oil) against smoking-related COPD in the Atherosclerosis Risk in Communities study. The study reported that after adjustment for smoking exposure and other possible confounders, the prevalence odds of COPD were inversely related to the docosahexaenoic acid (but not to the eicosapentaenoic acid) content of plasma lipid components in most of the models (Shahar 1999, Shahar 1994). Other epidemiological studies found possible protective effects against asthma in children but not in adults, and a cross-sectional analysis of data from the first National Health and Nutrition Examination Survey reported an improvement in forced expiratory volume at 1 s (FEV1) between adults with high compared with low fish consumption (Schwartz 2000). Similar findings were observed in a Japanese study on high intake of omega-3 and omega-6 PUFA in their diet (Hirayama 2010) and in a Chinese study on dietary fish or supplemental omega-3 PUFA intake (Ng 2014). On the basis of serum phospholipid concentrations, Kompauer *et al.* found a statistically significant positive correlation between docosahexaenoic acid concentration and FEV1 and forced vital capacity, but a negative correlation between concentrations of dihomo-gamma-linolenic acid and palmitoleic acid in serum phospholipids and these respiratory parameters (Kompauer 2008). Overall, a clear correlation between atherogenic blood lipoprotein profiles and COPD was not confirmed (Basili 1999). A recent Chinese study found increased levels of oxLDL and decreased levels of HDL-cholesterol in a small cohort of COPD patients, whereas serum TAG, TC, and LDL-cholesterol were not different from the control group (Can 2015). In contrast, the large CARDIA study found a positive correlation between lung function (FEV1) and HDL-cholesterol levels and physical activity, and a negative correlation with serum TAG, smoking, and other factors (Higgins 1991). Paradoxically, overweight and metabolic syndrome have been associated with less severe COPD symptoms and better survival of patients in spite of their abnormal metabolic and inflammatory profiles that predispose them to CVD (Poulain 2008).

3.1 Surfactant changes in smoking and lung diseases

Lung surfactant is a complex mixture of proteins (mainly the four surfactant-associated proteins SP-A, SP-B, SP-C, and SP-D) and lipids formed by alveolar type II cells (see Figure 3). Surfactant is a crucial element for lung function. It reduces alveolar surface tension by maintaining a specific molecular orientation at the air–water interface, with

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the hydrophilic head groups interacting with the aqueous side and the hydrophobic lipid tails facing the air. Phospholipids comprise 80%–90% of surfactant lipids, and phosphatidylcholine is the most abundant class (70%–80%) (Lang 2005). Dipalmitoylphosphatidylcholine, a disaturated phosphatidylcholine species (PC 16:0/16:0), is the major contributor to surfactant surface activity, and is highly conserved across mammalian species (Postle 2001). The anionic phospholipids phosphatidylglycerol and phosphatidylinositol each contribute 10% to the total phospholipids present, while cholesterol, a neutral lipid, comprises 10%–20% of the total lipids (Veldhuizen 2004). The fatty acid composition within the phosphatidylglycerol and phosphatidylinositol classes is variable among species, and in humans, mainly monounsaturated species 16:0/18:1, 18:1/18:1 and 18:0/18:1 are observed. Compared with human surfactant, all animal surfactant phosphatidylglycerol contains increased concentrations of PG 16:0/16:0 and PG 16:0/18:2, and their phosphatidylinositols are essentially monounsaturated except for a high content of PI 18:0/20:4 (29%) in the rat (Postle 2001). The hydrophobic proteins, SP-B and SP-C, together with dipalmitoylphosphatidylcholine, confer surface tension-lowering properties to the material (Han 2015).

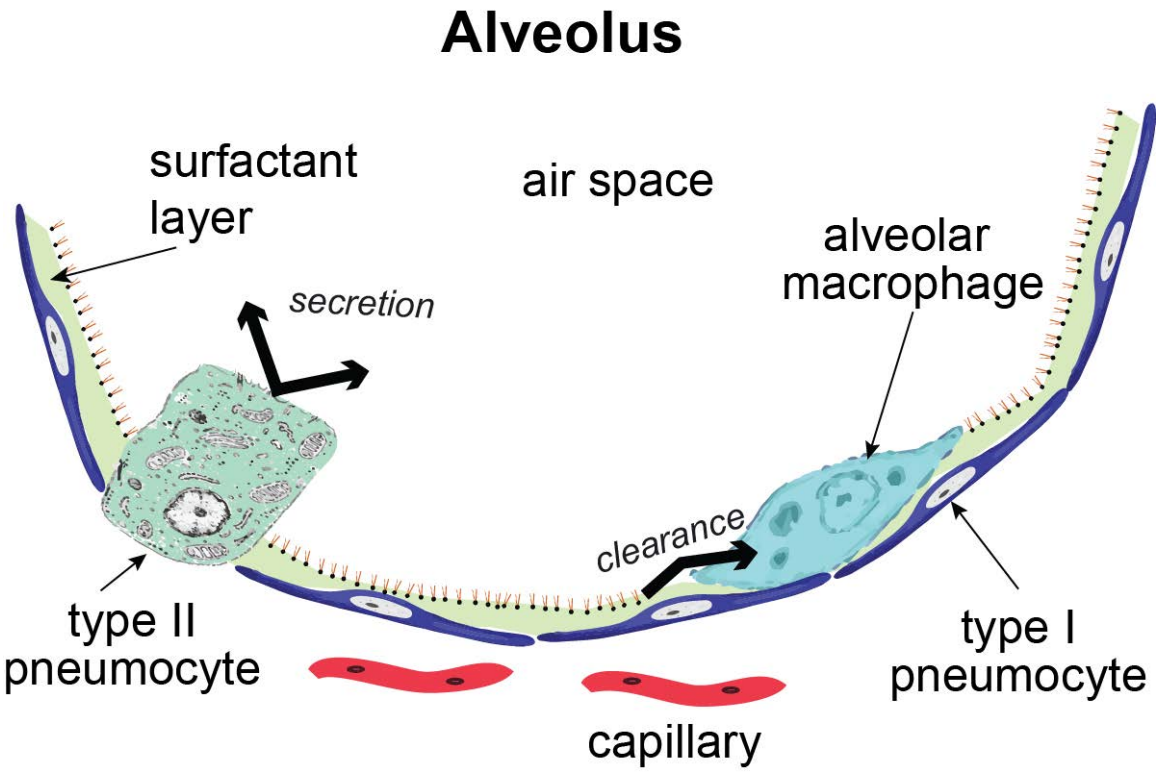


Figure 3. Lung surfactant

The composition of lung surfactant secreted by type II pneumocytes (also called alveolar type II cells), is critical to lung function.

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Surfactant alterations were reported in several lung diseases including COPD (Griese 2015, Lomas 2009, Ohlmeier 2008, Winkler 2011), and cigarette smoking directly affects surfactant levels (Hirama 2007, Kida 1997, Mazur 2011b). Lusuardi *et al.* reported a six- to seven-times lower total phospholipid level in bronchoalveolar lavage fluid (BALF) from smoking COPD patients compared with healthy nonsmokers, with similar composition except for phosphatidylglycerol-cardiolipin, which appeared significantly increased in COPD patients (Lusuardi 1992). Recently, Morissette *et al.* presented an intriguing model for how oxidative damage of surfactant lipids that are taken up by alveolar macrophages can contribute to the induction of an inflammatory response (Morissette 2015b). In developing rat lungs, the concentration of palmitoylmyristoyl-phosphatidylcholine (16:0/14:0PC) increased during alveolarization, while it specifically decreased in rats and humans with bronchopulmonary dysplasia, a chronic defect with reduced alveolarization, and also in humans with emphysema compared with healthy individuals (Ridsdale 2005).

Surfactant lipid composition and homeostasis can also be altered by changes in lung protein functions: ATP-binding cassette A3 (ABCA3) is a lipid transport protein in alveolar type II cells required for synthesis and storage of pulmonary surfactant and its deletion in mice or mutational inactivation in a rare form of human interstitial lung disease causes changes in surfactant lipid composition, such as strong reductions in saturated and monounsaturated phosphatidylcholine species with 30 and 32 carbons and almost all phosphatidylglycerol species (Besnard 2010, Griese 2015). The surfactant proteins SP-B and SP-C can be directly modified by oxidative degradation of amino acid residues and deacetylation, respectively, when exposed to an experimental environmental tobacco smoke surrogate in an artificial surfactant film (Stenger 2009). Specific reductions in SP-B concentrations in BALF, but no SP-B gene expression changes in lung tissue, were observed in rats chronically exposed to cigarette smoke (Subramaniam 1996).

3.2 Sphingolipids in smoking and lung disease

Sphingolipids have been shown to play important roles in the pathobiology of lung diseases with the pro-apoptotic effects of ceramides amongst the best documented (Petrache 2013). Ceramides have been implicated as crucial mediators of alveolar destruction. Inhibition of *de novo* ceramide synthesis prevented alveolar cell apoptosis and oxidative stress in a rodent model of emphysema (Petrache 2005). In tumor-free airway epithelial cells from lung adenocarcinoma patients, a lipid class distribution shift towards unusually high ceramide levels was observed, as well as in a mouse model of allergic asthma (Zehethofer 2015). Higher ceramide expression correlated with increased autophagy and apoptosis rates and with decreased gas exchange in the lungs of COPD patients compared with non-COPD smokers. However, ceramides also increased in idiopathic pulmonary fibrosis patients, but correlated with increased proliferation instead of autophagy and cell death, indicating that ceramide increases are a non-specific marker of lung damage (Scarpa 2013). Bodas *et al.* observed significantly elevated lactosylceramide (LacCer)-accumulation in human COPD lungs with increasing severity of emphysema over non-emphysema controls. Increased expression of the defective autophagy marker p62 in lung tissue of severe COPD patients suggest that LacCer-

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induced aberrant-autophagy may contribute to the pathogenesis of cigarette smoke-induced emphysema. LacCer-accumulation as a mechanism to initiate aberrant-autophagy (p62-accumulation) and apoptosis were also demonstrated in both bronchial-epithelial cells (BEAS2B) and murine macrophages (Raw264.7) exposed to cigarette smoke extract, and the effect was rescued by pharmacological inhibitor of LacCer-synthase (Bodas 2015). Likewise, the increase in ceramides and impairment in lung function during early elastase-induced lung inflammation in a mouse model of emphysema could be attenuated by a specific serine palmitoyltransferase inhibitor, but not by a sphingomyelinase inhibitor. However, for cigarette smoke-induced oxidative stress leading to ceramide generation and subsequent apoptosis in human airway epithelial cells, an alternative mechanism has been proposed by Chung *et al.:* neutral sphingomyelinase 2 is the sole sphingomyelinase activated during cigarette smoke-induced oxidative stress of human airway epithelial cells, leading to ceramide generation and subsequent apoptosis of affected cells. The tyrosine kinase Src regulates this ceramide generation, leveraging the activity of p38 MAPK, which phosphorylates neutral sphingomyelinase 2 and thereby modulates its activity during cigarette smoke-induced oxidative stress, both *in vitro* (human airway epithelial cells) and *in vivo* (mice) (Chung 2015).

Sphingosine-1-phosphate (S1P) and S1P receptors have been characterized as mediators in the pathobiology of fibrosis and acute lung injury (Natarajan 2013), and animal models suggest a role for ceramide and S1P in the pathogenesis of COPD as well (Tibboel 2014). In contrast to the pro-apoptotic effects of ceramide, S1P suppresses apoptosis (Maceyka 2002) and is regarded as vital for lung homeostasis and maintenance of alveolar septal integrity (Brinkmann 2006). Importantly, administration of sphingosine to mouse lungs with vascular endothelial growth factor blockade-induced emphysema decreased the ceramide to S1P ratio and inhibited alveolar space enlargement along with activation of pro-survival signaling pathways and decreased lung parenchyma cell apoptosis. However, this protection against ceramide-induced apoptosis was only seen in endothelial, not epithelial, cells *in vitro* (Diab 2010).

The important role that these molecular lipid species play in tissue homeostasis and pathobiology of lung disease (for a recent review, see (Lee 2015)) has attracted significant attention in the development of drugs targeting sphingomyelinases, sphingosine kinases, and S1P receptors for the treatment of various respiratory diseases (Yang 2011). In a recent paper, Barnawi *et al.* investigated phagocytosis-impaired macrophages derived from bronchoalveolar lavages of smoking COPD patients compared with macrophages from healthy nonsmokers and reported significant changes in several members of the S1P signaling system (mRNA of sphingosine kinases (SPHK1/2), S1P receptors (S1PR1-5), and S1P-degrading enzymes (SGPP1, SGPP2, SGPL1)). Cigarette smoke extract significantly increased mRNA expression of SPHK1, SPHK2, S1PR2, and S1PR5 by THP-1 macrophages, confirming the results in patient-derived macrophages (Barnawi 2015). A similar panel of probes had been applied already to investigate the S1P signaling system in tissue samples from COPD and non-COPD lungs, and revealed a lower expression of S1PR5 and other S1P receptors in COPD tissues while the enzymatic activity of sphingosine kinase was increased (Cordts 2011).

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3.3 Oxylipins/eicosanoids in smoking and lung disease

Eicosanoids derived from arachidonic acid, as well as compounds derived from the oxygenation of PUFA of different chain lengths, are grouped under the term oxylipins. These are a set of substances involved not only in respiratory pathology (Boyce 2008, Dahlen 1987, Lundstrom 2011, Samuelsson 1987), but also in cardiovascular and renal diseases (Hao 2007, Roman 2002, Shearer 2009). The major types of eicosanoids, hydroxyeicosatetraenoic acids (HETE), thromboxanes, prostaglandins/prostanes and leukotrienes, and their general pro- and anti-inflammatory activities have already been described in the CVD section. Prominent changes in tissue levels of eicosanoids are important in the development of COPD (Lundstrom 2011), and various lipid mediators including eicosanoids (e.g., PGE2 and prostaglandin D2 (PGD2)) have been implicated in the inflammatory component that accompanies emphysematous disease (Balgoma 2013). In general, the CYP2C/CYP2J-derived oxidation products of arachidonic acid, EET and dihydroxyeicosatrienoic (DHET) acids, elicit anti-inflammatory effects, whereas CYP4A/CYP4F-derived 20-HETE is pro-inflammatory (Theken 2011). 15-HETE regulated MUC5AC expression via modulating metalloproteinase-9, MEK/ERK/Sp-1, and PPARγ/PTEN/Akt signaling pathways in phorbol 12-myristate 13-acetate-treated human bronchial carcinoma cells (Song 2015). EET reduced lung immune responses (Morin 2009, Norton 2012).

EET and DHET have also been implicated in pulmonary vasoregulation. Their acute (5–45 min) induction by agents mimicking bacterial infections caused pulmonary vasoconstriction, and the concomitant increase in leukotrienes and prostanoids was responsible for vascular leakage and edema in isolated perfused human and rabbit lungs (Kiss 2010). Hypoxia also increased 14,15-EET in mice thereby causing pulmonary vasoconstriction and higher right ventricular systolic pressure (both symptoms of pulmonary hypertension) (Kandhi 2015). In contrast to the initial increase in these eicosanoids, a decrease in their levels was observed in the lungs of mice at 24 h after lipopolysaccharide stimulation, and baseline levels were reached again at 48 h (Theken 2011). Cigarette smoke-exposed mice had less pulmonary inflammation after 10 days of inhalation when processing of anti-inflammatory EET to pro-inflammatory DHET was blocked by pharmacological inhibition or EPHX2 activity (Podolin 2013), while short cigarette smoke exposure (3 days) did not reduce leukocyte recruitment to the lungs (Davis 2011).

In a 7-month cigarette smoke inhalation study on C57BL/6 mice, we previously observed, in addition to numerous cigarette smoke-related changes in other lipid classes (ceramides, phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols), marked increases in the abundance of several eicosanoids (e.g., PGD2, PGE2 and TXB3) and sphingosine and sphingosine-1-phosphate, as well as decreases in arachidonic acid. Within the class of gangliosides, GD1 and GM1 increased in abundance, while certain GM3 species increased and others decreased. All lipid changes were fully reversible within a 5-month cessation period following 2 months of cigarette smoke exposure (Titz 2016).

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Accumulation of saturated fatty acids (14:0, 15:0, 18:0) as well as arachidonic acid, the substrate of pro-inflammatory leukotriene B4 (LTB4) and PGD2, has been reported in erythrocyte membranes of patients with chronic bronchitis and COPD (Novgorodtseva 2013). COPD patients during late phase exacerbations also present reduced production of lipoxin A4 (LXA4) and an increased LTB4/LXA4 ratio, reflecting a pro-inflammatory balance (Balode 2012).

Pharmacological blockade of the PGD2 G protein-coupled receptor DP2 inhibited smoke-induced inflammation, mucus cell metaplasia, and epithelial hyperplasia in mouse lungs (Stebbins 2010). PGE2, another arachidonic acid derivative, induces lung fibroblast senescence, a phenomenon associated with COPD. Senescence and inflammation stimulated by PGE2 are enhanced in COPD patients owing to upregulation of PGE2 receptors EP2, EP4 (Dagouassat 2013), and PGE2 acted as a bronchodilator on human airway rings selectively via the EP4 receptor subtype (Benyahia 2012). In contrast, TXA2 induces bronchoconstriction via the thromboxane receptor on smooth muscle cells but only when muscarinic acetylcholine receptor M2 is also expressed (Allen 2006). In a mouse model of bleomycin-induced lung fibrosis, suppression of cysLTs/PGE2 and TXA2 pathways allowed the control of airway inflammation and pulmonary fibrosis (Kurokawa 2010). TXA2 signaling can also increase mucus hypersecretion. In rats exposed to cigarette smoke, the hyper-production of airway mucus was attenuated by seratrodast, a TXA2 receptor antagonist (An 2013). Thromboxane metabolite levels in the plasma and urine of COPD patients are elevated, possibly via hypoxia-induced thromboxane release from platelets, and thereby may contribute to increased atherothrombosis risk in COPD patients (Fimognari 2008). Animal studies showed that eicosapentaenoic acid or gamma-linolenic acid supplementation of animals exposed to endotoxins results in decreased effects on TXB2 and pulmonary vascular resistance. However, the results of epidemiological studies on the protective effect of dietary n-3 fatty acid consumption against COPD were mixed (Schwartz 2000, Stebbins 2010). Taken together, the opposing effects of cigarette smoke on PGD2/PGE2 and EET/DHET synthesis and on thromboxane release may contribute to COPD-related lung inflammation, airway remodeling, and pulmonary hypertension.

There is increasing evidence that mast cells, in addition to their well-recognized role in allergic respiratory diseases, can also contribute to the pathogenesis of COPD and other smoking-related lung diseases (Crotty Alexander 2015, Cruse 2015, Erjefalt 2014). In asthma, they can elicit leukocyte recruitment by producing histamine, leukotrienes, and prostaglandins, thus increasing vascular permeability and permitting rapid influx of neutrophils and other leukocytes such as monocytes. Through interactions with lung epithelial cells, neutrophils produce lipoxins, in particular, leukotriene A4, which interact with their receptor(s) regulating neutrophil apoptosis and promoting phagocytosis of apoptotic neutrophils by monocyte-derived macrophages to clear inflamed tissue (Barnig 2013, Barnig 2015, Levy 2014, Serhan 2007). In COPD patients, an enrichment of activated mast cells was observed in perivascular regions and their number correlated with improved lung function—this was interpreted as adaptive vascular remodeling as a consequence of chymase secretion by mast cells (Kosanovic 2014). In smokers undergoing lung resection for primary cancer, pre-operative lung function and an EBC

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test correlated with post-operative histological analysis of inflammatory cells (neutrophils, macrophages, mast cells) in non-tumor tissue, revealing the best correlation for mast cells with lung function parameters and 8-isoprostane in exhaled breath (Nussbaumer-Ochsner 2015). Cysteinyl leukotriene (LTD4) receptor expression in resident lung leukocytes increased with severity of COPD and was highest in mast cells of bronchial biopsies from patients with severe exacerbations (Zhu 2012), and the release of PGD2 from a subgroup of mast cells from chronic bronchitis patients compared with mast cells from healthy individuals (van Overveld 1989). Inhaled corticosteroids were effective at reducing the frequency of mast cells in bronchial biopsies from COPD patients (Gizycki 2002). LTD4 and TXA2 both contribute to the stimulation of airway smooth muscle cell proliferation during airway remodeling in COPD and asthma, and also *in vitro* (Capra 2014b).

The formation of immunoresolving agonists, such as lipoxins, resolvins, protectins and maresins, appears to be defective in several common lung diseases that are characterized by excess or chronic inflammation (Levy 2014). As already discussed in the CVD context, certain isoprostanes, particularly the cyclopentenone A2-isoprostane, can act in an anti-inflammatory manner. In human lung epithelial cells *in vitro*, A2-isoprostane was efficient at blocking ozone-induced NFκB activation and downstream IL-8 secretion by covalently modifying the active Cys(179) domain in inhibitory kappaB kinase, and A2-isoprostane levels were higher in NQO1-deficient mice, thereby protecting their lungs against ozone toxicity (Kummarapurugu 2013).

Less frequently, the roles played by isoprostanes or prostaglandin-like compounds non-enzymatically generated via peroxidation of membrane phospholipids by ROS, in particular F2-isoprostanes, in pulmonary pathophysiology have been highlighted (Rolin 2006). However, leveraging these molecules as general markers of oxidative stress (biomarkers of biological effect) has been more common than assigning to them an active role in the pathogenesis of COPD.

3.4 Lipids in induced sputum and EBC

The analysis of sputum or EBC has gained attention as a non-invasive end point in human studies that provides a rich source of exfoliated superficial cells from the airways and deeper lung, as well as dissolved biomarker molecules including cytokines, ROS/reactive nitrogen species and lipid mediators. The initial difficulties in standardization of sample generation and quantification of the highly diluted biomarkers, particularly in EBC, have become less prominent with the availability of highly sensitive liquid chromatography–tandem mass spectrometry or liquid chromatography–quadrupole time-of-flight mass spectrometry methods during the last decade (Antus 2012, Antus 2015, Malerba 2012, Rahman 2005, t'Kindt 2015).

Telenga *et al.* investigated the sputum lipidome in cigarette smokers, COPD patients, and subjects after smoking cessation using a sensitive lipidomics approach. Out of a total of more than 1500 lipid species from all classes, the most significant difference between smokers with and without COPD was seen in the sphingolipids. Out of 168 elevated sphingolipids in smokers, the 13 lipids with the highest increases in smokers correlated with lower lung function and higher inflammatory markers in sputum, and smokers

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without COPD could be distinguished from never smokers based on 20 (glyco)sphingolipids; moreover, a 2-month cessation period caused reduced levels in 26 sphingolipids (t'Kindt 2015, Telenga 2014). In a systems biology network building approach, Jamalkandi *et al.* investigated sputum lipidomics, metabolomics, and proteomics data from the literature and were able to identify, in clinical data, increased levels of sphingolipids that upregulated arachidonic acid metabolism and correlated with hypoxia via glucose-derived energy supply (Azimzadeh Jamalkandi 2015).

EBC collection from exhaled breath in a cooled tube is a completely non-invasive method. EBC is mainly formed by water vapor but also contains aerosol particles in which a variety of biomolecules including leukotrienes, prostaglandins, and isoprostanes, as well as hydrogen peroxide, NO-derived products, and hydrogen ions, have been measured in healthy and diseased subjects. Some inflammatory mediators are elevated in patients with COPD (Montuschi 2005). Compared with induced sputum analysis, methodological factors and the lack of standardized data that can be compared across studies are still inconsistent with clinical routine applications (Loukides 2011, Malerba 2012, van Beurden 2002). Fritscher *et al.* found higher levels of PGE2, LTB4, 11-12 EET, and arachidonic acid, while lower levels of LXA4, 11DHyTxB2, 11HETE, and 8,9EET were detected in EBC from COPD and asthmatic subjects when compared with healthy controls. The authors concluded that the analysis of EBC through mass spectrometry was mixed and did not distinguish between asthma and COPD (Fritscher 2012).

Oxidative stress markers such as hydrogen ions, nitrosothiols, nitrite/nitrate hydrogen peroxide, 8-isoprostanes, and thiobarbituric acid reactive products (such as acrolein, MDA, and 4-HNE), have been identified in EBC of COPD patients. The most studied substance is hydrogen peroxide, and its level in EBC is elevated in numerous inflammatory diseases; 8-isoprostane was also frequently measured in EBC (Lee 2009, Vass 2003). However, strenuous physical activity-induced hyperpnea could also increase the level of 8-isoprostane in EBC, as demonstrated in competitive swimmers (Morissette 2015a).

As was demonstrated for induced sputum lipidomics, co-evolution of standardized sampling methods and high-sensitivity/high-resolution liquid chromatography–mass spectrometry-based methods may enable the use of lipid analysis in EBC in forthcoming clinical studies.

4 ASSESSMENT OF LIPID-RELATED DISEASE MECHANISMS IN *IN VIVO* MODELS

4.1 Biomarkers reflecting overall oxidative damage in *in vivo* models

To our knowledge, there is no single biomarker capable of reflecting the complexity of cellular stress responses to exogenous stressors such as cigarette smoke. Therefore, to be able to monitor cellular stress responses in smokers, a combination of biomarkers is required, and this combination will most likely consist of markers traditionally employed to measure oxidative stress and multi-molecule signatures or profiles derived from large-scale data sets such as those provided by transcriptomics, proteomics, or lipidomics.

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In the context of tobacco product assessment, the 2001 Institute of Medicine report "Clearing the Smoke" provides a starting point for the selection of biomarkers that are reflective of cigarette smoke-induced harm and might be indicative of heightened disease risk in smokers (Stratton 2001b). Among the listed markers are 8-hydroxy-2′-deoxyguanosine (8-OHdG) and F2-isoprostanes for the assessment of oxidative damage to DNA and lipids, respectively. In addition, the report also proposes transcriptomics, proteomics, and methylation analysis as useful tools for the assessment of novel tobacco products.

8-OHdG is a major and ubiquitous product of DNA oxidation (Helbock 1999), and quantification of 8-OHdG can serve as an indicator of the extent of oxidative DNA damage (Cooke 2008). In addition, formation of lipid peroxidation products or protein modifications resulting from interaction of ROS with intracellular macromolecules can be utilized to assess damage resulting from uncompensated oxidative stress at the organism level, preferably in body fluids such as blood plasma and urine, while at the organ level, they can be determined immunohistologically in tissues or biochemically in homogenates.

Similar to the assessment of oxidative damage in humans, biomarkers such as 8-OHdG and F2-isoprostanes can also be used in animal models to estimate oxidative damage to DNA and lipids, as has been shown in models of acute inflammation or oxidative stress (Basu 2008). 8-iso-PGF2α levels were shown to increase in urine of male Apoe$^{-/-}$ mice following exposure to cigarette smoke for 4 days (Catanzaro 2007), in urine and serum of male Sprague-Dawley rats exposed to cigarette mainstream smoke for 56 days (Chan 2009) or side-stream smoke for 4 days (Visioli 2000), and in serum of female Hartley guinea pigs following 6-month exposure to cigarette smoke (Churg 2012). Further, a study by Guan *et al.* showed not only elevated 8-OHdG and 8-iso-PGF2α BALF levels, but also an increase in BALF 3-nitrotyrosine, a marker of oxidative protein damage, when female Balb/c mice were exposed to cigarette smoke for 5 days (Guan 2013). In C57Bl/6 mice, acute, 1-h cigarette smoke exposure resulted in a transient increase in oxidative stress, as seen by a significant increase in 8-OHdG levels in BALF at 1-h post-exposure, which declined to undetectable levels at 24-h post-exposure. Oxidative lung damage was further confirmed by positive immunostaining for 8-OHdG and 4-HNE, a lipid peroxidation end product, in bronchiolar and alveolar epithelium of exposed mice (Aoshiba 2003).

We conducted an evaluation on published reports where treatment or genetic changes resulted in significantly increased urinary isoprostane concentrations (Supplementary Table 2). This survey revealed high variability in daily amounts excreted in urine from untreated wild type control mice. Typical values ranged from 0.15 to 4 ng/day when calculated for 25 g body weight and 1 mL urine/day, and single studies reported even higher normal levels. Not only differences in analytical methods but also difficulties in obtaining the most representative, complete, and well-preserved urine samples from mice may have contributed to these large differences. It is essential that these parameters are kept as constant as possible within a study. A smaller number of published murine studies reported 8-iso-PGF2α levels in plasma and normal levels in control mice varied from 17 to 508.5 pg/mL (Supplementary Table 2). Values could be increased up to 30-fold under

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increased oxidative stress, e.g., in vitamin D-receptor-deficient mice treated with the radical initiating drug 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (Yoshida 2008).

It has been reported that in animal models, unlike in humans, a high fraction of isoprostanes can be generated enzymatically by prostaglandin-endoperoxide synthases, which are significantly induced in inflammation. In rats, this prostaglandin-endoperoxide synthase-dependent 8-iso-PGF2α could make up 97% of the plasma concentration; therefore, it was suggested to estimate the amount of isoprostanes that were exclusively the result from radical oxidation processes by calculating the ratio of 8-iso-PGF2α/PGF2α (van 't Erve 2015). Although the absolute values varied significantly, these studies indicate that in addition to 8-OHdG, F2-isoprostane and 4-HNE or other lipid peroxidation products can be successfully employed in animal models as indicators of oxidative damage.

4.2 *In vivo* CVD models and lipid changes

While providing significant new insights into isolated mechanisms underlying atherogenesis in humans, *in vitro* models can never fully reproduce the interplay between the different cell types in an organism and the spectrum of biological processes occurring during the development of atherosclerosis. However, many gaps in our understanding of these processes can be filled by knowledge gained from animal models, and CVD research has employed such models since the early 1900s (Leong 2015).

While atherosclerosis can be observed in many species fed a high-fat diet including hamsters, guinea pigs, rabbits, dogs and pigs, the mouse remains the most frequently used animal model owing to its easy maintenance and low cost (Getz 2012). However, mice are rather resistant to diet-induced atherogenesis, and it is known that, unlike in humans, HDL-cholesterol is the main lipoprotein in mice and mice do not express cholesteryl ester transfer protein (CETP), a plasma protein facilitating exchange of esterified cholesterol and TAG from lipoproteins (Getz 2012). In addition, atherosclerotic lesions in the mouse also differ from human disease in other aspects. Lesions in mice preferentially form in the aortic root (resembling the situation seen mainly in atherosclerosis patients suffering from familial hypercholesterolemia) and typically do not develop a fibrous cap, so unstable plaques are uncharacteristic in the mouse (Getz 2012). Several of these disadvantages, however, could be addressed by the generation of genetically modified mice.

Since there is no single ideal animal model that completely displays all features of human disease, various animal models have been developed to address specific aspects of human CVD, of which the ApoE knockout ($Apoe^{-/-}$) model has become the most widely studied atherosclerosis model (Jawien 2012, Zaragoza 2011). Developed in the early 1990s, this model was shown to be hypercholesterolemic even on a normal chow diet and to exhibit the full spectrum of lesions occurring during the development of atherosclerosis in humans (Nakashima 1994, Plump 1992, Zhang 1992). It should be noted that the hyperlipidemic lipoprotein profile in the $Apoe^{-/-}$ mouse is enriched in LDL, VLDL, and atherogenic lipoprotein remnants while HDL is strongly attenuated compared with wild type mice (Hofker 1998). Hence, a direct comparison of the $Apoe^{-/-}$ mouse lipoprotein

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profile with those from humans or wild type mice is not meaningful. Other murine atherosclerosis models leverage, e.g., knockout of the LDL receptor, thereby increasing LDL levels in response to cigarette smoke exposure more explicitly than observed in Apoe$^{-/-}$ mice (Han 2012), or transgenic expression of the human APOE*3 allele in "ApoE-Leiden" mice (Zadelaar 2007). More recently, the human CETP gene has been added to the APOE*3 transgene to more closely mimic human lipoprotein behavior (Wang 2011). Studies using the APOE*3-Leiden mouse, or the Apoe$^{-/-}$ mouse with additional genetic modifications (knockout of Rag2 or P-selectin, or overexpression of C-reactive protein), confirmed that chronic inflammation plays a major role in the development of atherosclerotic lesions (Daugherty 1997, Dong 2000, Khedoe 2013, Paul 2004).

Experimental evidence suggests that cigarette smoke exposure consistently accelerates atherosclerosis in Apoe$^{-/-}$ mice, with increased plaque volume and plaque area in exposed animals relative to control animals (Catanzaro 2007, Gairola 2001, Gairola 2010, Kunitomo 2009, Tani 2004, Von Holt 2009).

In a model of endothelial injury, cigarette smoke-exposed Apoe$^{-/-}$ mice had accelerated thrombosis and developed increased thickness of neointima lesions due to smooth muscle cell hyperplasia in the carotid artery (Schroeter 2008). In Apoe$^{-/-}$ mice chronically (6 or 8 months) exposed to cigarette smoke, global lipid profiling by lipidomics demonstrated substantial accumulation of sterol lipids (such as cholesteryl esters), glycerophospholipids, and sphingolipids (ceramides and sphingomyelins) in the aortic wall when compared with air-exposed mice, while only inconsistently changed levels of a few glycerolipids, glycerophospholipids, and ceramides were observed in the plasma (Boué 2012, Phillips 2015b). Importantly, in Apoe$^{-/-}$ mice that were first exposed to cigarette smoke for 3 or 2 months before being switched to air exposure for another 3 or 6 months (mimicking smoking cessation), the aortic plaque area was smaller than that in mice continuously exposed for 6 or 8 months, with aortic arches containing approximately 50% less TC and overall reduced lipid levels (by lipid class) in plaques and plasma in both studies (Lietz 2013, Phillips 2015b), indicating that this mouse model also recapitulates the cardiovascular benefit of smoking cessation that was seen in humans (Bakhru 2005, Gepner 2011).

Consistent with the leading role of the liver in lipid metabolism, the hepatic lipidome from Apoe$^{-/-}$ mice exposed to cigarette smoke for 6 months was also perceptibly affected. A trend of increased levels of some glycerolipids, glycerophospholipids, and sterol lipids, as well as statistically significantly higher levels of diacylglycerophosphoinositols and a number of sphingomyelin species were reported (De Leon 2013), and similar trends were also observed in a recent 8-month cigarette smoke exposure study (Lo Sasso2016). Further, we and others also noted a distinct pulmonary inflammatory response to cigarette smoke in the Apoe$^{-/-}$ mouse, characterized by influx of leukocytes into the lungs, typical lipid changes, and elevated levels of pro-inflammatory mediators in BALF, eventually culminating in alveolar destruction reminiscent of emphysema (Arunachalam 2010, Boue 2013, Phillips 2015b). This suggests that the Apoe$^{-/-}$ mouse model may also prove useful in COPD research, in particular, when addressing questions regarding cigarette smoke-related comorbidities. Details will be discussed in the next chapter.

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For completeness, it should be mentioned that numerous experimental, genetic, and transgenic rat models have been developed to address cardiac hypertrophy, arrhythmia, myocardial infarction, and other heart diseases (Bader 2010, Szekeres 1986). With regard to the context of this documentation, relatively little work has been published on the effects of lipids on these cardiac parameters, particularly in the context of cigarette smoke exposure. A few examples of direct lipid effects on cardiac function are interventions against arrhythmia with a linoleic acid-rich diet to change the phospholipid composition of cardiac cell membranes (and their electrical properties) (Szekeres 1986), or the role of LDL in the transport of infarct size-increasing C-reactive protein to the site of infarction (Singh 2008). Although the spontaneously hypertensive rat model has elevated free fatty acid levels, the three published papers on cardiac cigarette smoke effects in this model do not indicate any relationship to blood lipids or lipid mediators (Meurrens 2007, Tanaka 2004, Valenti 2010).

4.3 *In vivo* COPD models: opportunities and limitations

COPD is a heterogeneous disorder with varying contributions of lung inflammation, chronic obstructive bronchiolitis, mucus plugging, and emphysema, and with a systemic inflammatory component in its advanced stage. *In vitro* models are very helpful in addressing mechanistic aspects at the cellular/tissue level; however, the pathophysiological complexity in an organismal context can only be investigated in *in vivo* disease models. As recently reviewed by Vlahos & Bozinovski, many species have been used, including rodents, dogs, guinea-pigs, monkeys, and sheep. Mice offer the greatest ability to investigate the pathogenic pathways of disease given the low cost, the ability to produce animals with genetic modifications that shed light on specific processes within COPD, the plethora of information about the mouse genome, the abundance of antibody probes, and the availability of numerous naturally occurring mouse strains with different reactions to smoke. Although mice and humans share many basic physiological processes, animal models should be one component of the process for studying human disease, and should be integrated with human *in vitro* and *in vivo* studies (Vlahos 2014). The availability of systems-wide technologies (e.g., transcriptomics, proteomics, and metabolomics/lipidomics) have enabled pulmonary disease processes following cigarette smoke exposure *in vivo* or *in vitro* to be analyzed using complex global data sets encompassing thousands of differentially expressed molecular species. These investigations have made significant inroads into our understanding of the temporal and cell-type specific complexities of COPD disease processes. With support from computational modeling, such systems-wide datasets and knowledge bases may provide important cues for non-clinical to clinical translation (Adair-Kirk 2008, Azimzadeh Jamalkandi 2015, Cabanski 2015b, Cano 2014, Heguy 2006, Mercer 2015, sbv 2015).

Table 1 from Vlahos & Bozinovski (Vlahos 2014) provides an overview of COPD features that can or cannot be modeled in mice. Detailed aspects are discussed in the following paragraphs.

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Table 1 Features of COPD that can/cannot be modelled in cigarette smoke-exposed mice (from (Vlahos 2014))

Can be modelled	Cannot be modelled
BALF/lung inflammation, including neutrophilia, accumulation of macrophages and T-cells, and lymphoid aggregates/follicles	Chronic bronchitis
Increase in BALF/lung inflammatory mediators, including cytokines, chemokines and proteases	Severe disabling disease observed in GOLD stage 3 to 4
Increased oxidative stress	
Emphysema	
Small airway and vascular remodelling	
Pulmonary hypertension	
Mucus hypersecretion	
Impaired lung function	
Systemic co-morbidities (e.g. cardiac dysfunction and peripheral skeletal muscle wasting)	
Increased BALF/lung inflammation in response to respiratory pathogens associated with infectious exacerbations	
Persistence of BALF/lung inflammation following smoking cessation	

Our current knowledge about the role of inflammation in cigarette smoking-related lung disease is built on decades of research into the molecular underpinnings of specific inflammatory processes including epithelial pro-inflammatory responses or macrophage activation following exposure of individual cell types to cigarette smoke (de Boer 2000, Mio 1997, Murugan 2009, Parsanejad 2008, Russell 2002).

Many cellular and molecular mechanisms involved in the pathogenesis of COPD in humans are also observed in murine pulmonary emphysema models (Brusselle 2006), with several features shared between human early stage disease and mouse disease development that primarily recapitulate the emphysema component (Churg 2011). Lesions in mice caused by chronic cigarette smoke exposure resemble centrilobular human emphysema both morphologically and physiologically (Churg 2008). Despite these anatomical differences, cigarette smoke-related lung function impairment in emphysematous mice with decreased elastic recoil and increased compliance resembles typical emphysematous features in the human lung (March 2006, Phillips 2015c, Vanoirbeek 2010). Small airway remodeling, an important cause of airflow obstruction in cigarette smokers with COPD (Hogg 2004), was also observed in cigarette smoke-exposed mice (Churg 2008). As observed in humans, the small airways from smoke-exposed C57BL/6 mice exhibited persistent up-regulation of type I pro-collagen and pro-fibrotic cytokine gene expression, which would be expected to contribute to airway remodeling (Churg 2006). In addition, cigarette smoke exposure activates the innate immune response cascade in mice, leading to protease/anti-protease imbalances in lung tissue and eventual alveolar wall degradation, suggesting that murine models can also replicate these features of human COPD (Yoshida 2007). The aspect of persisting lung inflammation after smoking cessation is also listed by Vlahos & Bozinovski under the pathologies that can be reproduced in mice (Table 1). However, this appears to depend on the mouse strain and the duration of the cessation period. We observed in mouse cigarette smoke inhalation studies a return to sham control levels for inflammatory markers in BALF and a strong decline in inflammatory bronchoalveolar lavage cells when post-exposure periods were sufficiently long, i.e., 2 to 7 months, although a low but significantly elevated degree of pigmented alveolar macrophages and macrophage nests

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persisted (Boue 2013, Phillips 2015b, Phillips 2015c). These histological effects were more pronounced in BALB/c mice (Hansen 2014) and in A/J mice (Stinn 2013).

Notwithstanding, murine models of COPD/emphysema have several limitations. First, these models only display a subset of disease phenotypes and do not fully recapitulate airflow limitations from chronic small airway obstruction, which defines COPD (Hogg 2004).

Second, there are considerable differences in respiratory physiology and anatomy between mice and humans, making disease modeling difficult. In contrast to humans, mice are obligate nose breathers and filter tobacco smoke differently. Additionally, the submucosal glands in mice are limited to the trachea, and mice lack a cough reflex, which further delineates the pulmonary physiology between the two species (Groneberg 2004). The airways are only scarcely populated with goblet cells, and no mucus plugging occurs. Importantly, pulmonary emphysema in humans is classified according to the distribution of the airspace enlargement within the acini in centriacinar emphysema, and in cigarette smokers with COPD, significant airspace enlargement occurs primarily across the three generations of respiratory bronchioles (Hogg 2004). However, rodents lack clearly defined respiratory bronchioles and distinct lobular architecture, further obscuring anatomically equivalent comparisons of disease development (March 2000).

Third, key species differences also exist in innate and adaptive immune responses, which play a critical role in the initiation and maintenance of disease. These include differences in the expression of defensins and toll-like and chemokine receptors, as well as a unique repertoire of active chemokines in each species. Differences in adaptive immunity include immunoglobulin subtypes, Th1/Th2 differentiation dynamics, and the roles and ratios of $CD4^+$ and $CD8^+$ T cell populations, together with co-stimulatory molecule expression and function (Mestas 2004).

Fourth, unlike in humans, cigarette smoke exposure-induced emphysema in mice does not progress after smoking cessation. It has been recognized that in human COPD, the characteristic feature of an abnormal inflammatory response with self-perpetuating properties is associated with an increased number and/or proportion of CD8+ T cells (Baraldo 2007, Saetta 1999, Saetta 2003, Tetley 2005), while disease progression after cessation was not usually observed in cigarette smoke-exposed mice. However, a study with CD8+ T cell-deficient mice demonstrated a blunted inflammatory response and failure to develop emphysema (Maeno 2007), and the transfer of CD4+ and CD8+ T cells from cigarette smoke-exposed mice was sufficient to induce inflammation and alveolar destruction in unexposed mice (Eppert 2013). The theory of an autoimmune component to advanced COPD in humans contributing to the self-perpetuating nature of the disease could not be reproduced in mice (Taraseviciene-Stewart 2005), while Hodge *et al.* observed elevated numbers of CD8+ T cells in COPD patients as well as in lungs from cigarette smoke-exposed mice (Hodge 2011). The involvement of B cell follicle formation was observed in emphysematous human lungs as well as in mouse lungs with cigarette smoke-induced alveolar damage, and suppression of B cell activation could suppress pulmonary inflammation and alveolar destruction in cigarette smoke-exposed mice (Seys 2015, van der Strate 2006). All these observations support the involvement of

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autoimmune mechanisms in COPD pathogenesis in humans and in mice, although the phenotypic manifestation of this component seems to be less pronounced in mice.

Finally, mice do not develop spontaneous exacerbations or the more severe COPD symptoms characteristic of the human Global Initiative on Obstructive Lung Disease (GOLD) stages III and IV. Despite these differences, the observed features of cigarette smoke-related lung disease suggest that mouse models can aid our understanding of key aspects, such as inflammation, underlying human COPD pathophysiology.

Though all symptoms of COPD may not be recapitulated in rodent lungs, cigarette smoke-exposed mice are particularly well-suited to model the early phases of human COPD (GOLD stages 1 and 2) (Churg 2008, Pauwels 2004, Rabe 2007), and it is expected that more aspects of this disease, including infectious exacerbations, will be recapitulated in *in vivo* models in the near future (Fricker 2014, Vlahos 2014).

4.4 Lipid-related biomarkers of tobacco heating system 2.2 aerosol vs. cigarette smoke exposure in rodent inhalation studies

In our *in vivo* studies, we investigated the effects of inhaled aerosols from the tobacco heating system (THS)2.2 or THS2.2 Menthol (THS2.2M) compared with conventional cigarette smoke-induced changes using subchronic (rats) or chronic (mice) inhalation study designs without or with various cessation periods of up to 6 months. In addition to the standard set of apical end points typical for Organisation for Economic Co-operation and Development (OECD) TG 413 studies (such as gross pathology, clinical chemistry, lung function, histopathology), we included transcriptomics, proteomics, and lipidomics analyses of the major target tissues and organs of cigarette smoke effects. Hence, we characterized the respiratory tract, blood and vascular system, and the liver to investigate molecular underpinnings of the disease mechanisms leveraging a systems toxicology approach (Sturla 2014). As a measure for overall oxidative damage, urinary levels of 8-iso-PGF2α and other lipid peroxidation products (4-HNE, MDA, and others) were determined. A general disadvantage of urinary biomarkers in mice is the difficulty to obtain reproducibly a significant amount of urine that is representative of the metabolic processes during the collection period (usually 24 h; in inhalation studies, often 18 h after end of exposure to avoid contamination with substances from the inhalation atmosphere).

Besides lipidomics, classical assays were applied to measure the lipid content and lipoprotein profiles considered to be relevant for atherosclerosis in rat and murine blood plasma. Routinely, clinical autoanalyzer methods were applied, and in the Apoe/THS2.2 switching study, an ultracentrifugation-based method was also applied to overcome the limitation of VLDL identification.

In the lung, cigarette smoke-induced effects on lipid metabolism were part of a broader exposure response including xenobiotic and oxidative stress responses and other metabolic adaptations. Among these lipid-related effects, we studied surfactant sphingolipid metabolism and several classes of ceramides in more detail.

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4.4.1 Rat-THS2.2 Menthol-TG413

To characterize toxicity and assess the hazard of the THS2.2M product, a battery of chemical and toxicological assays was conducted. Among the studies performed, the 90-day rat inhalation study, as described in OECD guideline 413 (OECD (Organisation for Economic Co-operation and Development) 2009), aimed to characterize the adverse effects caused by repeated daily inhalation exposure to THS2.2M for 90 days. This duration represents approximately 10% of the lifespan of a rat.

Data derived from subchronic inhalation toxicity studies are an essential element for quantitative risk assessments (regulatory toxicology) as part of the Evidence Package for the THS2.2 product. This is in accordance with the requirements for due diligence, i.e., to investigate whether human adult smokers could be exposed to additional hazards to those presented by smoking conventional cigarettes. Therefore, the study included groups of rats exposed (nose-only) to filtered fresh air (sham control) and to mainstream smoke of the reference cigarette 3R4F, and of two mentholated DDA3 reference cigarettes with a delivery of 1.2 mg and 2.4 mg menthol per cigarette, matched to the medium and high THS2.2M delivery, corresponding to 23 µg nicotine/L, or to a low, medium, and high concentration of the aerosol from THS2.2M, corresponding to 15, 23, and 50 µg nicotine/L, respectively, for 90 days, 6 h per day, 5 days/week (for details, see "15025 THS SR Part 1").

As in the previous OECD TG 413 study on THS2.2, the inhalation toxicology of THS2.2M based on classical end points such as in-life observations and exposure biomarkers, respiratory physiology, clinical observations and gross pathology, pulmonary inflammation (BALF analysis) and histopathology of the respiratory tract and non-respiratory tract organs and tissues revealed considerably lower exposure effects in rats exposed to THS2.2M compared with rats exposed to 3R4F or the mentholated reference cigarettes. Particularly, THS2.2M aerosol caused very limited inflammatory changes in the lung compared with cigarette smoke from the three conventional cigarettes. The reduced biological effects were consistent with the systems biology-related findings. Transcriptomics, proteomics, and lipidomics data from the lung and lipidomics data from blood plasma were also suggestive of reduced perturbation of toxicity pathways. Within the focus of this document, only the lipidomics results will be presented and discussed. For a detailed description of all results, see the study report of Rat-THS2.2M-TG413, which is included in this package: 15025_176500 THS SR.

Samples from the following groups were analyzed by lipidomics: sham, THS2.2M_23 (23 µg nicotine/L), THS2.2M_50 (50 µg nicotine/L), 3R4F_23 (23 µg nicotine/L), and DDA3_2xMIS_23 (23 µg nicotine/L).

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4.4.1.1 Lipids measured in blood plasma

As shown in the volcano plots for the combined male and female measurements (Figure 4 A–D), conventional cigarette smoke did not cause any increase in concentrations of blood lipids, but did induce a pronounced and statistically significant decrease in numerous lipid species, which was particularly strong for the class of glycerolipids and with a high degree of similarity between the mentholated and the non-mentholated reference cigarettes (Figure 4 A, B). For the THS2.2M aerosol, at the same nicotine level as the reference cigarettes, no increase was observed, and a considerably lower number of lipids, again predominantly glycerolipids, was observed but at a reduced magnitude and also with less significant p-values (Figure 4 C). Doubling the THS2.2M aerosol concentration to 50 µg nicotine/L led to an increase in this response, but it was still less intense than the reference cigarette smoke effects at half the nicotine concentration (Figure 4 D). The apparently significant single increase in one sterol lipid at the high THS2.2M concentration disappeared when male and female results were analyzed separately (Figure 5). This representation at the single lipid species level shows, like the sex-combined volcano plots, the same relationship for cigarette smoke with and without menthol with similar patterns in both sexes, with the exception of more pronounced decreases for a number of ceramides, Glc/GalCer, and LacCer in females. THS2.2M aerosol exposure caused similar decreases in a number of the same lipid species that were decreased in cigarette smoke-exposed rats, while other decreases did not reach statistical significance. The sex difference in the cigarette smoke response regarding Glc/GalCer was also seen in THS2.2M aerosol-exposed groups, though it was attenuated in the overall changes in THS2.2M groups.

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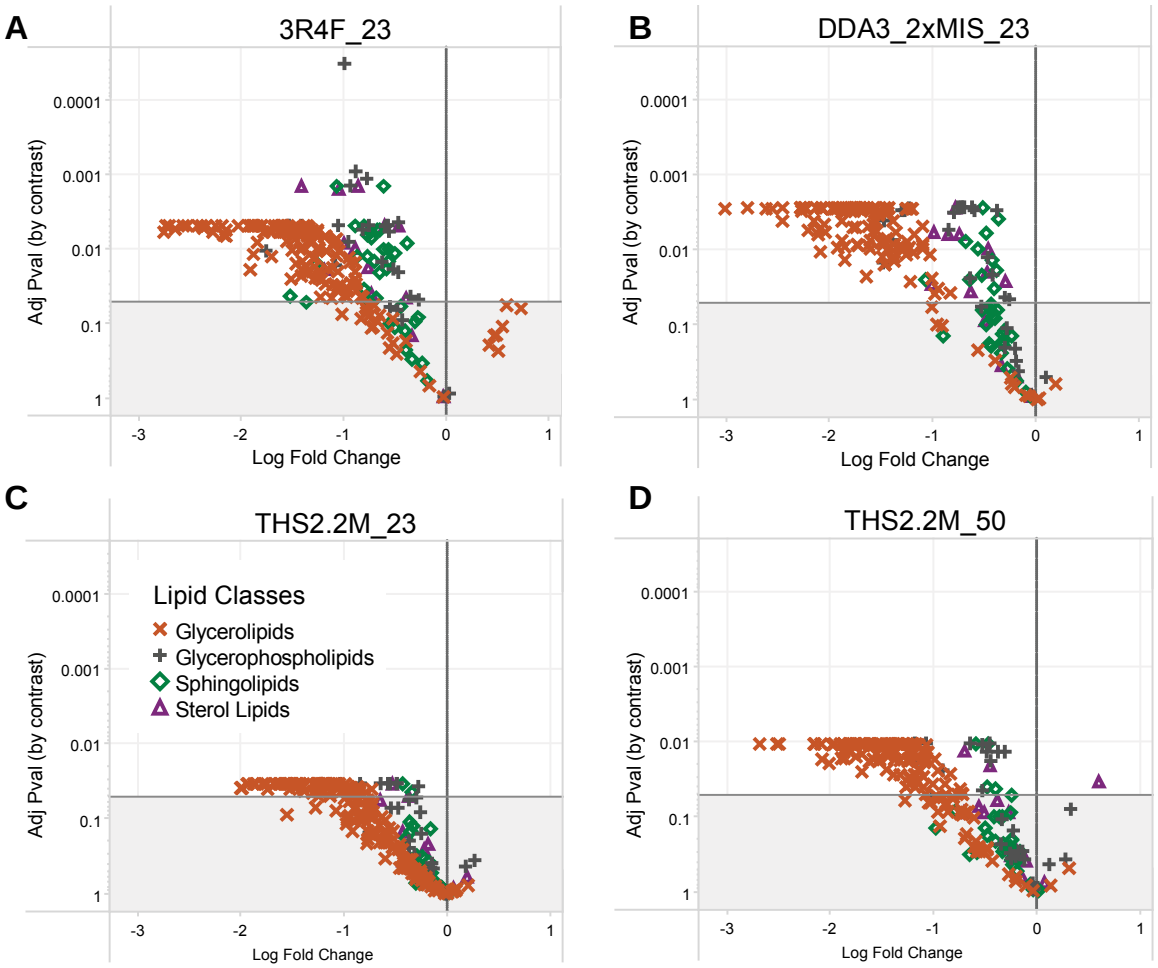


Figure 4. Systems response profiles of blood lipids

(A–D) Volcano plots show the amplitude (log_2 fold-change, x-axis) and significance ($-log_{10}$ Benjamini–Hochberg (BH)-adjusted p-value, y-axis) for exposure groups compared with the appropriate sham-exposed groups (male and female rats combined). **(A)** 3R4F_23. **(B)** DDA3_2xMIS_23. **(C)** THS2.2M_23. **(D)** THS2.2M_50.

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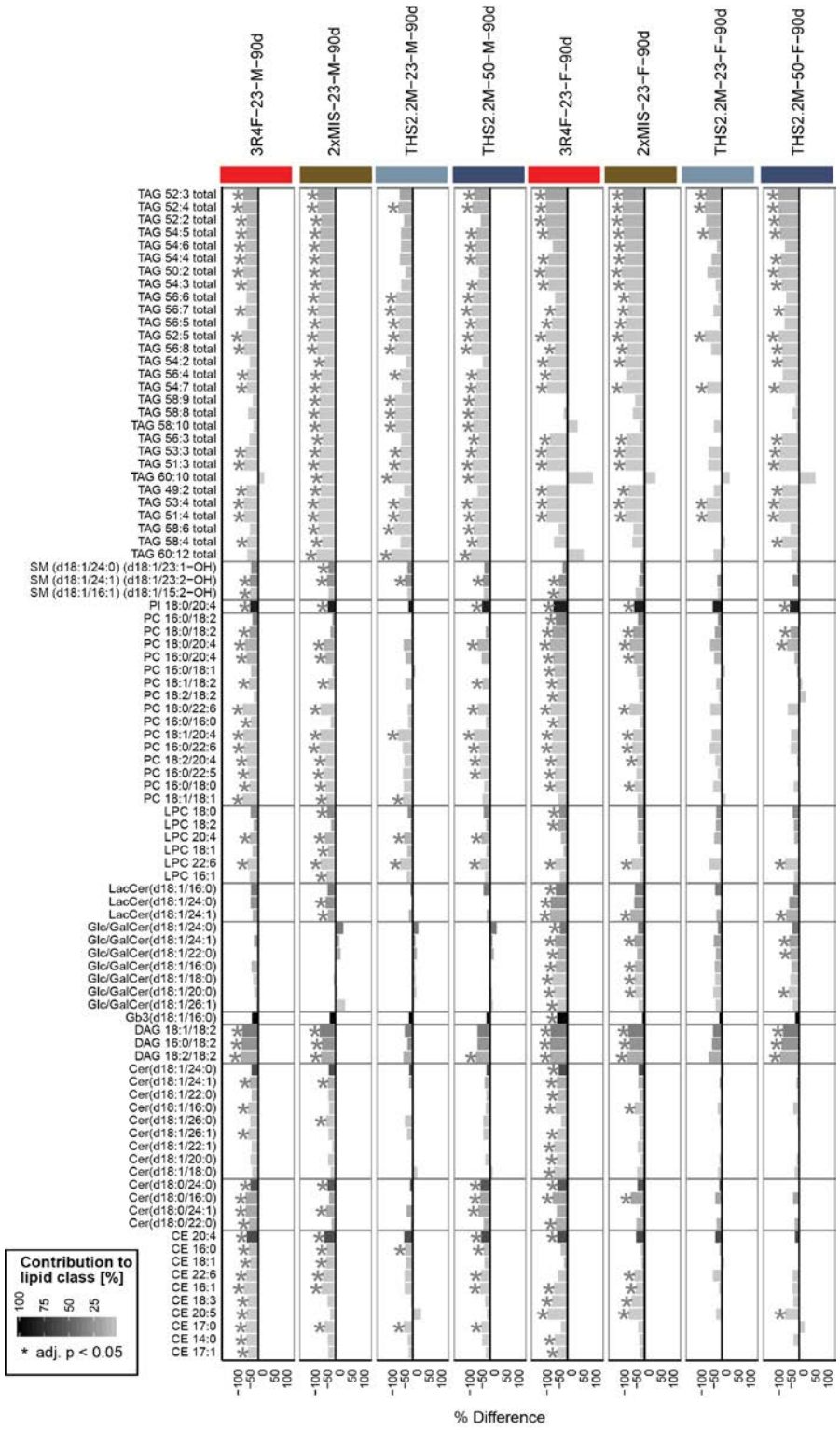


Figure 5. Differential abundance profiles for plasma lipid species

Each row shows one lipid species with a statistically significant change in at least one condition. Each column represents an exposure condition compared with the respective sham group after 90 days of

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exposure. Changes in lipid abundance compared with sham exposure are shown as % difference. Lipids with a differential abundance above the BH-adjusted p-value threshold line of 0.05 are considered statistically significant (*).

Compared with blood lipid changes in mice (4.4.2), the cigarette smoke (3R4F)-induced changes are markedly different: the bulk of glycerolipids downregulated in rats was not observed in mice, a few glycerolipid species were even slightly higher than in sham mice, and the few decreased lipids in Apoe$^{-/-}$ mice were mainly sphingolipids. It is likely that the hyperlipidemic status of Apoe$^{-/-}$ mice completely overrides any possible glycerolipid-lowering effects of the various exposures.

4.4.1.2 Lipids measured in lung tissue

Compared with blood lipid changes, a less intense pattern of cigarette smoke effects (without and with menthol) was observed, and more increases than decreases were evident (Figure 6). While only a few glycerolipids were affected, the majority of differentially abundant lipids were glycerophospholipids and sphingolipids. At the comparable nicotine level, THS2.2M aerosol caused only a marginally significant increase in one glycerophospholipid; all other changes did not reach statistical significance (Figure 6 C). Doubling the THS2.2M aerosol concentration significantly increased several more glycerophospholipids, as well as some sphingolipid species (Figure 6 D). Strikingly, in none of the three female groups investigated after the 42-day post-exposure period was there any detectable change in lung lipid concentration, indicating the reversibility of these lipid perturbations upon stopping exposure to either product, conventional cigarette smoke, or THS2.2M aerosol.

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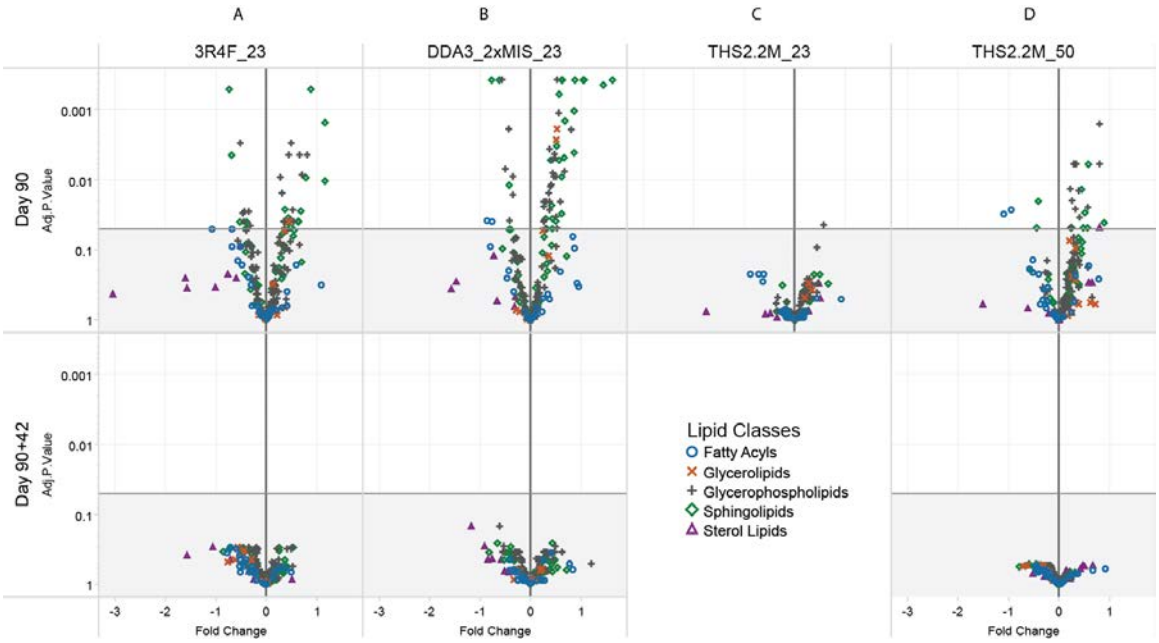


Figure 6. Systems response profiles of lung lipids

(A–D) Volcano plots show the amplitude (log_2 fold-change, x-axis) and significance ($-log_{10}$ Benjamini–Hochberg (BH)-adjusted p-value, y-axis) for exposure groups compared with the appropriate sham-exposed groups (male and female rats combined). **(A)** 3R4F_23. **(B)** DDA3_2xMIS_23. **(C)** THS2.2M_23. **(D)** THS2.2M_50. The top row shows the results for the 90-day and the bottom row for the 90+42-day time point. Note that for the 90+42-day time point only the response of females was assessed.

Figure 7 shows detailed results of lipid species levels separately for each sex. Cigarette smoke exposure-related response profiles were very similar with or without menthol, with changes being more pronounced for cigarette smoke with menthol in male rats (only two significant changes without menthol), and vice versa in female rats. In post-exposure groups (only assessed in females), all changes reverted to sham levels. While only a few sterol lipids and one glycerolipid were affected, the majority of differentially abundant lipids were glycerophospholipids and sphingolipids, with typical patterns in Glc/GalCer and Gb3 changes seen in both sexes. The most consistently increased lipids in cigarette smoke-exposed rat lungs were various ceramides, Glc/GalCer, and Gb3 species (Figure 7). At the comparable nicotine level, THS2.2M aerosol caused only one significant change in each sex, and two significant changes per sex in the high THS2.2M aerosol concentration group, which were different between males and females. With one exception, these individual lipid abundances changed in the same direction as for cigarette smoke. The exception was an increase in the sterol CE 20:4 in female rats exposed to the high THS2.2M aerosol concentration, which was not observed in cigarette smoke-exposed groups, and also not in male rats from THS2.2M groups. An increase in cholesterol ester levels has also been observed in lungs from rats exposed to aerosolized nicotine (Phillips 2015a).

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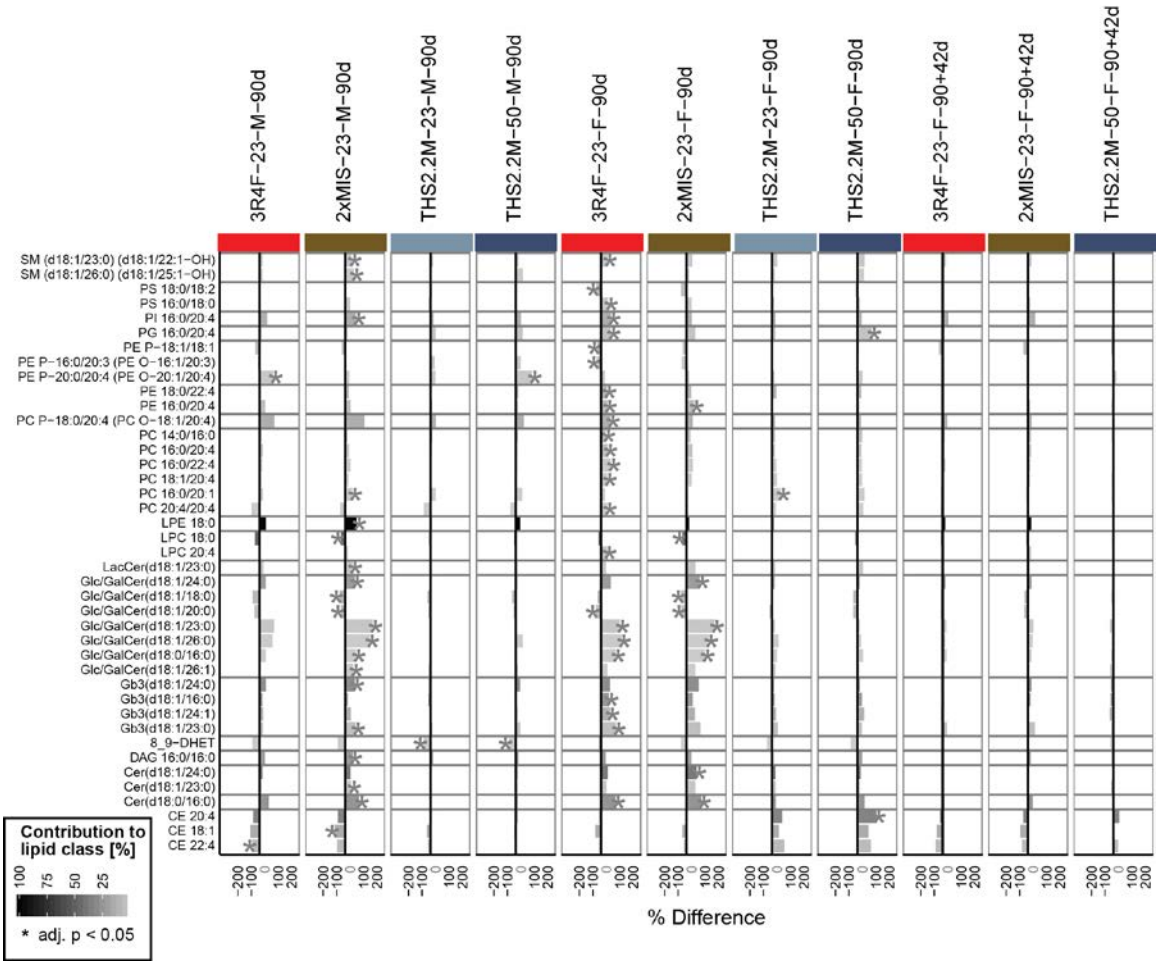


Figure 7. Differential abundance profiles for lung lipid species

Each row shows one lipid species with a statistically significant change in at least one condition. Each column represents an exposure condition compared with the respective sham group after 90 days of exposure or after 90 days of exposure plus 42 days post-exposure (females only). Changes in lipid abundance compared with sham exposure are shown as % difference. Significantly abundant differences are indicated (*=BH-adjusted p-value <0.05).

4.4.1.3 Lipid changes in the liver

Compared with blood plasma and lung tissue, in the liver only a low number of lipids were differently abundant between sham controls and exposed rats (Figure 8). Although trends were similar, statistical significances were only achieved for lipid changes in male rats exposed to THS2.2 (at both concentrations). All eight changes observed in the high THS2.2M aerosol exposure group mirrored the changes seen in the menthol cigarette smoke-exposed reference group with half the nicotine concentration, and seven of eight changes seen in the male non-mentholated reference (3R4F) group. Although the magnitude of THS2.2M-related changes was often similar to or even lower than that of the cigarette smoke reference groups, significance was only achieved in THS groups.

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This points to less variability in THS2.2M-exposed liver samples but not necessarily to a stronger effect compared with the cigarette smoke effect.

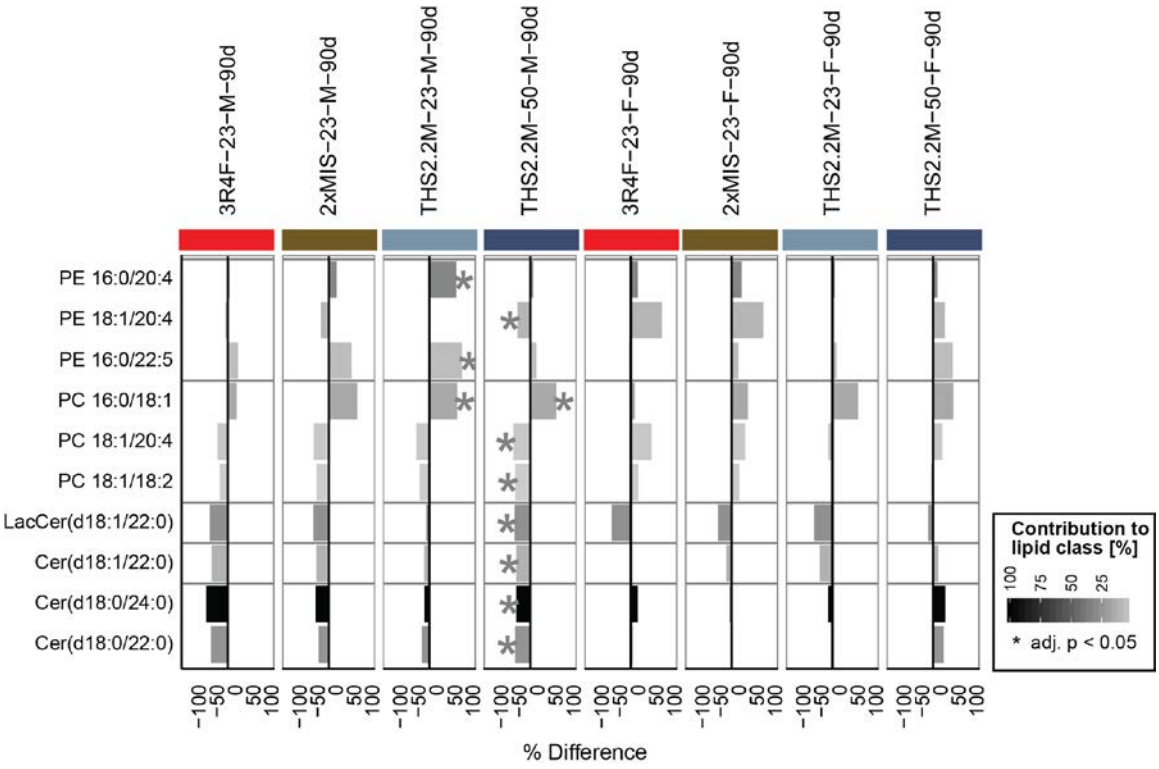


Figure 8. Differential abundance profiles for liver lipid species

Each row shows one lipid species with a statistically significant change in at least one condition. Each column represents an exposure condition compared with the respective sham group after 90 days of exposure. Changes in lipid abundance compared with sham exposure are shown as % difference. Significantly abundant differences are indicated (*=BH-adjusted p-value <0.05).

In summary, at a nicotine concentration equivalent to the highest tolerated conventional cigarette smoke concentration, THS2.2M aerosol caused significant changes in a lower number of lung and plasma lipid species that also changed in cigarette smoke groups. The doubled concentration of THS2.2M caused more changes, but still fewer than in either cigarette smoke groups. Changes in the liver were similar in quality and magnitude for cigarette smoke and THS2.2M groups, but reached statistical significance only for male THS2.2M groups. The addition of menthol to a conventional cigarette at the two-fold use level resulted in an overall similar lipid response profile with many shared and several additional differentially abundant lipid species. All lipid changes seen after the 90-day exposure period were transient and disappeared after a 42-day post-exposure period.

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4.4.2 Apoe$^{-/-}$ THS2.2 switching

The development and progression of COPD and CVD were evaluated in Apoe$^{-/-}$ mice following chronic exposure to mainstream cigarette smoke or to an aerosol from THS2.2. Female Apoe$^{-/-}$ mice were exposed to cigarette smoke from conventional cigarettes (3R4F) at a target nicotine concentration of 29.9 µg/L, to an aerosol from THS2.2 at a matched nicotine concentration, or to filtered fresh air for 3 h per day, 5 days per week, for up to 8 months. After 2 months of exposure to 3R4F cigarette smoke, designated groups of mice were switched to THS2.2 aerosol exposure (switching group), or exposed to filtered air (cessation group) for up to 6 months following the same daily exposure regimen (Figure 9). At designated time points (months 1, 2, 3, 6, and 8 after starting exposure), mice were examined for multiple parameters to comprehensively assess the development and progression of CVD or COPD indicators. These included hematology and clinical chemistry, pulmonary inflammation measurements, assessment of pulmonary function, lung histopathology and morphometry, evaluation of atherosclerotic plaque formation in the aortic arch and descending aorta, and examination of molecular changes in various tissues throughout the exposure period by transcriptomics, proteomics, and lipidomics.

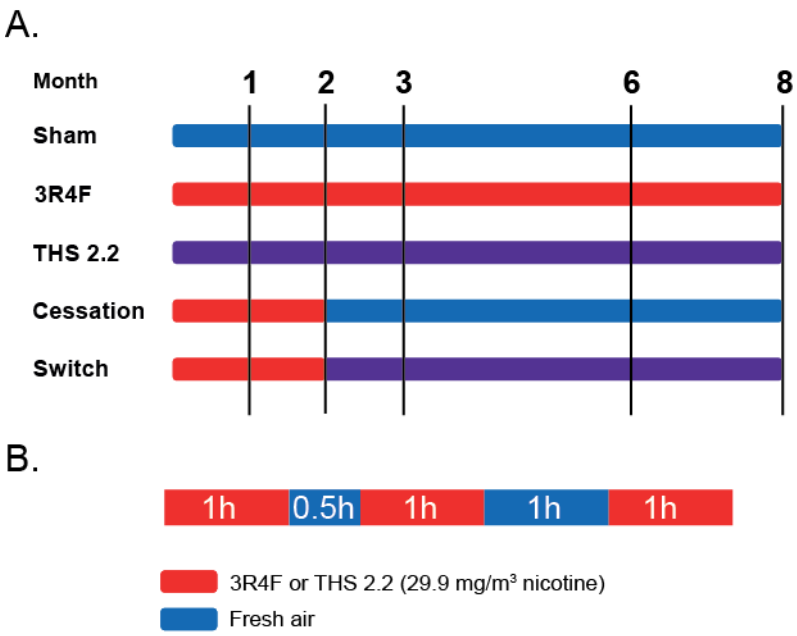


Figure 9. Study design

(A) Groups and exposures. **(B)** Daily exposure schedule. Details are given in the text.

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Within the focus of this documentation, we provide deeper insight into the effects of cigarette smoke exposure on the plasma, aortic, and lung lipidomes and on the lipid-related lung proteome, and compare these with effects of cessation, exposure to THS2.2 aerosol, and switching to THS2.2. Special emphasis is given to lipid metabolism revealed by integrated analysis of the lung lipidome and proteome response profiles in the lungs. For a broader context, method description, and additional endpoints, the reader is referred to our overview publications about these two studies (Phillips 2015b, Titz 2016).

4.4.2.1 Lipid peroxidation markers in the urine

As markers of systemic oxidative stress and damage, 8-OHdG, MDA, 4-HNE, and several isoprostanes were determined from the same urine samples that were also used to analyze nicotine metabolites and adducts from non-nicotinic harmful and potentially harmful constituents. For most of the analytes, variability was high and interfered with possible exposure effects. This was probably because of the general difficulties in obtaining sufficiently similar and representative urine samples during the 18-h collection following the exposure period. Figure 10 shows that urinary 4-HNE and MDA values in 3R4F groups had a consistent trend to be higher than sham values, and differences were statistically significant at months 6 and 8. Cessation, THS2.2, and switching groups were not different from sham controls.

For eicosanoids, including isoprostanes, no significant changes could be determined (data are compiled in Supplementary Results File 1).

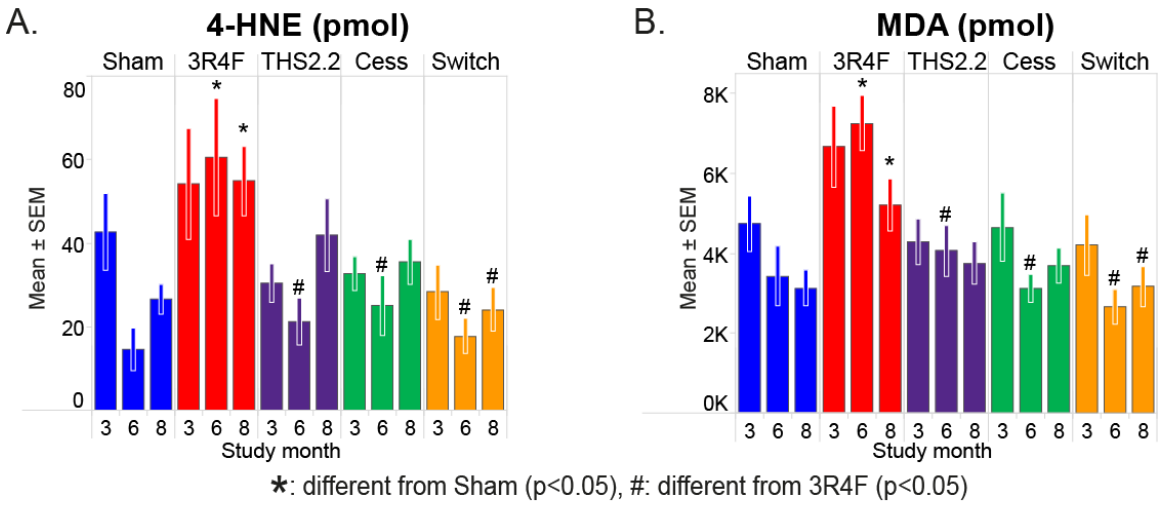


Figure 10. Urinary biomarkers of oxidative damage

Urine was collected throughout an 18-h post-exposure period, and the respective metabolites were determined. Measurements are expressed as the amount of metabolites excreted per mouse to account for the variable water content of the urine (physiological variations and evaporation). **(A)** 4-HNE (4-hydroxy-2-nonenal). **(B)** MDA (malondialdehyde). Mean ± SEM, *n*=8. p-values against sham obtained by *t*-tests accounting for variance heterogeneity (*different from sham, p<0.05; #different from 3R4F, p<0.05).

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4.4.2.2 Lipids measured in the circulation

Analysis by an autoanalyzer of non-fasted plasma samples using enzyme/biochemical assays revealed a statistically significant increase in total cholesterol (TC) and triacylglycerols/triglycerides (TAG) in the cigarette smoke-exposed group at months 1 and 3, with a decrease to sham levels after 1 month of either cessation or switching. Accompanying the increase in TC was a similar increase in HDL-cholesterol, although TC, TAG, and HDL values were no longer statistically significantly different from sham at month eight. There were also no obvious differences in LDL levels for any groups relative to sham controls at any time point (Figure 11).

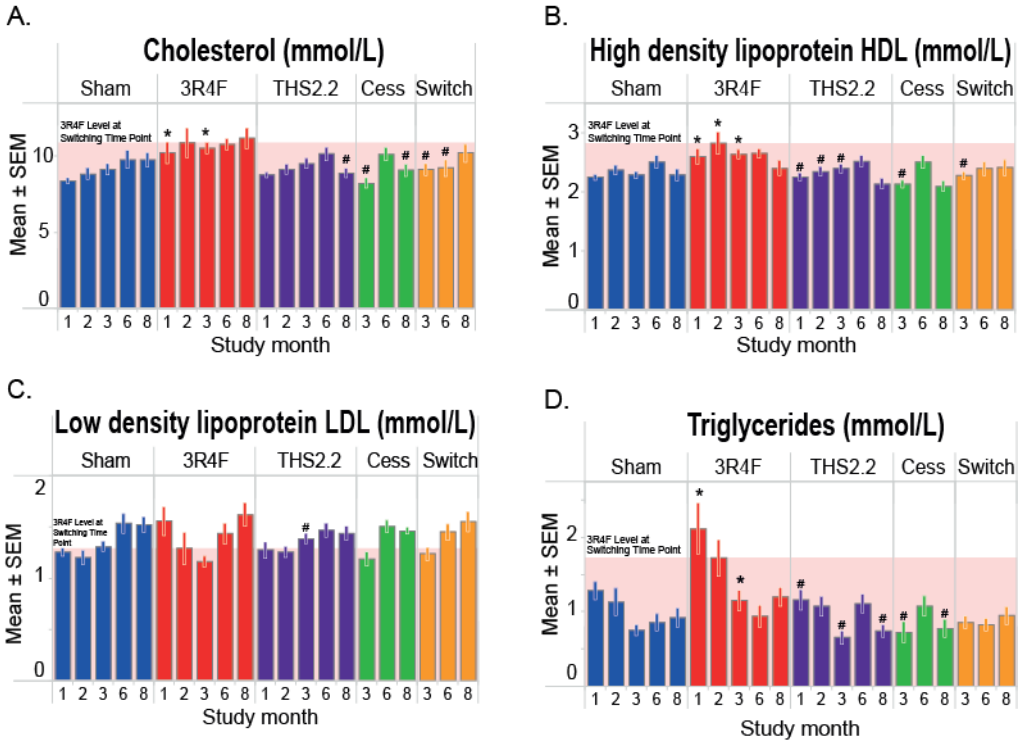


Figure 11. Lipid profiles determined by autoanalyzer

(A) Total cholesterol. **(B)** HDL. **(C)** LDL. **(D)** Triglycerides. Samples were measured in an autoanalyzer using Beckman/Coulter test kits. Total cholesterol and HDL-cholesterol were both based on enzyme/biochemical assays. Data are mean ± SEM, *n*=8–10. Statistics describe comparisons with sham-exposed mice (*) or with 3R4F-exposed mice (#). */# p<0.05. Red shaded area indicates the 3R4F value at the switching/cessation time point (month 2).

An independent evaluation of lipoproteins using samples from a different cohort of mice and the vertical auto profile ultracentrifugation method (Figure 12) confirmed the trends seen in the autoanalyzer-derived results for TC (marginally elevated for 3R4F from month 1 to 6), HDL (inconsistently elevated for 3R4F at months 1 to 3), LDL (inconsistently elevated for 3R4F at months 1 and 6), and TAG (inconsistently elevated

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for 3R4F at months 1 and 3) (Figure 12A–D). Additionally, concentrations of VLDL (Figure 12E), which were significantly increased in 3R4F cigarette smoke-exposed mice at months 1, 2 and 6, showed the greatest difference compared with sham seen at month 1. Values for THS2.2 aerosol-exposed, cessation, and switch mice did not significantly differ from sham at any time point. Even if there were a few apparently time-related trends (e.g., TAG in the THS2.2 group in Figure 12D), they were inconsistent (compared with the switch group, which received the same THS2.2 exposure at months 3–8, and with the decreasing trend in autoanalyzer results; Figure 11 D), and did not reach statistical significance, given the high variability (for both methods) in all groups, including sham. Because of an age-related increase in VLDL levels in all groups, levels were similar at month 8, which neutralized the 3R4F effect. A more detailed summary of all lipoprotein results, including lipoprotein subfractions, is provided in Supplementary Results File 2.

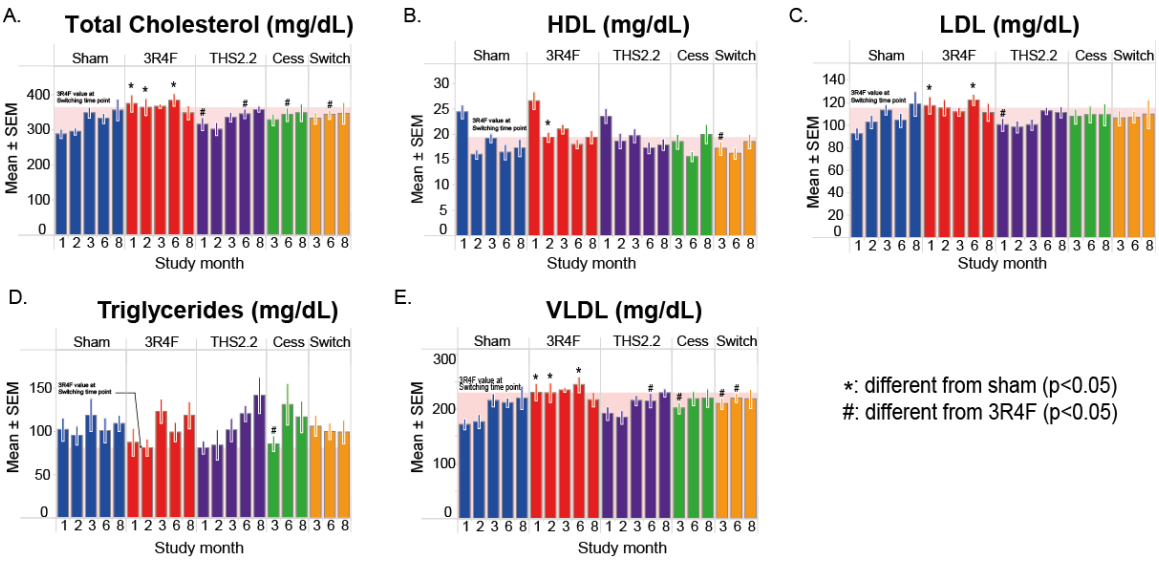


Figure 12. Lipid profiles determined by ultracentrifugation

(A) Total cholesterol. **(B)** HDL. **(C)** LDL. **(D)** Triglycerides. **(E)** VLDL. Samples were analyzed by Atherotech Inc., (Birmingham, AL) using the vertical auto profile method based on ultracentrifugation (Kulkarni 2006).

Both approaches show limitations, especially with their month-to-month variability, which is particularly obvious in TAG measurements (both methods) for all groups except the cessation group, but also in HDL and LDL measurements for sham and/or 3R4F groups (Figure 11 and Figure 12). Even for the overall weak effect of 3R4F *vs.* sham, our statistics for pairwise comparisons at each time point indicates, at the given variability, statistical significance for only a few changes (error bars indicate SEM, not SD). The apparent trend of increasing TAG concentrations (by vertical auto profile ultracentrifugation) over time in several treatment groups just missed statistical significance when calculating slopes for these values, mainly because of the high

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variations (coefficient of variation up to 40%). In this picture, the apparent trend of increasing TC values in the THS2.2 group over time, in the absence of significant differences to sham at any time point, is not plausible. Furthermore, it is not plausible with regard to the absent increase in the switch group, and the opposite trend (also not significant) seen for TAG (by the enzymatic detection method) for THS2.2, sham, and 3R4F.

Oxysterol analysis

A total of 10 oxysterols were evaluated and normalized to TC levels. Of these, three oxysterol species were shown to differ in the 3R4F exposure group relative to the sham group (Figure 13). Concentrations of 4β-hydroxycholesterol and 7β-hydroxycholesterol were lower in the 3R4F-exposed group and 25-hydroxycholesterol concentrations were higher. No significant changes were seen in the other exposure groups.

Also evaluated were 22-, 24- and 27-hydroxycholesterol, 5α6α-, 5β6β-, 7α-hydroxycholesterol, and 7-ketocholesterol, none of which showed significant differences ($p>0.05$) between 3R4F-exposed and sham-exposed animals. Previous studies evaluating oxidative stress in human smokers (Mol 1997, Seet 2011) showed a general increase in TC oxidation products in plasma from smokers, attributed to increased cigarette smoke-related oxidative stress. These results did not replicate the human findings, with only a single oxysterol product examined (25-hydroxycholesterol) showing a cigarette smoke-related increase. Further evaluation of the regulation of oxysterol metabolic enzymes at transcriptomics and lipidomics levels is in progress.

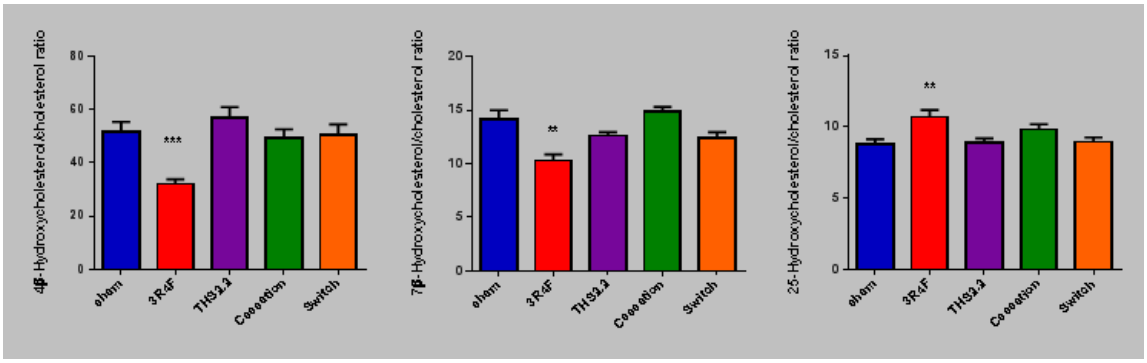


Figure 13. Oxysterol levels, month 8 (6-months post-exposure)

Mean ± SEM, *n*=10. Statistics describe comparisons with sham-exposed animals. *p<0.05; **p<0.01; ***p<0.001.

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As a third independent assessment of blood lipids, we assessed plasma lipidomics at month 8, providing among other lipid class concentrations, concentrations of TAG. Again, only in the 3R4F group did several TAG species display a statistically significantly higher level than sham, and some sphingolipids showed significant decreases. Two individual ceramides, LacCer(d18:1/18:0) and Gb3(d18:1/24:0), were lower in 3R4F-exposed mice. It is worth noting that the plasma lipidome of mice from cessation, THS2.2, and switching groups did not exhibit significant changes (Figure 14). Decreases in several ceramides/sphingolipids at the 8-month time point in the 3R4F group resemble some of our previously observed decreases at the 6-month time point (Boue 2012).

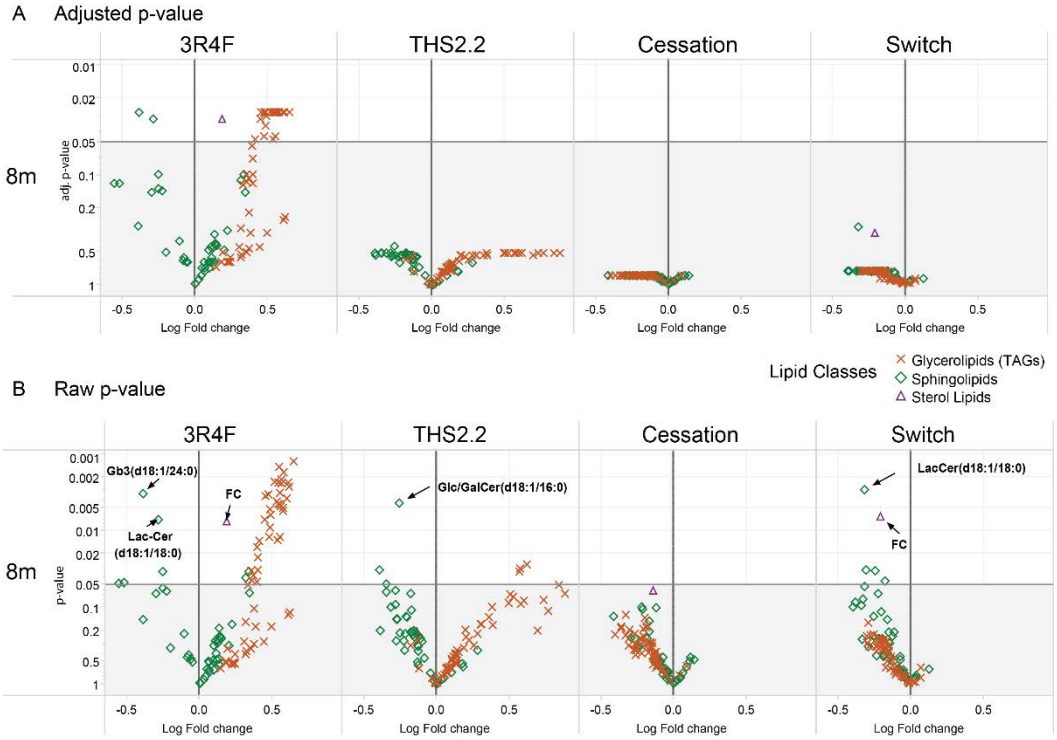


Figure 14. Plasma lipidomics

Lipidomics profiles of plasma in Apoe-/- mice exposed for 8 months to cigarette smoke (3R4F), THS2.2, or to a cessation or switch protocol (2 months 3R4F followed by 5 months fresh air or THS2.2, respectively). Volcano plots show amplitude ($\log_2$ fold-change, x-axis) and significance ($-\log_{10}$ BH-adjusted p-value, y-axis) for exposure groups compared with the 8-month sham exposed group. Lipids with a differential abundance above the BH-adjusted p-value threshold line of 0.05 are considered statistically significant. Each dot in the volcano plot represents a measured lipid. Classes of lipids, as defined by the Lipid Maps consortium, are color-coded and indicated in the legend.

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4.4.2.3 Lipids measured in the vascular wall

4.4.2.3.1 Aortic arch plaque formation

The most obvious pathological manifestation of perturbed lipid metabolism in Apoe$^{-/-}$ mice is the formation of lipid-rich plaques at defined sites of the aortic vasculature, which occurs spontaneously because of deficient reverse lipoprotein transport, and can be accelerated by a high-fat diet or cigarette smoke exposure (Von Holt 2009). Image analysis of the plaque area formed at the aortic arch revealed an expected and progressive increase in plaque burden in all groups over the 8-month study period. From month 2 onwards, a significantly increased aortic plaque area was observed in mice exposed to 3R4F cigarette smoke compared with either sham or THS2.2 aerosol-exposed animals. In contrast, exposure to THS2.2 aerosol resulted in plaque areas that did not significantly differ from those seen in sham animals, except at month 6 when the plaque area was increased relative to sham, albeit less than in 3R4F-exposed mice. In animals that were switched to either air or THS2.2 aerosol, the plaque area was significantly smaller than in mice from the 3R4F-exposure group at months 3 and 8, although plaques remained slightly, but not significantly, larger than those of sham or long-term THS2.2 aerosol-exposed mice (Figure 15).

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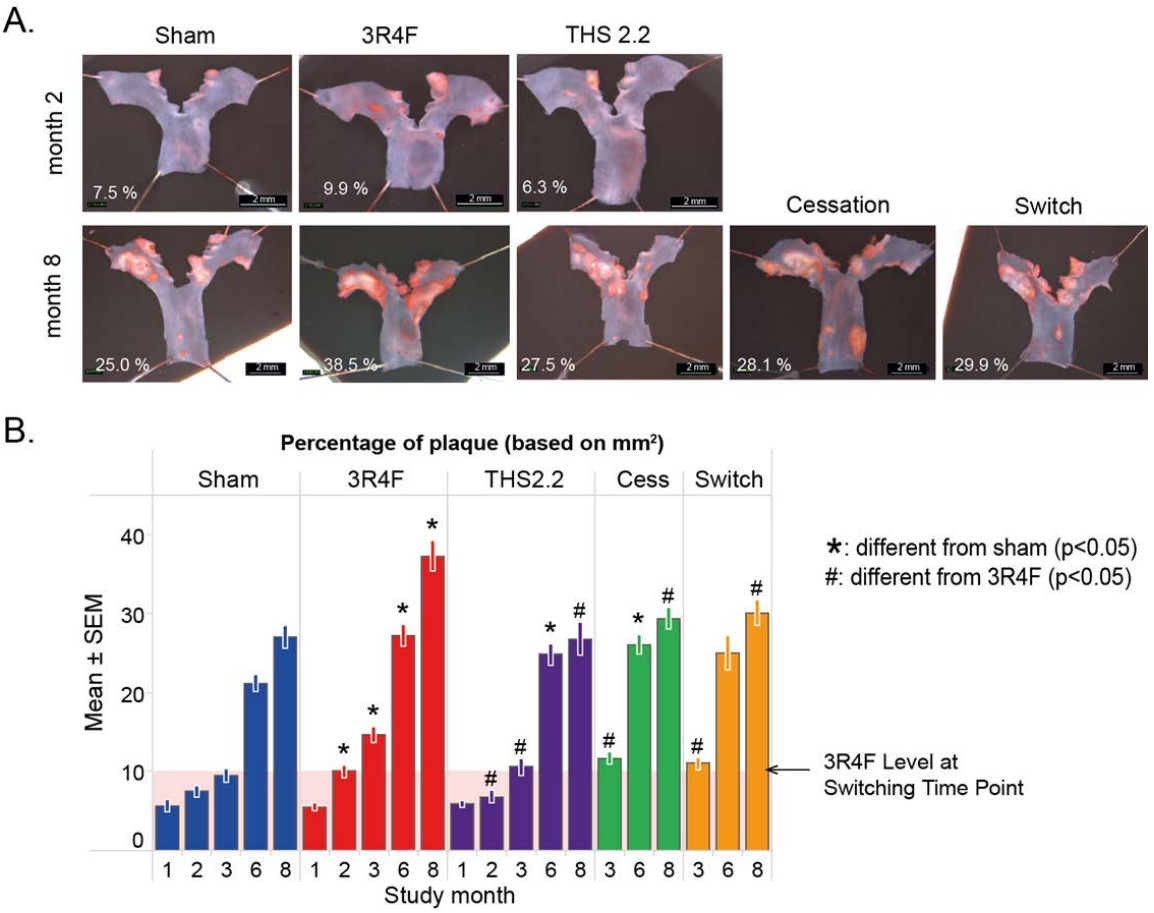


Figure 15. Aortic arch (isolated) plaque area measurements

(A) Representative images from the dissection microscope. **(B)** Plaque size determined by planimetry in the dissected aortic arch. Data are mean ± SEM. *p<0.05 compared with sham; #p<0.05 compared with 3R4F. Red shaded area indicates the 3R4F value at the switching/cessation time point (month 2).

A quantitative micro-computed tomography investigation in a separate cohort of animals at the 7-month time point confirmed these findings, showing significantly higher plaque volume, plaque area, and aortic occlusion in the aortic arch of 3R4F-exposed mice, while values from the THS2.2 group or cessation and switching arms did not differ significantly from those in the sham control group (Figure 16).

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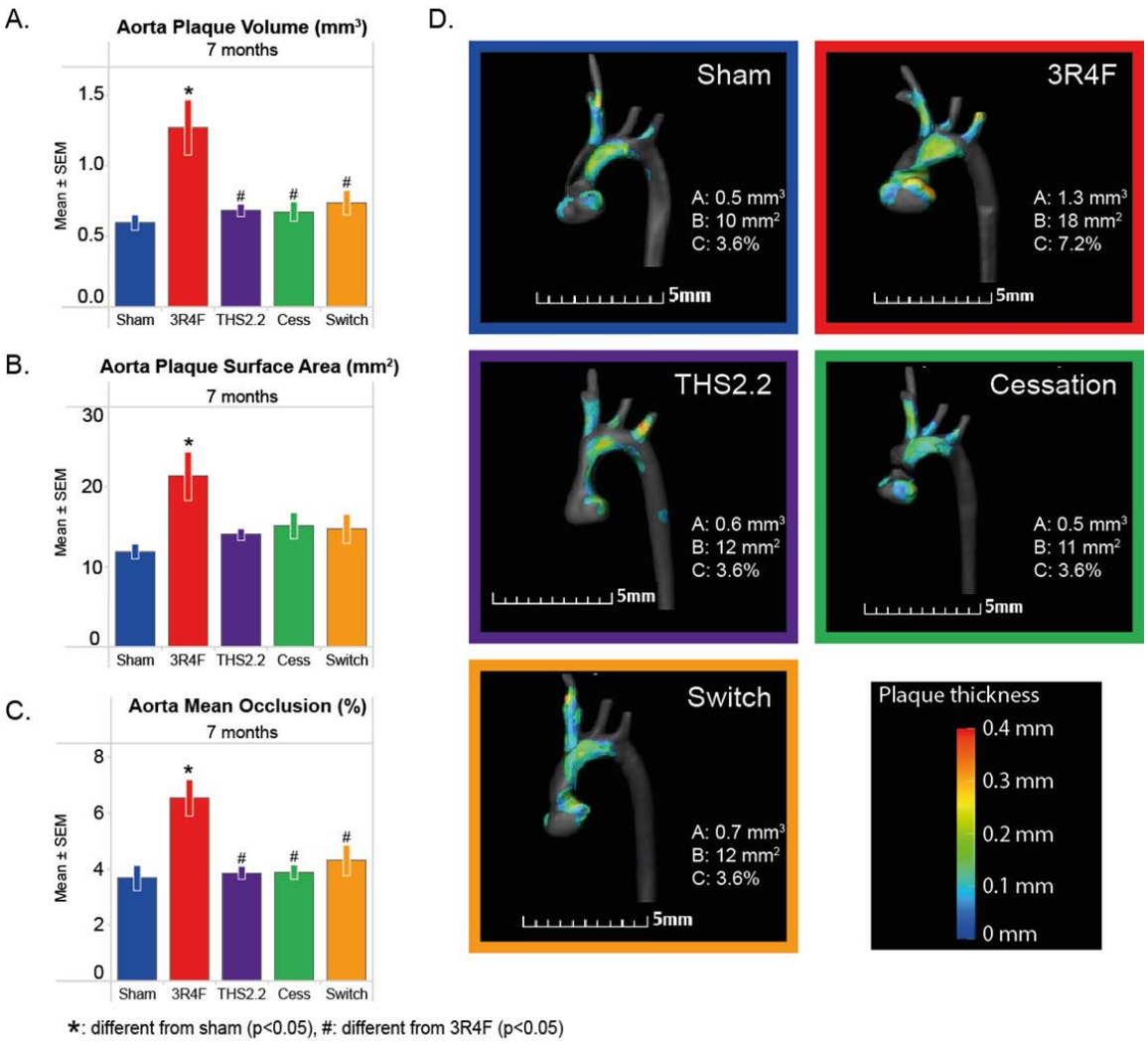


Figure 16. Micro-computed tomography-based aortic arch (in situ) plaque measurements

(A) Plaque volume. **(B)** Plaque surface area. **(C)** Aortic occlusion (mean ± SEM). **(D)** Representative micro-computed tomography images. *p<0.05 compared with sham; #p<0.05 compared with 3R4F.

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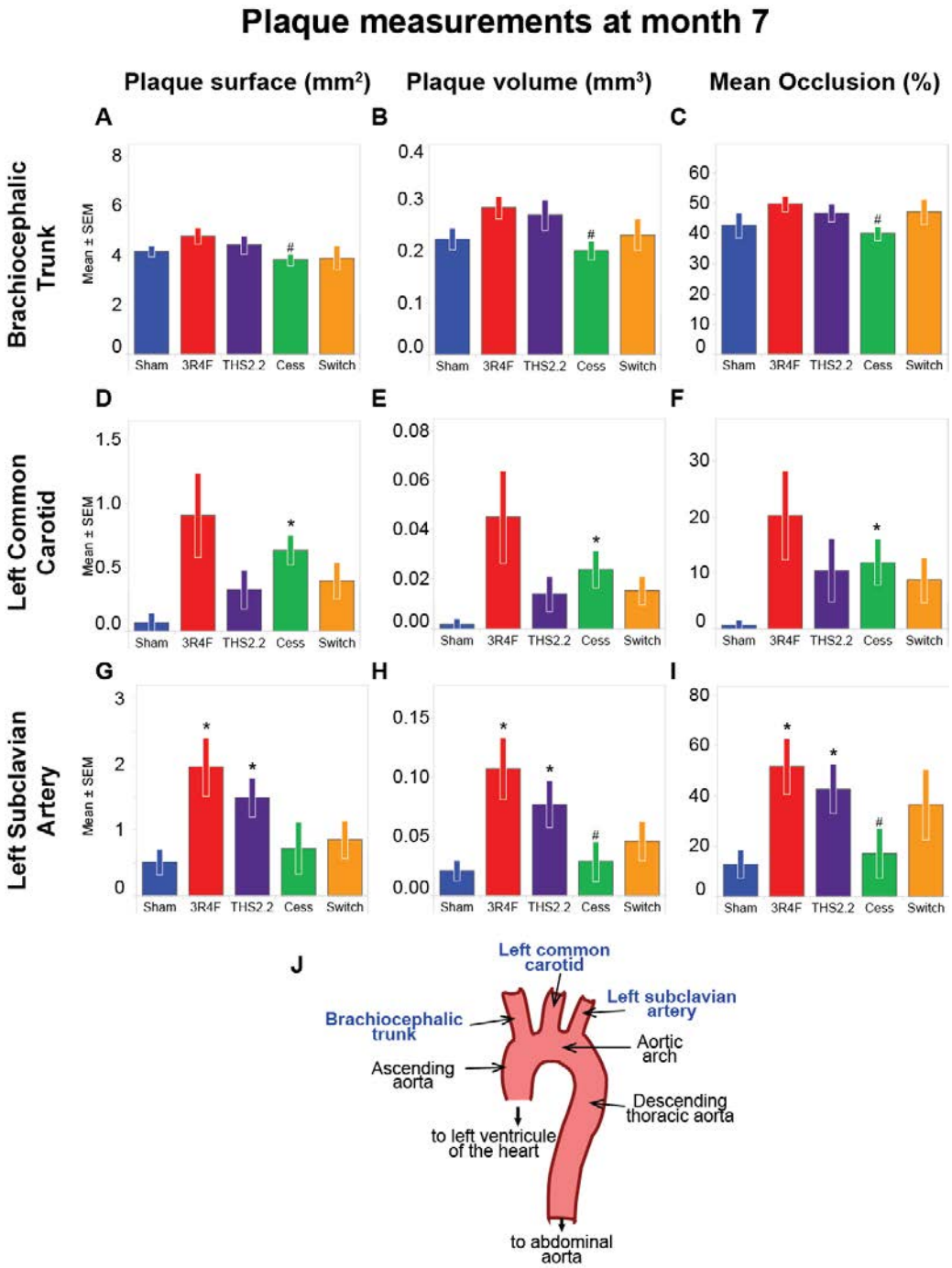


Figure 17. Micro-computed tomography-based (in situ) plaque measurements at additional sites of the aortic tree

(A, B, C) Brachiocephalic trunk. **(D, E, F)** Left common carotid. **(G, H, I)** Left subclavian artery. **(J)** Sites for micro-computed tomography measurements. **(A, D, G)** Plaque volume. **(B, E, H)** Plaque surface area. **(C, F, I)** Aortic occlusion. Mean ± SEM. *p<0.05 compared with sham; #p<0.05 compared with 3R4F.

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Additional measurements at three other known sensitive sites of the aortic tree (Figure 17 J) showed no significant effects at the brachiocephalic trunk (Figure 17 A–C), a significant increase in the three parameters at the left common carotid for the cessation group; the even higher trend in the 3R4F group did not reach statistical significance because of very high variability (Figure 17 D–F) and significant effects for both 3R4F and THS2.2 at the left subclavian artery (Figure 17 G–I). Paradoxically, no significant increases in plaque size and volume were seen at this site for the switching group. Taken together, a significant cigarette smoke effect was observed at two out of four micro-computed tomography measurement sites, and less consistent effects were observed for cessation and THS2.2 groups at one site each, while no effects were seen at any site in the switching group.

4.4.2.3.2 Aortic arch lipidomics

In aortic arch tissue of 3R4F-exposed mice, levels of glycerophospholipids, sphingolipids, and sterol lipids were significantly higher than in sham animals, while no statistically significant differences were seen in cessation, THS2.2, and switching groups relative to sham (Figure 18).

We speculate that the accumulation of sphingolipids in the vascular wall could at least in part lead to their reduced levels in plasma.

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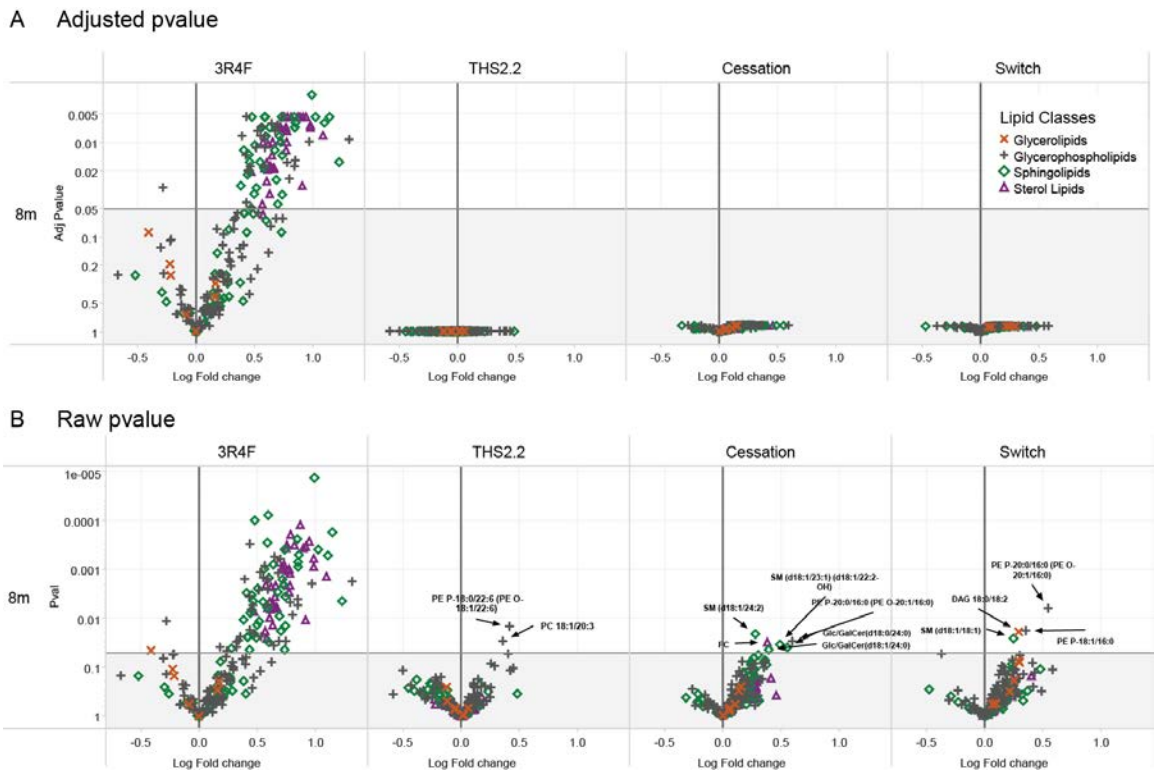


Figure 18. Aortic arch lipidomics

Lipidomics profiles of aortic arch in Apoe-/- mice exposed for 8 months to cigarette smoke (3R4F), THS2.2, or to a cessation or switch protocol (2 months 3R4F followed by 5 months fresh air or THS2.2, respectively). Volcano plots show amplitude (log_2 fold-change, x-axis) and significance ($-log_{10}$ BH-adjusted p-value, y-axis) for exposure groups compared with the 8-month sham exposed group. Lipids with a differential abundance above the BH-adjusted p-value threshold line of 0.05 are considered statistically significant. Each dot in the volcano plot represents a measured lipid. Classes of lipids, as defined by the Lipid Maps consortium, are color-coded and indicated in the legend.

Stegemann *et al.* analyzed the lipid composition of human atherosclerotic plaques from carotid endarterectomies and reported a list of plaque-enriched lipid species, also with a strong contribution from sterol lipids, sphingolipids, and glycerophospholipids (Stegemann 2011). In Figure 19, Apoe$^{-/-}$ murine aortic arch lipid species that are common with human plaque-enriched lipids have been compared. With the exception of two phosphatidylcholines and one phosphatidylethanolamine, all these common lipids were also significantly higher in 3R4F group vascular tissue compared with tissue from sham-exposed controls. For cessation and switch groups, weaker but strikingly similar trends were seen, but they did not reach statistical significance. In contrast, only marginal differences, also lacking statistical significance, were seen for THS2.2 aerosol-exposed mice. This correlation between human and murine plaque-enriched lipids is suggestive of related pathomechanisms in plaque formation in this mouse model of atherosclerosis.

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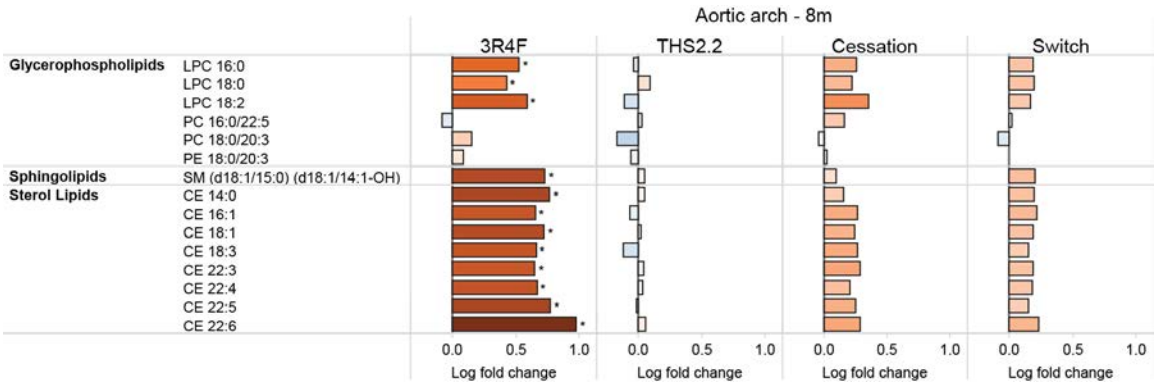


Figure 19. Aortic arch lipids from exposed ApoE mice vs. sham controls shared with human plaque-enriched lipids, as reported by Stegemann et al. (Stegemann 2011)

Differential abundance profiles for lipid species with significant differential abundance in any exposure to sham comparison (*BH-adjusted p-value <0.05). X-axis and color code indicate $\log_2$(Fc).

4.4.2.4 Lipid metabolism changes in the liver

Consistent with previous results (Whitehead 1995), overt hepatotoxic effects were not detected for any exposure groups when classical markers and histopathological parameters were investigated (for details, see (Lo Sasso 2016)). Despite the time-dependent increase of parenchyma fat accumulation, which is obviously caused by disruption of reverse cholesterol transport in Apoe$^{-/-}$ mice, no statistically significant differences between groups were observed. However, at the molecular level, cigarette smoke exposure clearly affected several functional categories, which included induction of a xenobiotic and oxidative stress response program and rearrangements in liver lipid metabolism. In contrast, these cigarette smoke-induced effects were either absent or greatly reduced in mice exposed to THS2.2 aerosol. In addition, these effects quickly approached sham group levels upon cessation or switching.

For integrative analysis of exposure effects on the proteome and transcriptome, we applied multivariate canonical sparse partial-least-squares analysis, which is conceptually related to principal component analyses. Where the first two identified correlated components were unable to separate exposure groups, the third component demonstrated a clear separation between cigarette smoke-exposed mice and other groups, highlighting also a time-dependent trend with increased scores for the later time points. To understand which molecules were driving this separation, a functional clustering approach was applied (Figure 20). This analysis showed that lipid metabolism and xenobiotic phase 1 and 2 proteins/genes tended to be more highly expressed in cigarette smoke-exposed mice (i.e., showed a positive association with cigarette smoke-exposed samples) (Figure 20 A), while interferon-γ responses and cell cycle proteins/genes tended to be downregulated in cigarette smoke-exposed groups relative to other groups (Figure 20 B) (for details, see (Lo Sasso 2016)).

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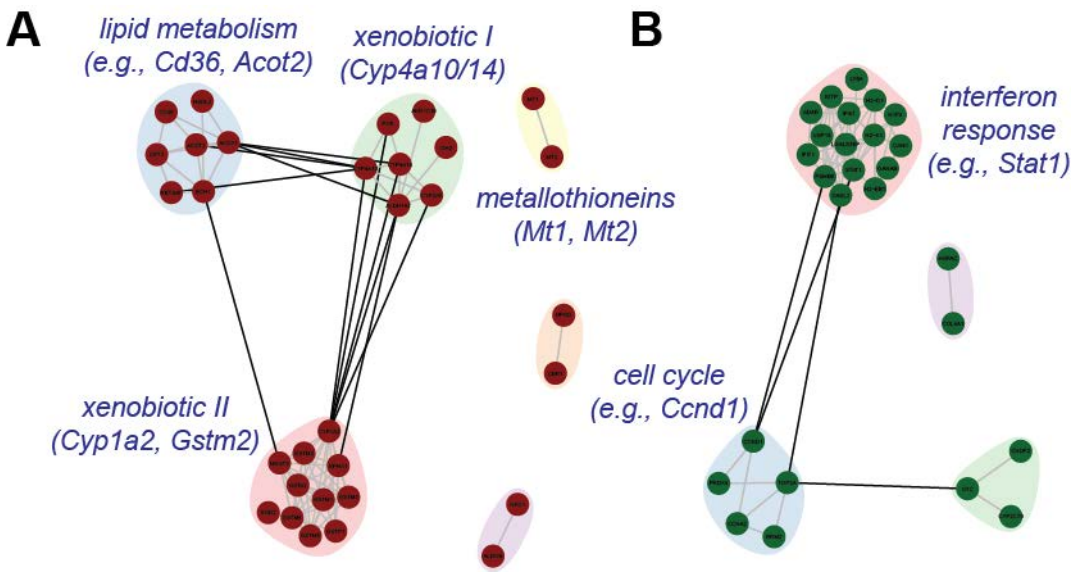


Figure 20. Cigarette smoke exposure induces a molecular response in the liver

(A) Clustered functional association network for proteins/genes positively contributing to component 3 (i.e., with an upregulation trend in 3R4F groups). Identified clusters were manually annotated, guided by ToppGene (Chen 2009). **(B)** As in (A), but for genes/proteins with a negative contribution to component 3 (i.e., with a downregulation trend in 3R4F groups).

Liver lipidomics showed very high intra-group variability, especially in the sham group, rendering interpretation of the results very difficult. In Figure 21, a heat map representing the different detected lipid species in the different groups compared with sham is shown. Of note, few classes of ceramide (Cer d18:0, Cer d18:1, LacCer) significantly decreased at late time points in THS2.2, switch, and cessation groups. Glycerophospholipid classes showed a fluctuating trend. In fact, lysophosphatidylethanolamine, phosphatidic acid, and phosphatidylglycerol showed an increasing trend in the 3R4F group. THS2.2, cessation, and switching groups showed a general decreasing trend, with only the latter two groups significantly affected. Conversely, phosphatidylcholine's abundance showed a general increase in all groups, reaching statistical significance in the cessation group, while lysophosphatidylcholines seemed to decrease across groups. Finally, diacylglycerol (DAG) and TAG showed dissimilar trends. Thus, while DAG seemed to decrease over time, TAG show significant decreases in 3R4F and THS2.2 groups compared with sham at early time points, with an increasing trend over time, as observed earlier for 3R4F (Boué 2012, De Leon 2013). It is worth highlighting that lack of consistent detection of several TAG species at later time points and in the switch group may undermine any speculative conclusion on the impact of cigarette smoke on liver TAG accumulation (Figure 21). Overall, these results seem to support the idea that in the healthy liver, or at least in the absence of chronic liver disease, cigarette smoke does not affect the liver lipidome profoundly.

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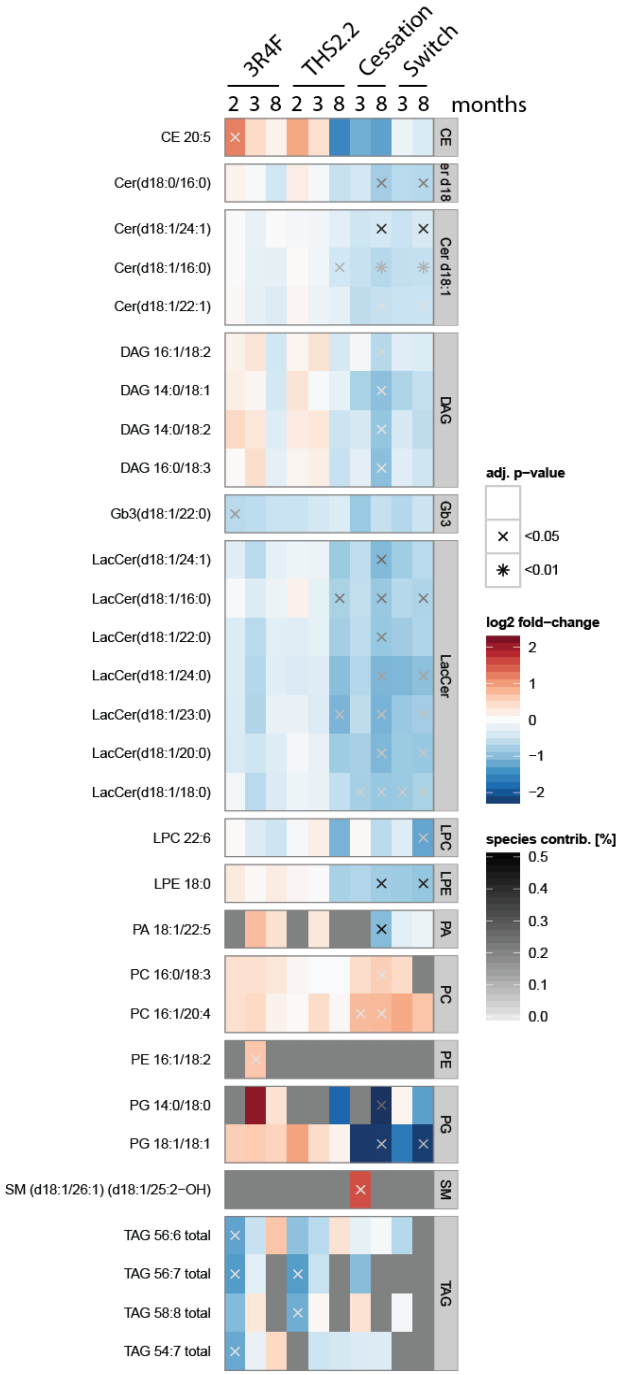


Figure 21. Liver lipidomics: results for individual lipid species

Differential abundance profiles for lipid species with significant differential abundance in any exposure to sham comparison (BH-adjusted p-value <0.05). Each row shows one lipid species. Each column shows an exposure condition compared with the sham group at the respective time point. Fold-change compared with sham exposure is color-coded (see color key). Significantly abundant differences are indicated (x=BH-adjusted p-value <0.05, *=BH-adjusted p-value <0.01). In addition, the molar contribution of significantly abundant lipid species to their lipid class is indicated by the shade of the significance indicator ("species fractions").

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In the above mentioned gene/protein clustering approach, a lipid metabolism protein/gene cluster contributed to the separation observed between 3R4F and the other groups (Figure 20). Thus, to expand the analysis of the effects on lipid metabolism, the response of molecules in three lipid-related hallmark gene sets from the Molecular Signatures Database were analyzed (fatty acid metabolism, bile acid metabolism, and cholesterol homeostasis) (Mootha 2003, Subramanian 2005) (Figure 22). Based on liver gene expression and protein abundance data, cigarette smoke affected the three main lipid metabolic pathways: fatty acid, cholesterol, and bile acid metabolism. Although no significant gene expression changes were observed, an abundance of proteins involved in the three pathways was significantly affected in the 3R4F group, while no significant changes were found for the other groups. Upregulation of CD36 and acyl-CoA thioesterases 2 (Acot2) in the 3R4F group suggested an increase in fatty acid uptake and intracellular acyl-CoA and free fatty acid levels, while hydroxyacyl-CoA dehydrogenase (Hadh) downregulation likely mirrored reduced fatty acid oxidation (Figure 22). Overall, these changes in protein abundance may reflect the time-dependent TAG increasing trend observed in the liver of the 3R4F group (Figure 21). Of note, some proteins in the hallmark set of fatty acid metabolism are also important components of the xenobiotic elimination pathways (Cbr1, Ephx1, and Fmo1) (Figure 22) (Hassett 1994, Oppermann 2007, Phillips 1995).

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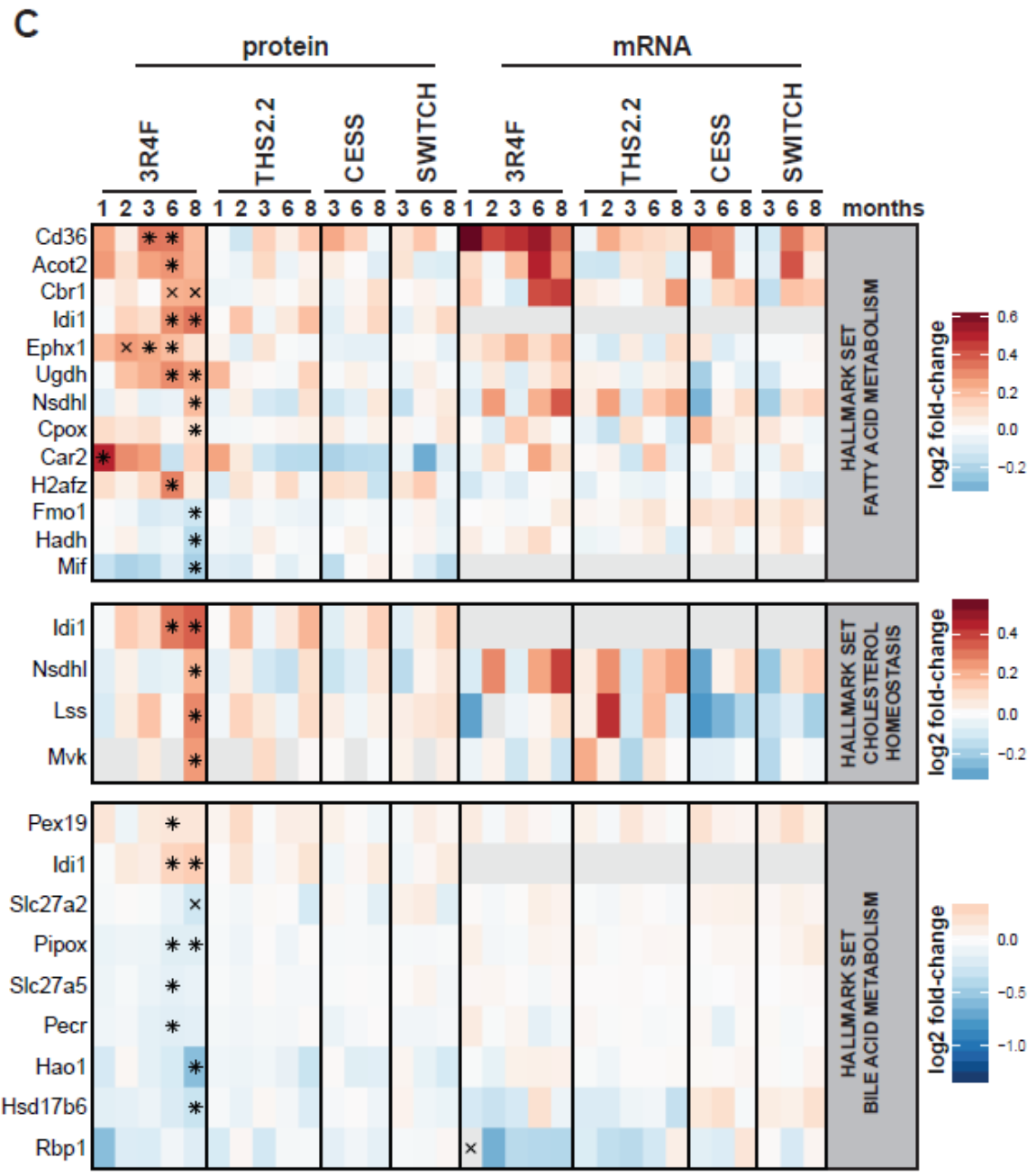


Figure 22. Cigarette smoke exposure affects lipid metabolism in Apoe$^{-/-}$ mice

Protein and gene expression profiles for three liver lipid-metabolism-related panels. Profiles for significantly affected proteins or genes (BH-adjusted p-value <0.05) of three hallmark gene/protein sets in any of the comparisons are shown: fatty acid metabolism, cholesterol homeostasis, and bile acid metabolism (www.broadinstitute.org/gsea/msigdb) (Liberzon 2011). Fold-change compared with the respective sham groups is color-coded and statistical significance is marked (BH-adjusted p-value, *≤0.05, x≤0.01). Missing values are grey.

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Cholesterol synthesis was affected in the 3R4F group at later time points. Several enzymes involved in different steps of cholesterol biosynthesis such as isopentenyl-diphosphate delta isomerase 1 (Idi1), NAD(P)H steroid dehydrogenase-like (Nsdhl), lanosterol synthase (Lss) and mevalonate kinase (Mvk) (Russell 1992) were upregulated. These results have already been observed in the rat liver (Latha 1988) and may explain increases in cholesterol esters observed in the liver (Figure 21) and free cholesterol in plasma (Figure 19).

Finally, proteins belonging to the hallmark set of bile acid metabolism also showed significant changes (Figure 22) in the liver of 3R4F-exposed mice. The majority of these regulated proteins were microsomal enzymes showing decreased abundance (Pipox, Pecr, Hao1, and Hsd17b6), although Pex19, which is required for the early stage of peroxisome biogenesis, increased.

Peroxisomes play an important role in the biosynthesis of bile acids, because a peroxisomal β-oxidation step is required for the formation of mature C24-bile acids from their C27-bile acid intermediates (Ferdinandusse 2006). Additionally, two solute carrier family proteins (Slc27a2, involved in both bile and fatty acid metabolism, and Slc27a5, which activates secondary bile acid entering the liver from the enterohepatic circulation), were significantly downregulated in the 3R4F group. Overall, this cigarette smoke-dependent downregulation of bile acid synthesis and catabolism pathways may contribute, on one hand, to the reduced bile acid liver content observed previously (Latha 1988). On the other hand, it may contribute to the observed increase and accumulation of cholesterol (Boué 2012, De Leon 2013, Phillips 2015a).

Taken together, although liver lipidomics analysis showed high variability and incomplete results, genome and proteome analysis showed extensive dysregulation of liver lipid metabolism pathways upon cigarette smoke exposure, confirming previous results (Altamirano 2010, Boue 2012, De Leon 2013, Latha 1988). In particular, while fatty acid and cholesterol metabolism enzymes showed consistent upregulation, bile acid synthesis and transformation were negatively affected by cigarette smoke. In contrast, THS2.2 demonstrated more limited effects on lipid metabolism pathways in the liver, as well as cessation and switch groups appearing to quickly revert cigarette smoke-induced effects.

4.4.2.5 Lipids measured in the lung

Quantified lipid classes of the lung lipidome covered a range of concentrations (Figure 23). Phosphatidylcholine (PC) had by far the highest concentration in the lung lipidome, most likely because of its role as a surfactant lipid (see below). Phosphatidylcholine is followed in abundance by several lipid classes with important structural roles, including sphingomyelin (SM), phosphatidylserine (PS), and phosphatidylglycerol (PG). Lipids present at the lower end of the concentration range include sphingosine-1-P and sphinganine-1-P, important lipid signaling mediators. The Apoe$^{-/-}$ study showed generally good agreement with the previous C57BL/6 study in concentrations of these lipid classes, with some exceptions (Titz 2016). For example, cholesterol ester (CE) concentrations were greater in the Apoe-/- study because of the general perturbation of cholesterol metabolism in this mouse strain (Nakashima 1994).

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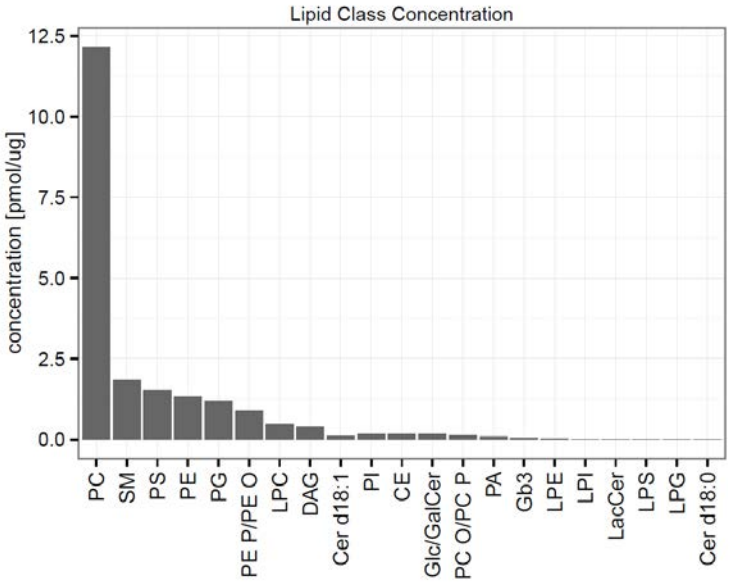


Figure 23. Lipid class concentrations in the lung

Sums of median lipid species concentrations of a class across the whole study are shown. For abbreviations, see Supplementary Table 1.

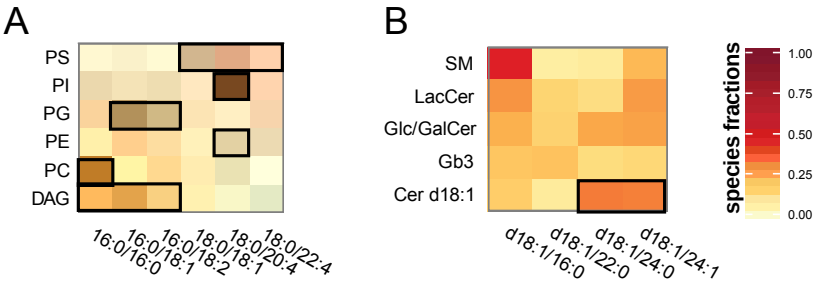


Figure 24. Lipid composition profiles

(A) Detailed composition profiles for glycerophospholipids and DAG. Only lipid compositions contributing at least 20% to the total concentration of either lipid class were included. **(B)** Composition profiles for d18:1 Cer, Glc/GalCer, LacCer, and Gb3.

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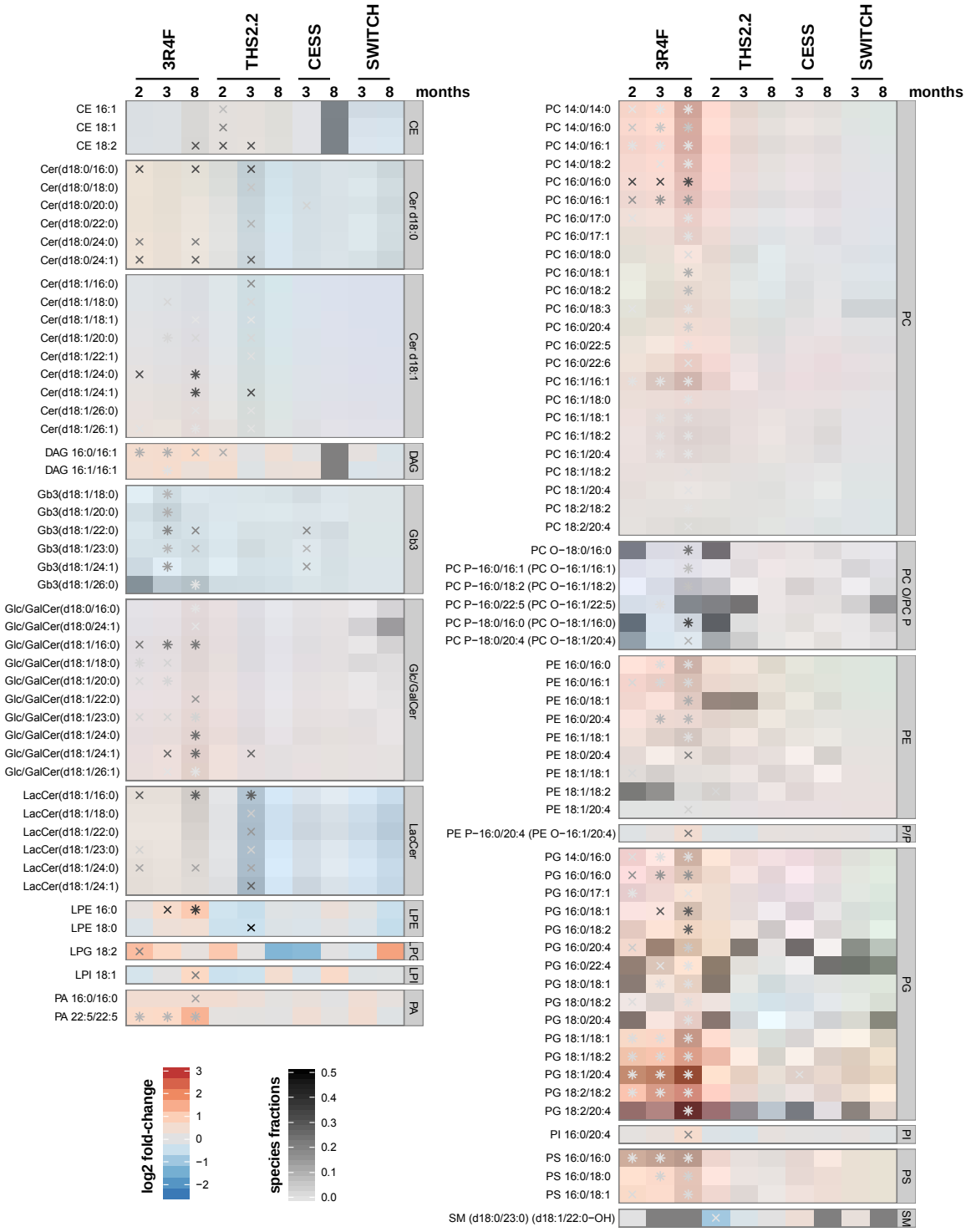


Figure 25. Differential abundance profiles for lung lipid species with significant differential abundance

Each row shows one lipid species. Each column shows an exposure condition compared with the sham group at the respective time point. Only lipids with significant differential abundance in any exposure to sham comparison are included. Fold-change compared with sham exposure is color-coded (see color key).

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Significantly abundant differences are indicated (x=BH-adjusted p-value <0.05, *=BH-adjusted p-value <0.01). The molar contribution of significantly abundant lipid species to their lipid class is indicated by the shade of the significance indicator ("species fractions").

Our liquid chromatography–tandem mass spectrometry approach enabled resolution of the exact composition of the analyzed lipid species. Glycerophospholipids and DAG showed an especially intriguing fatty acid composition pattern, with different composition profiles for the different lipid classes (Figure 24). Dipalmitoylphosphatidylcholine (PC 16:0/16:0) dominated the class of phosphatidylcholine lipids, which is most likely because of the dominant role of this lipid as a component of lung surfactant (Batenburg 1992). The phosphatidylglycerol class was enriched for PG 16:0/18:1 and PG 16:0/18:2 lipid species, which, similarly, is likely to reflect their role as surfactant lipids (Postle 2001). Both phosphatidylethanolamine (PE) and phosphatidylinositol had a relatively abundant 18:0/20:4 lipid species. The 20:4 fatty acid most likely represents arachidonic acid and a particularly high abundance of this lipid in phosphatidylinositol and phosphatidylethanolamine has been noted previously (Balgoma 2010). Therefore, PI 18:0/20:4 and PE 18:0/20:4 represent important substrates for the release of arachidonic acid by phospholipases, e.g., to initiate prostaglandin signaling in the lung. In contrast to these other classes, ceramide lipids had a less distinct pattern of composition (Figure 24B). However, as discussed further below, an enrichment of longer-chain ceramide species (Cer d18:1/24:0 and Cer d18:1/24:1) was apparent. These distributions agreed very well with the lipid distributions seen in wild type C57BL/6 mice (for a comparison, see (Titz 2016)).

We next investigated how the quantified lipid species were affected by various exposure conditions. These data are expressed as changes in abundance of each analyte, as compared with its levels in lungs from sham-treated mice at the same time point. A range of lipids was significantly affected by cigarette smoke exposure and the effect size tended to increase with exposure duration (Figure 25). These included several major lipid classes, such as glycerophospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, and lipid ethers of phosphatidylcholine and phosphatidylethanolamine), sphingolipids (Cer, Glc/GalCer, LacCer, and Gb3) and cholesteryl esters. The majority of these significantly affected lipid species were upregulated by cigarette smoke exposure. In contrast to the effects of cigarette smoke exposure, there was a much more limited and transient response of these lipids in mice from THS2.2 exposure, cessation, and switching groups. In the most prominent such effect, after 3 months exposure to THS2.2, levels of only a few ceramides, LacCer, and one Glc/Gal ceramide species, were changed in Apoe$^{-/-}$ mice. Furthermore, changes in these lipids were primarily in the opposite direction of changes occurring with cigarette smoke exposure, and were transient and completely disappeared at month 8. Likewise, no statistically significant changes were seen in cessation and switching groups at this time point. These temporal patterns point to a cumulative cigarette smoke exposure effect on lung lipid composition over the 8-month exposure period, while the 2-month cigarette smoke effect was fully reversible within 6 months of

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cessation or switching to THS2.2. In contrast, THS2.2 aerosol caused fewer and only transient changes at month 3, which were no longer apparent at month 8.

In summary, lipidomics profiling of lung tissue demonstrated a strong and consistent effect of cigarette smoke exposure on many lipid classes. Upon cessation or switching to THS2.2, these increases mostly returned to sham levels by the end of each study, whereas THS2.2 exposure by itself had only limited effects that were transient and disappeared at month 8.

4.4.2.6 Lung lipid metabolism: contributions from proteomics

We investigated if the global alterations induced by cigarette smoke exposure in the lung lipidome are reflected by changes in proteins involved in lipid-related processes. For this, we leveraged quantitative lung proteomics data from five time points (1, 2, 3, 5, and 8 months) (for the overall proteomics results, see (Phillips 2015b)). Cigarette smoke (3R4F) exposure induced strong changes in the lung proteome, whereas the THS2.2 aerosol-exposed group showed no statistically significant effects and the minimally elevated protein levels in the cessation and switching groups seen at month 3 returned to sham levels over the course of the study (Figure 26). Interestingly, the subset of proteins involved in lipid-related processes (lipid metabolic process (GO:0006629) and lipid homeostasis (GO:0055088)) demonstrated a response similar to this global profile, with a robust differential abundance response for all 3R4F exposure time points.

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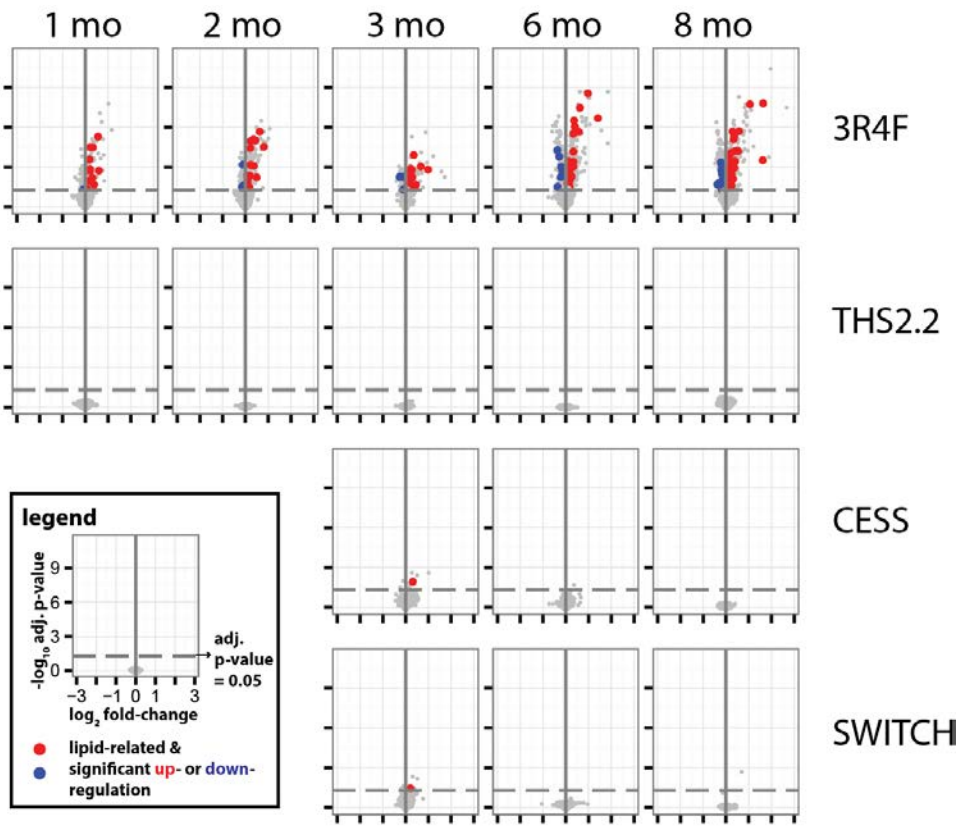


Figure 26. Cigarette smoke effect on the whole and lipid-related lung proteome

Volcano plots show amplitude (log_2 fold-change, x-axis) and significance ($-log_{10}$ BH-adjusted p-value, y-axis) for exposure groups compared with the appropriate sham-exposed groups. Lipid-related proteins (lipid metabolic process (GO:0006629) and lipid homeostasis (GO:0055088)) are indicated with colored dots and remaining proteins with grey dots. Proteins with a differential abundance above the BH-adjusted p-value threshold line of 0.05 are considered statistically significant.

To gain an initial view of which functional categories are involved in the overall cigarette smoke-induced response of lipid-related proteins, we conducted a set enrichment analysis focused on lipid metabolism pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa 2014). Many lipid metabolism pathways were significantly affected by cigarette smoke exposure, including several pathways with links to the lipidome alterations discussed in the previous section (Figure 27). Examples of these pathways include glycerophospholipid metabolism, sphingolipid metabolism, and arachidonic acid metabolism, all showing consistent upregulation with cigarette smoke exposure. In contrast, effects observed for the other exposure groups, modified risk tobacco product (MRTP)-exposed, cessation, and switching, were more limited or showed inconsistent time dependence.

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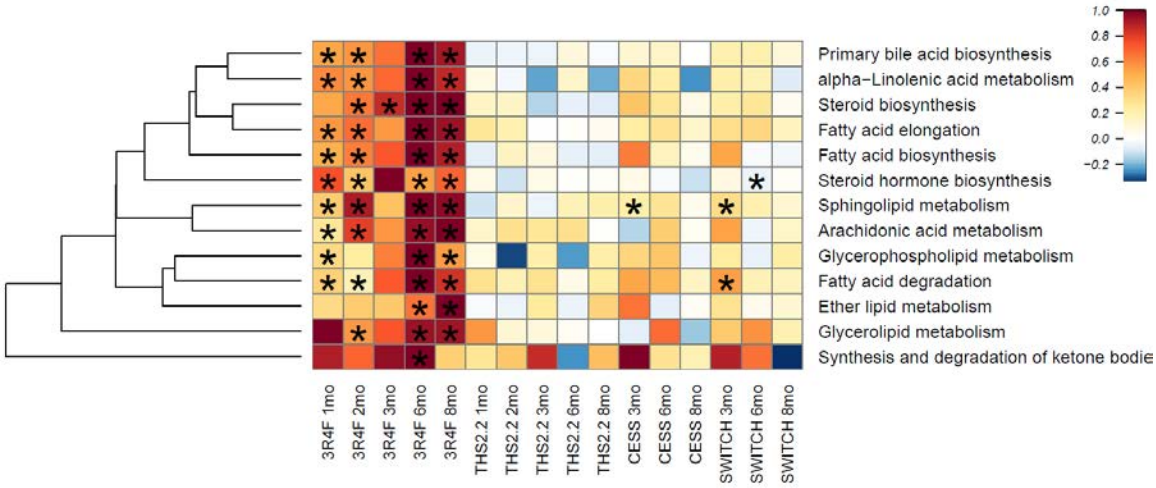


Figure 27. Protein set enrichment for lipid-related pathways from the KEGG database

Fold-change was used as the protein-level metric and mean as the protein set statistic (Väremo 2013). Protein pathways were obtained from the KEGG database (Kanehisa 2014). Only lipid-related pathways with a BH-adjusted p-value <0.05 in any of the comparisons are shown. Enrichment score is color-coded (see key) and the statistical significance of an enrichment is indicated with "*" (BH-adjusted p-value <0.05).

The broad effects of cigarette smoke on the lung lipidome likely require *de novo* synthesis of lipids. The fatty acid biosynthesis pathway was significantly upregulated, as indicated by several key enzymes (Figure 28). Cigarette smoke exposure resulted in significant upregulation of fatty acid synthase (Fasn) as well as of acetyl-CoA carboxylase α (Acaca), which catalyzes the rate-limiting step in the biogenesis of long-chain fatty acids. In addition, the long-chain fatty-acid-CoA ligases 4/5 (Acsl4/5) were upregulated, whereas isoform 1 (Acsl1) was downregulated. Acsl enzymes conjugate fatty acids to coenzyme-A, facilitating the incorporation of these fatty acids into glycerolipids and glycerophospholipids. This is important, for example, in the synthesis of surfactant lipids or synthesis of arachidonic acid-conjugated glycerophospholipids. In this context, it would be interesting to further investigate the intriguing finding that cigarette smoke exposure induced opposing effects on Acsl1 and Acsl4/5. This observation might be related to mechanisms for channeling individual fatty acids to specific synthetic pathways (Robichaud 2015).

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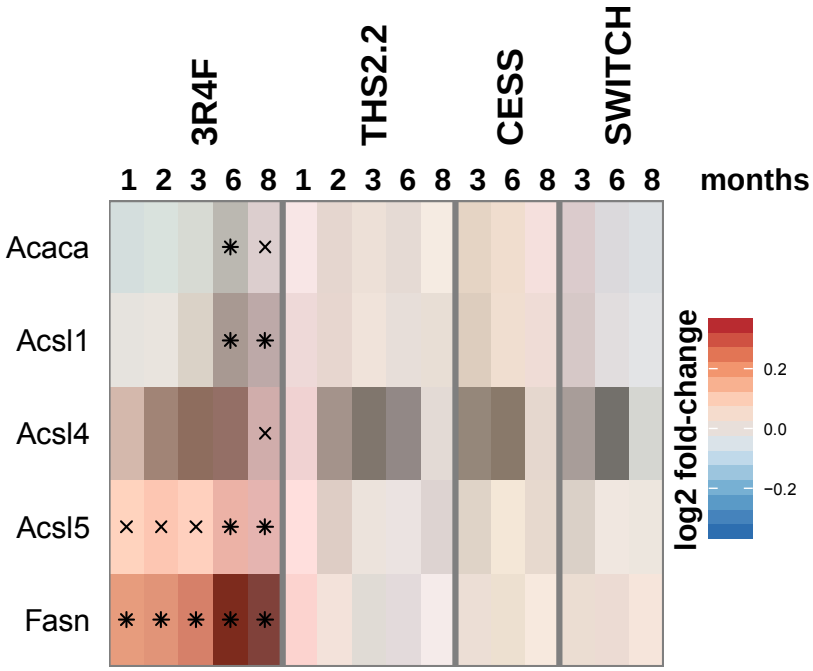


Figure 28. Protein abundance profiles for the KEGG fatty acid biosynthesis pathway

Fold-changes and statistical significance of differential expression are color-coded and marked with a symbol, respectively (see key, BH-adjusted p-value, x≤0.05, *≤0.01). Missing values are grey.

Simultaneously, several fatty acid degradation enzymes were upregulated (e.g., Acaa1a, Acads, Acox1, and Hadha) (Figure 29), which could reflect the need to dispose of lipids that have, for example, been damaged by cigarette smoke-linked ROS (Morissette 2015b).

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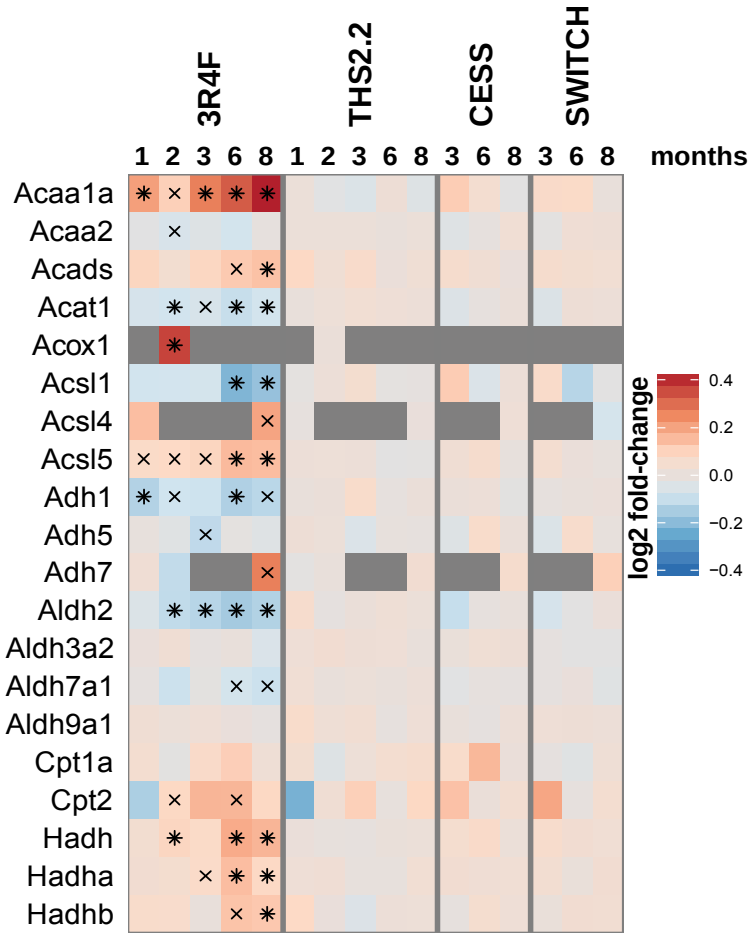


Figure 29. Protein abundance profiles for the KEGG fatty acid degradation pathway

Fold-changes and statistical significance of differential expression are color-coded and marked with a symbol, respectively (see key, BH-adjusted p-value, x≤0.05, *≤0.01). Missing values are grey.

To further relate the exposure effects on the lung lipidome to those in the proteome, we applied a multivariate sparse partial-least-squares canonical analysis approach, as described for the liver lipidomics and proteomics (see above). Clusters with a positive contribution to the first component, that is, proteins tending toward having higher expression in cigarette smoke-exposed animals, involved a range of functions. These included those involved in lipid-related functions (cluster 1, e.g., Fasn, Acsl5, Abcd3), components of the pentose-phosphate pathway (cluster 2), immune-related proteins (cluster 4), surfactants (cluster 5), and xenobiotic response proteins (cluster 6) (for details, see (Titz 2016)). Overall, this analysis clearly illustrates the complexity of the major and correlated responses to cigarette smoke exposure in these data sets. Lipid metabolism and the other functional categories are tightly connected components of the overall exposure response. These protein and lipid connections, as they apply to surfactant regulation, immune-related processes, and the arachidonic acid/eicosanoid pathway, are discussed below.

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4.4.2.6.1 Cigarette smoke-induced surfactant lipid–protein complexes

Pulmonary surfactant is an especially important lipid–protein complex for lung function and homeostasis. It stabilizes alveoli by reducing surface tension (Figure 3) (Perez-Gil 2010) and functions as an innate component of the lung immune system (Chroneos 2009).

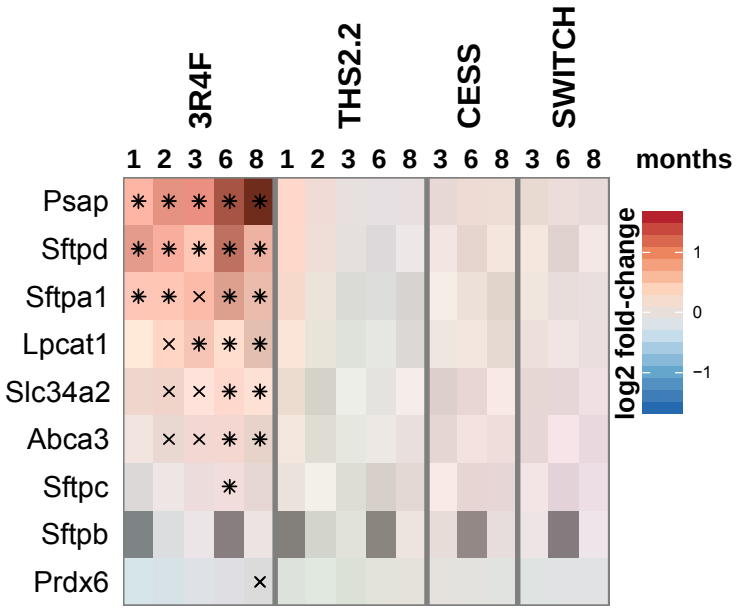


Figure 30. Protein abundance profiles of surfactant-related proteins in the lung

Fold-change is color-coded and statistical significance is marked (BH-adjusted p-value, x≤0.05, *≤0.01). Missing values are grey.

Quantitative proteomics showed that cigarette smoke exposure had statistically significant effects on several surfactant and surfactant metabolism proteins (Figure 30). These effects were substantially lower and did not reach statistical significance in the THS2.2-exposed, cessation, and switching groups. Specifically, Sftpa1 and Sftpd, two surfactant proteins involved in immune defense and surfactant homeostasis (Chroneos 2009, Ikegami 2009), were strongly induced by cigarette smoke exposure. Although Sftpc and Sftpb, the main structural protein components of surfactant (Perez-Gil 2010), showed minimal or no changes, respectively, a strong increase in prosaposin (Psap) was observed. The precursor molecule of Sftpb has strong sequence homologies with Psap (Patthy 1991, Zaltash 1998). Lpcat1, a critical enzyme for the synthesis of dipalmitoylphosphatidylcholine (PC 16:0/16:0), the dominant surfactant lipid in mice (Bridges 2010, Nakanishi 2006), was significantly upregulated by cigarette smoke exposure. In contrast, Prdx6, which acts as a lysosomal-type phospholipase A2 for surfactant lipid (Fisher 2005), was significantly downregulated at month 8. In addition, ABCA3, a transporter critically important for surfactant lipid secretion (Fitzgerald 2007), and Slc34a2, a marker of alveolar type II cells that secrete surfactant (Xu 2010), were upregulated by cigarette smoke exposure.

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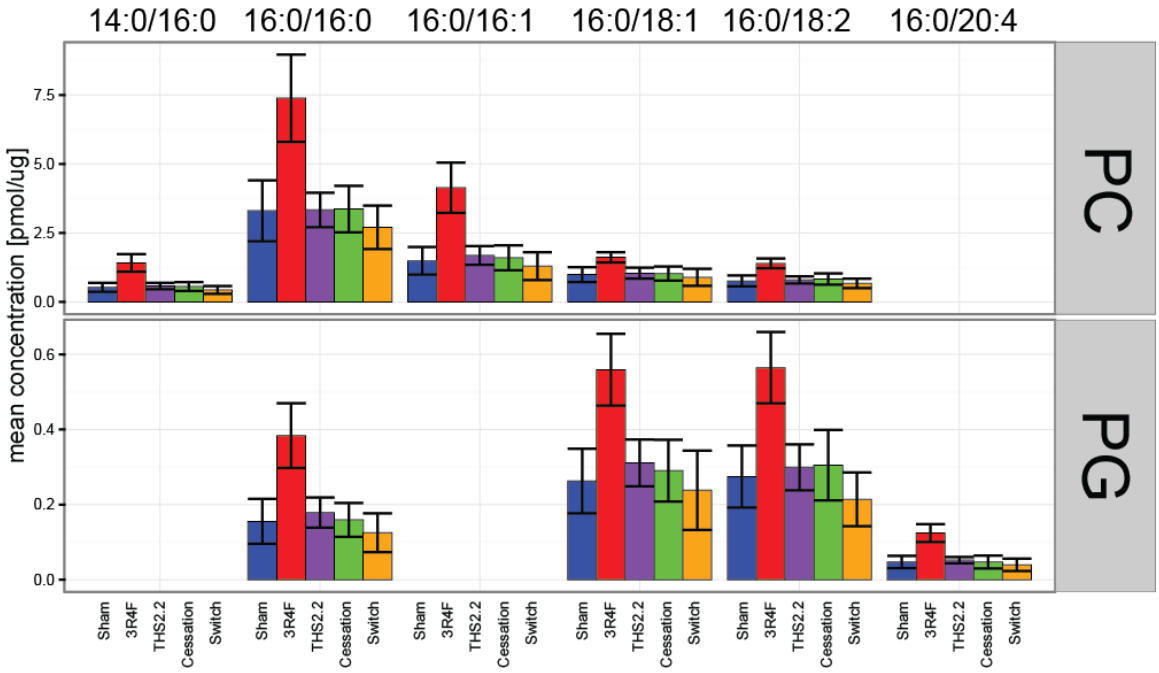


Figure 31. Comparison of lipid concentration profiles for surfactant-associated phosphatidylcholine and phosphatidylglycerol lipid classes

Mean and 95% confidence intervals of measured lipid concentrations are shown for the 8-month time point. Only those lipid species with fatty acid chain compositions contributing at least 5% to the total lipid class concentration of either phosphatidylcholine (PC) or phosphatidylglycerol (PG) are included.

These alterations in surfactant metabolism likely contribute to the cigarette smoke-induced changes in lipidome profiles of surfactant lipids (Figure 31). Consistent with its dominant effects on surfactant, cigarette smoke exposure resulted in the highest measured concentrations within the phosphatidylcholine class for PC 16:0/16:0 at the latest time points of both studies. A strong cigarette smoke-induced increase was also observed for PC 16:0/16:1 and PC 16:0/14:0, both known to contribute to surfactant (Goss 2013). In addition to phosphatidylcholine, phosphatidylglycerol is also relevant in surfactant. While phosphatidylcholine lipids are particularly effective at reducing surface tension, phosphatidylglycerol represents a minor component in human and animal surfactants (about 10% of total surfactant lipid content), which enables surfactant spreading over the inner surface of alveoli (Postle 2001). Those phosphatidylglycerol species most significantly changed by cigarette smoke exposure were PG 16:0/18:1, PG 16:0/18:2, and PG 16:0/16:0. This observation is consistent with previous analyses of mammalian surfactant lipids (Postle 2001).

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Overall, this integrative analysis of surfactant changes revealed consistent and aligned surfactant protein and surfactant lipid responses to cigarette smoke exposure that were reversible when cigarette smoke exposure was discontinued, and these effects in Apoe$^{-/-}$ mice strongly correlated with cigarette smoke exposure effects in C57BL/6 mice (Titz 2016). Cigarette smoke exposure strongly affected both protein and lipid components of surfactant, while THS2.2 exposure, like exposure to prototypic MRTP in the C57BL/6 study, did not induce such changes and the cessation and switching groups rapidly returned to sham levels of these proteins and lipids.

4.4.2.6.2 Cigarette smoke-induced changes in arachidonic acid, eicosanoids, and sphingolipid metabolism-related proteins

Eicosanoids and sphingolipids are key signaling mediators regulating the immune response and cellular processes, such as differentiation, survival, and apoptosis (Gilroy 2010, Lee 2015). Several sphingolipids also play structural roles within cells. In this study, the eicosanoid response was only measured at the protein level (e.g., changes in enzyme levels) (Figure 32), while lipidomics results were not available. However, protein profiles were very similar to those from the C57BL/6 mouse study, where eicosanoid lipid response profiles were measured in the same mice, which suggested an overall similar eicosanoid response between these studies (for a detailed comparison, see (Titz 2016))

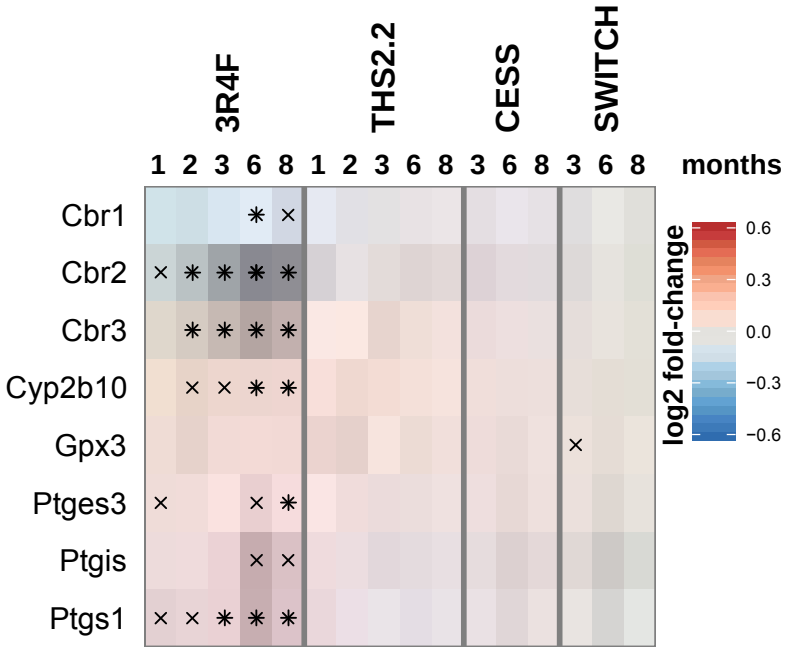


Figure 32. Protein abundance profiles for the KEGG arachidonic acid metabolism pathway

Fold-changes and statistical significance of differential abundance are color-coded and marked with a symbol, respectively (see key, BH-adjusted p-value, x≤0.05, *≤0.01). Missing values are grey.

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The enzyme prostaglandin-endoperoxide synthase 1 (Ptgs1, also known as COX1) representing the metabolic branch leading to PGD2 and PGE2 was strongly induced by cigarette smoke exposure. In cigarette smoke-exposed C57BL/6 mice, this correlated with particularly high PGD2 upregulation (Titz 2016).

Conversely, abundance of the enzyme Cyp2b10, belonging to the branch leading from arachidonic acid via EET to DHET (8,9-DHET, 11,12-DHET, 14,15-DHET) was reduced. EET, in contrast to PGDs, reduce the lung immune response (Morin 2008, Norton 2012). Taken together, the opposing effects of cigarette smoke on PGD2/PGE2 and EET/DHET synthesis may have contributed to the strong inflammatory response in the lungs of cigarette smoke-exposed mice.

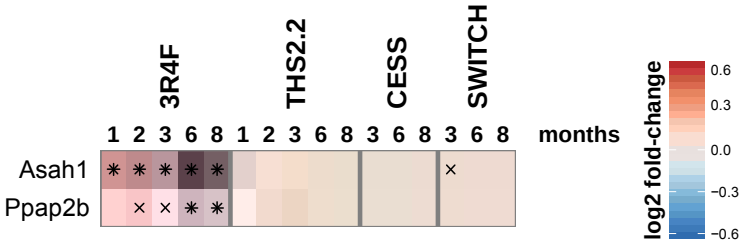


Figure 33. Protein abundance profiles for sphingosine and sphingosine-1-phosphate metabolic enzymes

Fold-changes and statistical significance of differential abundance are color-coded and marked with a symbol, respectively (see key, BH-adjusted p-value, x≤0.05, *≤0.01). Missing values are grey.

Regarding sphingolipid metabolism (Figure 33), we identified the acid ceramidase Asah1s as a likely candidate enzyme causing cigarette smoke-mediated increase in sphingosine synthesis, finding that it was strongly and consistently upregulated upon cigarette smoke exposure in this as well as in the C57BL/6 study, where it correlated with significant increases in sphingosine and S1P (Titz 2016). Interestingly, this enzyme has been implicated in cigarette smoke-mediated inhibition of apoptotic cell clearance, or efferocytosis, by alveolar macrophages (Petrusca 2010). Importantly, the balance between pro-apoptotic ceramide and sphingosine and pro-survival S1P, the "sphingolipid rheostat", was demonstrated as being vital for lung homeostasis and maintenance of alveolar septal integrity (Lee 2015). In addition, Ppap2b, a lipid phosphate phosphatase acting on S1P and other substrates, was strongly downregulated by cigarette smoke exposure. It has been proposed that this lipid phosphatase, through its effect on S1P levels, is involved in changing vascular permeability (Ren 2013). This suggests that Asah1 upregulation and Ppap2b downregulation are likely to contribute to the complex response of sphingolipids to cigarette smoke exposure in our study, and to be implicated in disease-related effects of cigarette smoke exposure on the lung, including suppressed apoptotic cell clearance and vascular disturbances. While the specific effects of these extensive changes in eicosanoid and sphingolipid metabolism will be difficult to unravel, it is likely that, overall, they contribute to disease development in cigarette smoke-

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exposed mice, including the emphysematous effects observed in our studies (Phillips 2015a, Phillips 2015b, Phillips 2015c).

Taken together, the proteome alterations observed upon cigarette smoke exposure are consistent with a major shift in lipid metabolism of the lung. This, in turn, likely forms the basis for our observed cigarette smoke-induced alterations in the lung lipidome. Again, the THS2.2-exposed and cessation and switching groups exhibited reduced effects, as compared with the effects of cigarette smoke exposure, on these lipid metabolism pathways.

4.4.2.6.3 Cigarette smoke exposure affects lipid metabolism regulators and induces associated metabolic alterations

Having demonstrated that cigarette smoke induces extensive alterations in lung lipid metabolism in both mouse strains, we investigated whether a subset of these alterations could be attributed to effects on transcriptional regulators of lipid metabolism. Activity of transcriptional regulators can be deduced though expression of their downstream genes. We applied an established scoring approach for transcriptomics data to quantitatively deduce activities of transcriptional regulators related to lipid metabolism (Martin 2012). Specifically, we employed this approach to evaluate activities of transcriptional regulators of the sterol responsive element binding proteins (SREBP), C/EBP, and PPAR families, all implicated in lipid metabolic processes (Jeon 2012, Varga 2011). We also included Ahr and Nfe2l2 (Nrf-2), key regulators of xenobiotic and oxidative stress responses, respectively. We found that, among these, only the activities of Srebf1a/Srebf1c and Cebpb/Cebpd/Cebpe were predicted to be significantly upregulated in Apoe$^{-/-}$ mice (Figure 34). In a previous investigation comparing common transcription factors predicted from their downstream gene expression to be activated in five mouse models of cigarette smoke-related COPD, we also predicted increased transcriptional activity of Cebpd and Cebpe and confirmed their increased abundance in Apoe$^{-/-}$ mice by targeted proteomics (Cabanski 2015a).

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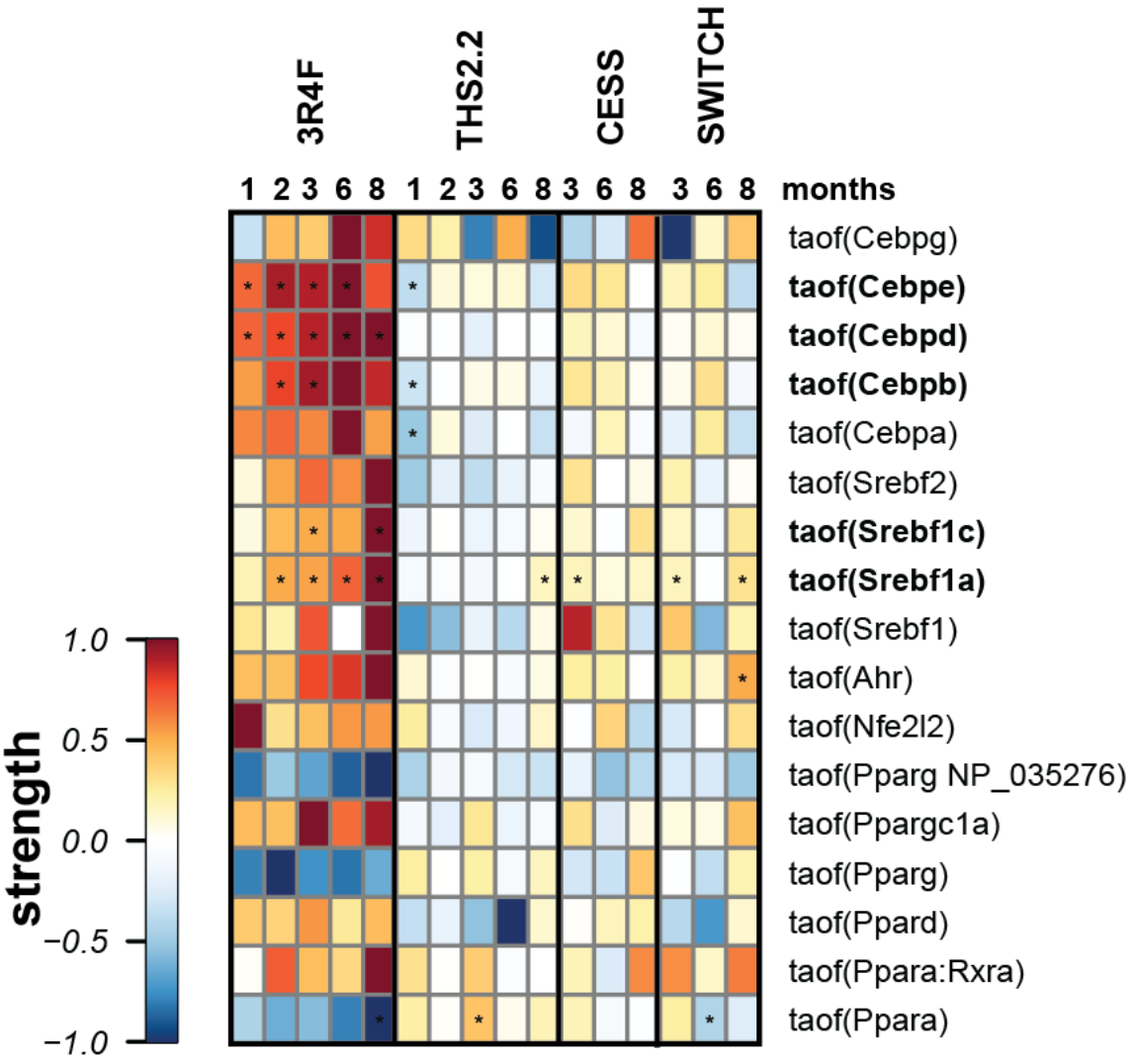


Figure 34. Transcriptional regulation of lipid metabolism

Transcription regulator activity analysis indicates upregulation of SREBP and C/EBP transcriptional activities with cigarette smoke exposure. Each row of the heat map represents the predicted activity of a transcriptional regulator (taof() = transcriptional activity of ()). Each column represents an exposure group compared with sham exposure at the same time point. Perturbation strength is color-coded and statistical significance is indicated (*=BH-adjusted p-value <0.05).

SREBP, including Srebf1a/c, are central hubs in the regulation of lipid metabolism (Bommer 2011). Interestingly, Srebf1c and Cebpb were recently linked to a control mechanism of early adipocyte differentiation (Ayala-Sumuano 2011). In lung tissue, SREBP and C/EBP have been implicated in the regulation of surfactant-related lipogenesis (Zhang 2004), in transcriptional upregulation of the surfactant transporter ABCA3 (Besnard 2007), and as components of a key regulatory module of surfactant

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lipid homeostasis (Xu 2010). Taken together, these results suggest that upregulation of surfactant synthesis by cigarette smoke, which appears to represent the largest quantitative effect on lipid biosynthesis identified in our data, is controlled by activation of SREBP and C/EBP transcriptional complexes.

Nrf-2 is a key regulator of the oxidative stress response (Hayes 2014, Ishii 2002, Muller 2012). Nrf-2 transcriptional activity only showed a trend indicating induction by cigarette smoke exposure in both studies. For a more comprehensive assessment of effects on Nrf-2 signaling, we employed the network perturbation amplitude approach as previously reported for the Nrf-2 signaling network (Boué 2015, Martin 2014). Using this analysis, cigarette smoke exposure induced Nrf-2 signaling in Apoe$^{-/-}$ mice with the downstream node permutation showing marginal significance ($p<0.1$); however, a significant perturbation of Nrf-2 signaling after 5 and 7 months of cigarette smoke exposure was induced in C57BL/6 mice (Titz 2016).

Besides inducing expression of proteins to directly attenuate oxidative stress, Nrf-2 signaling also reprograms cellular metabolism toward NADPH production (Hayes 2014). For example, upregulated proteins include the rate-limiting enzymes of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase, Pgd) and NADPH-generating malic enzyme (Me1). Increasing reduction power in the cell, in the form of NADPH, by the pentose phosphate pathway is an essential component of both the cellular response to oxidative stress and of fatty acid biosynthesis. SREBP regulators have also been reported to facilitate lipid biosynthesis through upregulation of NADPH-generating enzymes including malic enzyme (Me1), glucose-6-phosphate dehydrogenase (G6pd), and 6-phosphogluconate dehydrogenase (Pgd) (Shimomura 1998).

In further support of a strong oxidative stress response of lung tissue upon cigarette smoke exposure, several oxidative stress enzymes significantly and specifically increased in abundance with cigarette smoke exposure. These proteins, which as a whole likely mitigate the detrimental effects of cigarette smoke-induced oxidative damage, included quinone NAD(P)H dehydrogenase 1 (Nqo1), superoxide dismutase 2 (Sod2), thioredoxin reductase 1 (Txnrd1) , and glutamate-cysteine ligase (Gclc) (Titz 2016).

Taken together, the extensive alterations in lipid metabolism induced by cigarette smoke exposure are controlled by a complex regulatory program, which, concomitantly, also mediates other adaptive tissue responses, such as protection against oxidative stress. Thus, changes in lipid metabolism are intrinsic to the broad response of the tissue to cigarette smoke exposure, including oxidative stress and xenobiotic stress responses, immune response, and other metabolic alterations.

4.4.3 Conclusion from ApoE/THS2.2 switching study

In conclusion, Apoe$^{-/-}$ mice were assessed for physiological, histopathological, and molecular evidence of cigarette smoke-related atherosclerosis, lung inflammation, and the development of emphysema. The results collectively show that cigarette smoke exposure accelerated the development of atherosclerotic plaques and emphysema. While 2-month exposure of mice to cigarette smoke resulted in early manifestations of emphysema and atherosclerosis endpoints, both switching and cessation exhibited a beneficial effect, as evidenced by partial (lung function, plaque area, and lung

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morphometry) or even complete (pulmonary inflammation) recovery to levels seen in sham controls. Importantly, chronic exposure of Apoe$^{-/-}$ mice to a THS2.2 aerosol (with nicotine concentration matched to that of cigarette smoke) had minimal biological impact, reflecting the potential of this prototype to positively modify the risk of CVD and COPD in an animal model.

Within the scope of this documentation, we performed integrative systems toxicology analysis of alterations in levels of lipids and lipid metabolism-related proteins in aortic blood vessels, blood plasma, liver, and lungs of cigarette smoke-exposed Apoe$^{-/-}$ mice. We found an extensive, highly consistent response of the lipidome and lipid-related proteome to cigarette smoke exposure. These lipid-related alterations are intrinsic to the overall response "program" of various tissues that may either be direct targets of cigarette smoke exposure or mediators of systemic exposure responses. Several direct links between protein and lipid alterations induced by cigarette smoke exposure became evident, e.g., in lung surfactant and eicosanoid metabolism. Using this model, we evaluated effects of smoking cessation, THS2.2 exposure, and switching from cigarette smoke to THS2.2 exposure. Consistent with the lack of significant morphological and functional adverse effects, exposure to THS2.2 aerosol alone showed little to no effect on lipid-related processes. Both the cessation and switching groups showed rapidly normalized lipid metabolism-related profiles with respect to sham-exposed animals, in correlation with halted progression in emphysema and atherosclerotic plaque formation, and a rapid normalization of inflammatory processes. Thus, our systems toxicology analysis supports an integrated product risk assessment of THS2.2, including the biological benefits of cessation or switching in this model, as compared with continued smoking. Moreover, this work forms the foundation for the identification of translational molecular markers for human risk assessment.

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5 OVERALL CONCLUSIONS

Oxidative stress and inflammatory processes are driving multiple mechanisms associated with smoking-related diseases such as CVD and COPD, and changes in the regulation, composition, and metabolism of lipids are essential macromolecular contributors to these disease mechanisms, and an increasing number of them has been suggested in the literature as potential biomarkers of exposure and primary biological effects (e.g., lipid peroxidation products) or as causally related disease biomarkers (e.g., pro-inflammatory eicosanoids and pro-apoptotic ceramides).

We have included a number of lipid-related biomarkers in our non-clinical studies conducted to assess our candidate MRTP to provide scientifically based evidence for their reduced exposure and harm properties. The lipid classes investigated and described in these studies focused on the mechanisms of cigarette smoke-related oxidative stress and inflammatory processes that are causally involved in the pathogenesis of CVD and COPD.

The reflected lipid changes in rodent disease models following cigarette smoke exposure are indicative of an altered surfactant metabolism and a perturbed balance between pro- and anti-inflammatory lipids in the lung, of a pro-atherosclerotic milieu in the vascular wall of the aortic arch, and an increase in systemic abundance of lipid peroxidation products. Following exposure to THS2.2 or THS2.2M aerosols, most of these changes were substantially lower or absent, and switching to THS2.2 aerosol for 6 months following an initial 2 months of cigarette smoke exposure resulted in a marked reduction of cigarette smoke-related lipid changes similar to the effect of smoking cessation.

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7 APPENDIX 1

7.1 Lipid nomenclature

7.1.1 Lipid classes

Supplementary Table 1 Lipid classes nomenclatures according to Lipid Maps.

Lipid MAPS ontology			Lipid Class
Glycerolipids	Diradylglycerols	Diacylglycerols	DAG
	Triradylglycerols	Triacylglycerols	TAG
Glycero-phospholipids	Glycerophosphocholines	1-(1Z-alkenyl),2-acylglycerophosphocholines	PC P
		1-(1Z-alkyl),2-acylglycerophosphocholines	PC O
		Diacylglycerophosphocholines	PC
		Monoacylglycerophosphocholines	LPC
	Glycerophosphoethanolamines	1-(1Z-alkenyl),2-acylglycerophosphoethanolamines	PE P
		1-(1Z-alkyl),2-acylglycerophosphoethanolamines	PE O
		Diacylglycerophosphoethanolamines	PE
		Monoacylglycerophosphoethanolamines	LPE
	Glycerophosphoglycerols	Diacylglycerophosphoglycerols	PG
	Glycerophosphoinositols	Diacylglycerophosphoinositols	PI
	Glycerophosphoserines	Diacylglycerophosphoserines	PS
Sphingolipids	Ceramides	Ceramides	Cer
	Neutral glycosphingolipids	GalNAcβ1-3Galα1-4Galβ1-4Glc- (Globo series)	Gb3
		Simple Glc series	Glc/GalCer
			LacCer
	Phosphosphingolipids	Ceramide phosphocholines (sphingomyelins)	SM
Sterol Lipids	Sterols	Free Cholesterol	FC
		Steryl esters	CE

7.1.2 Annotations of lipid species

Wherever possible, we report lipids following the Lipid Maps classification (Fahy 2009). Briefly, the different lipid species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG), and diacylglycerol (DAG) are listed with the two fatty acyl groups separated with a hyphen, e.g., PC 16:0-18:1, when positions are unknown and with a slash, e.g., PC 16:0/16:0, when known. LysoPC and lysoPE are abbreviated as LPC and LPE, respectively and cholesteryl esters are abbreviated CE. Ether-linked phospholipids are shown as PC O (alkyl), PC P (alkenyl), PE O (alkyl), and PE P (alkenyl). Fatty acyl groups of ether-linked lipids and N-amidated fatty acyl groups for SM, ceramide (Cer), sphingolipids, and gangliosides are shown after the slash. Sphingolipids are: sphingosine (SPH), sphingosine-1-phosphate (S1P), sphinganine-1-phosphate (SA1P), glucosylceramide (GlcCer), lactosylceramide (LacCer), and globotriaosylceramide (Gb3). Gangliosides are: GD1, GM1, GM3, GQ1, and GT2. Fatty acids and eicosanoids are: docosahexaenoic acid (DHA), arachidonic acid (AA), eicosapentaenoic acid (EPA), 12-hydroxyeicosatetraenoic acid (12-HETE), 11-

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hydroxyeicosatetraenoic acid (11-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE), 8-hydroxyeicosatetraenoic acid (8-HETE), 5-hydroxyeicosatetraenoic acid (5-HETE), prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), 13-hydroxyoctadecadienoic acid (13-HODE), 9- hydroxyoctadecadienoic acid (9-HODE), 6-keto prostaglandin F1α (6-keto-PGF1alpha), prostaglandin F2α (PGF2alpha), tromboxane B2 (TXB2), tromboxane B3 (TXB3), 12-hydroxyeicosapentaenoic acid (12-HEPE), 14,15-dihydroxyeicosatrienoic acid (14_15-DHET), 11,12-dihydroxyeicosatrienoic acid (11_12-DHET), 8,9-dihydroxyeicosatrienoic acid (8_9-DHET), 5,6-dihydroxyeicosatrienoic acid (5_6-DHET), hydroxyoctadecatrienoic acid (13-HOTrE), 12-oxo-eicosatetraenoic acid (12-oxoETE), 5-oxo-eicosatetraenoic acid (5-oxoETE), 5-hydroxyicosapentaenoic acid (5-HEPE), and 15-hydroxyicosapentaenoic acid (15-HEPE).

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7.2 Published 8-iso-prostaglandin F2α levels in rodent urine and blood plasma

Supplementary Table 2. Published concentrations of 8-iso-prostaglandin F2α in urine and blood plasma of mice and rats

This table includes experimental data from control animals and from animals following treatment inducing oxidative stress. To compare the values across the reports with different measurement units, a recalculation as ng/day (urine) or pg/mL (plasma) was done where required. If not otherwise indicated in the papers, the following assumptions were made for the conversion of urine concentrations to daily doses: 1 mL urine excreted per 24 h (Zhang 2011a); 0.4 mg creatinine excreted per day (Newaz 2014); body weight, 25 g. It should be noted that these assumptions are inapplicable for treated animals if treatment changed the amount of excreted urine and/or creatinine, and hence, converted dose values will be misleading in these cases.

Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	C57BL/6 and 129SV	wt	male	control diet	3	ng/mL	24 h	**3**	**ng/day**		(Yatera 2010)
mouse	urine	C57BL/6 and 129SV	wt	male	high-fat diet	6	ng/mL	24 h	**6**	**ng/day**		(Yatera 2010)
mouse	urine	C57BL/6 and 129SV	NOS (triple) ko.	male	control diet	11	ng/mL	24 h	**11**	**ng/day**		(Yatera 2010)
mouse	urine	C57BL/6 and 129SV	NOS (triple) ko.	male	high-fat diet	16	ng/mL	24 h	**16**	**ng/day**		(Yatera 2010)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	C57BL/6 and 129SV	wt	male	control	2.8 ± 0.2	ng/day	24 h	**2.8**	**ng/day**		(Kopkan 2010)
mouse	urine	C57BL/6 and 129SV	wt	male	high salt	3.8 ± 0.2	ng/day	24 h	**3.8**	**ng/day**		(Kopkan 2010)
mouse	urine	C57BL/6 and 129SV	eNOS ko.	male	control	2.4 ± 0.3	ng/day	24 h	**2.4**	**ng/day**		(Kopkan 2010)
mouse	urine	C57BL/6 and 129SV	eNOS ko.	male	high salt	4.6 ± 0.3	ng/day	24 h	**4.6**	**ng/day**		(Kopkan 2010)
mouse	urine	(C57BL/6 and 129SV)-Jackson	Apoe ko.	male	linoleic acid-rich diet	0.078	ng/µmol creatinine	24 h	**0.27**	**ng/day**	based on 3.5 µmol creatinine per 24 h	(Sato 2005)
mouse	urine	(C57BL/6 and 129SV)-Jackson	Apoe ko.	female	linoleic acid-rich diet	0.139	ng/µmol creatinine	24 h	**0.49**	**ng/day**	based on 3.5 µmol creatinine per 24 h	(Sato 2005)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	(C57BL/6 and 129SV)-Jackson	Apoe ko.	male	saturated fatty acid diet	0.058	ng/µmol creatinine	24 h	**0.2**	**ng/day**	based on 3.5 µmol creatinine per 24 h	(Sato 2005)
mouse	urine	(C57BL/6 and 129SV)-Jackson	Apoe ko.	female	saturated fatty acid diet	0.114	ng/µmol creatinine	24 h	**0.4**	**ng/day**	based on 3.5 µmol creatinine per 24 h	(Sato 2005)
mouse	urine	C57BL/6	Apoe ko.	n.i.	saline infusion	3.3	ng/mg creatinine	24 h	**1.3**	**ng/day**		(Sukhanov 2007)
mouse	urine	C57BL/6	Apoe ko.	n.i.	IGF-1 infusion	2	ng/mg creatinine	24 h	**0.8**	**ng/day**		(Sukhanov 2007)
mouse	urine	C57BL/6	wt	male	control	300	pg	24 h	**0.3**	**ng/day**		(Zhang 2011a)
mouse	urine	C57BL/6	wt	male	high salt and DOCA	1400	pg	24 h	**1.4**	**ng/day**		(Zhang 2011a)
mouse	urine	C57BL/6	wt	male	control	0.52	ng	24 h	**0.52**	**ng/day**		(Wang 2008)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	C57BL/6	wt	male	high salt, DOCA, uninephrectomy	1.27	ng	24 h	**1.27**	**ng/day**		(Wang 2008)
mouse	urine	C57BL/6	TRPV1-ko.	male	control	0.6	ng	24 h	**0.6**	**ng/day**		(Wang 2008)
mouse	urine	C57BL/6	TRPV1-ko.	male	high salt, DOCA, uninephrectomy	2.64	ng	24 h	**2.64**	**ng/day**		(Wang 2008)
mouse	urine	C57BL/6	wt	n.i.	control	6 to 8	pg/g bw	24 h	**0.175**	**ng/day**		(Ohashi 2009)
mouse	urine	C57BL/6	wt	n.i.	uretral obstruction + high salt	8 to 15	pg/g bw	24 h	**0.29**	**ng/day**		(Ohashi 2009)
mouse	urine	BALB/c	wt	n.i.	control	0.6 to 1	ng	24 h	**0.8**	**ng/day**		(Ohashi 2009)
mouse	urine	ddY (HIGA)	wt	n.i.	spont. IgA-induced nephropathy	1 to 1.3	ng	24 h	**1.15**	**ng/day**		(Ohashi 2009)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	C57BL/6	wt	male	control	2	ng/mg creatinine	24 h	**0.8**	**ng/day**		(Escano 2009)
mouse	urine	SJL/J	wt	male	control	3	ng/mg creatinine	24 h	**1.2**	**ng/day**		(Escano 2009)
mouse	urine	C57BL/6	wt	male	high salt	5	ng/mg creatinine	24 h	**2**	**ng/day**		(Escano 2009)
mouse	urine	SJL/J	wt	male	high salt	13	ng/mg creatinine	24 h	**5.2**	**ng/day**		(Escano 2009)
mouse	urine	B6D2	wt	n.i.	control (day 0)	5	ng/20 g bw	24 h	**4**	**ng/day**		(Zhang 2011b)
mouse	urine	B6D2	P2Y2R-ko.	n.i.	control (day 0)	7.5	ng/20 g bw	24 h	**6**	**ng/day**		(Zhang 2011b)
mouse	urine	B6D2	wt	n.i.	high salt (day 14)	8	ng/20 g bw	24 h	**6.4**	**ng/day**		(Zhang 2011b)
mouse	urine	B6D2	P2Y2R-ko.	n.i.	high salt (day 14)	14	ng/20 g bw	24 h	**11.2**	**ng/day**		(Zhang 2011b)
mouse	urine	C57BL/6	wt	male	control	2.52 ± 0.35	pg/day	24 h	**0.0025**	**ng/day**	100–1000 × too low	(Kawada 2002)
mouse	urine	C57BL/6	wt	male	Ang II	5.85 ± 0.78	pg/day	24 h	**0.0085**	**ng/day**	100–1000 × too low	(Kawada 2002)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	C57BL/6	wt	male	Ang II and tempol	3.53 ± 0.71	pg/day	24 h	**0.0035**	**ng/day**	100–1000 × too low	(Kawada 2002)
mouse	urine	C57BL/6	wt	male/ female	Ang II	3.1	µg/g creatinine	24 h	**1.24**	**ng/day**		(Kawada 2002)
mouse	urine	C57BL/6	wt	male/ female	control	2.2	µg/g creatinine	24 h	**0.88**	**ng/day**		(Kawada 2002)
mouse	urine	C57BL/6	wt	n.i.	control	8.10 ± 1.09	pg/min/g	30 min	**233.33**	**ng/day**	from short-term measurement	(Shahid 2008)
mouse	urine	C57BL/6	wt	n.i.	TNF	11.13 ± 1.34	pg/min/g	30 min	**320.54**	**ng/day**	from short-term measurement	(Shahid 2008)
mouse	urine	C57BL/6	wt	male	control	6	pg/day/g bw	24 h	**0.15**	**ng/day**		(Carlstrom 2009)
mouse	urine	C57BL/6	SOD k.o.	male	control	8	pg/day/g bw	24 h	**0.2**	**ng/day**		(Carlstrom 2009)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	C57BL/6	wt	male	high salt and ligation	8	pg/day/g bw	24 h	**0.2**	**ng/day**		(Carlstrom 2009)
mouse	urine	C57BL/6	SOD k.o.	male	high salt and ligation	15	pg/day/g bw	24 h	**0.38**	**ng/day**		(Carlstrom 2009)
mouse	urine	ICR	wt	male	control	10	ng/day	24 h	**10**	**ng/day**		(Hong 2014)
mouse	urine	ICR	wt	male	CsA-induced nephropathy/fibrosis	45	ng/day	24 h	**45**	**ng/day**		(Hong 2014)
mouse	urine	C57BL/6	wt	n.i.	control	5	mg/g creatinine/ day	24 h	**2**	**ng/day**	0.4 mg (or 3.5 µmol) creatinine/day	(Min 2014)
mouse	urine	C57BL/6	wt	n.i.	unilateral uretral obstruction	110	mg/g creatinine/ day	24 h	**44**	**ng/day**	0.4 mg creatinine/day	(Min 2014)
mouse	urine	BTBR	wt	female	control	1700	pg/mg creatinine	6 h	**0.7**	**ng/day**		(Askari 2014)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	BTBR	ob/ob	female	spont. renal damage	5500	pg/mg creatinine	6 h	**2.3**	**ng/day**		(Askari 2014)
mouse	urine	C57BL/6	Apoe / GPx1 ko.	male	control	0.69 ± 0.1	pg/µmol creatinine	n.i.	**0.0027**	**ng/day**	1000–10000 × too low	(Chew 2010)
mouse	urine	C57BL/7	Apoe / GPx1 ko.	male	stz. injection	0.93 ± 0.09	pg/µmol creatinine	n.i.	**0.003**	**ng/day**	1000–10000 × too low	(Chew 2010)
mouse	urine	C57BL/6	wt	n.i.	control	75	pg/mg creatinine	n.i.	**0.03**	**ng/day**	same data as (Fujita 2005)	(Kanaya 2004)
mouse	urine	KKAy	wt	n.i.	spont. diabetic	155	pg/mg creatinine	n.i.	**0.062**	**ng/day**	same data as (Fujita 2005)	(Kanaya 2004)
mouse	urine	C57BL/6	wt	male	control	75	pg/mg creatinine	24 h	**0.03**	**ng/day**	100–1000 × too low	(Fujita 2005)
mouse	urine	KKAy	wt	male	spont. diabetic	155	pg/mg creatinine	24 h	**0.062**	**ng/day**	100–1000 × too low	(Fujita 2005)
mouse	urine	ICR	wt	male	control	75	pg/mg creatinine	24 h	**0.03**	**ng/day**	100–1000 × too low	(Fujita 2005)

(table continues)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	urine	ICR	wt	male	stz. injection	180	pg/mg creatinine	24 h	**0.072**	**ng/day**	100–1000 × too low	(Fujita 2005)
mouse	urine	C57BL/6	db/+	male	control	45	ng/day	24 h	**45**	**ng/day**		(Fujii 2007)
mouse	urine	C57BL/6	db/db	male	spont. diabetic	180	ng/day	24 h	**180**	**ng/day**		(Fujii 2007)
mouse	plasma	C57BL/6	wt	male	normal and choline-deficient diet	4 to 5	nM	n.a.	**508.5**	**pg/mL**		(Yoshida 2006)
mouse	plasma	C57BL/6	wt	male	antioxidant -free diet	13	nM	n.a.	**1469**	**pg/mL**		(Yoshida 2006)
mouse	plasma	C57BL/6	wt	male	plus normal diet	0.8	nM	n.a.	**90.4**	**pg/mL**		(Yoshida 2008)
mouse	plasma	C57BL/6	Vit. D transporte r-ko.	male	plus Vit. D-deficient diet	7	nM	n.a.	**791**	**pg/mL**		(Yoshida 2008)
mouse	plasma	C57BL/6	wt	male	normal diet + AIPH	8	nM	n.a.	**904**	**pg/mL**		(Yoshida 2008)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	plasma	C57BL/6	Vit. D transporte r-ko.	male	Vit. D-deficient diet + AIPH	27	nM	n.a.	**3051**	**pg/mL**		(Yoshida 2008)
mouse	plasma	n.i.	wt	male	control	165 ± 20	pg/mL	n.a.	**165 ± 20**	**pg/mL**		(Newaz 2014)
mouse	plasma	n.i.	gp91phox	male	control	100 ± 15	pg/mL	n.a.	**100 ± 15**	**pg/mL**		(Newaz 2014)
mouse	plasma	n.i.	wt	male	glycerol injection	363 ± 22	pg/mL	n.a.	**363 ± 22**	**pg/mL**		(Newaz 2014)
mouse	plasma	n.i.	gp91phox	male	glycerol injection	152 ± 20	pg/mL	n.a.	**152 ± 20**	**pg/mL**		(Newaz 2014)
mouse	plasma	C57BL/6	wt	n.i.	control	330	pg/mL		**330**	**pg/mL**		(Min 2014)
mouse	plasma	C57BL/6	wt	n.i.	unilateral uretral obstruction	400	pg/mL		**400**	**pg/mL**		(Min 2014)
mouse	plasma	SV/129	wt	n.i.	control	88.5 ± 8.7	pg/mL	n.a.	**88.5 ± 8.7**	**pg/mL**		(Menini 2006)
mouse	plasma	SV/129	wt	n.i.	stz. injection	143.5 ± 11.2	pg/mL	n.a.	**143.5 ± 11.2**	**pg/mL**		(Menini 2006)

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Species	Matrix	Strain	Genotype	Sex	Treatment /Control	Value	Unit (original)	Collection period	Value (converted)	Unit (converted)	Remark	Ref.
mouse	plasma	SV/129	p66(Shc)-ko.	n.i.	control	79.2 ± 4.6	pg/mL	n.a.	**79.2 ± 4.6**	**pg/mL**		(Menini 2006)
mouse	plasma	SV/129	p66(Shc)-ko.	n.i.	stz. injection	94.4 ± 14.5	pg/mL	n.a.	**94.4 ± 14.5**	**pg/mL**		(Menini 2006)
mouse	plasma	CF-1	wt	male	control	17	pg/mL	n.a.	**17**	**pg/mL**		(Lambert 2010)
mouse	plasma	CF-1	wt	male	EGCG	190	pg/mL	n.a.	**190**	**pg/mL**		(Lambert 2010)
rat	urine	Sprague-Dawley	wt	male	control	12	ng/day	24 h	**12**	**ng/day**		(Montero 2000)
rat	urine	Sprague-Dawley	wt	male	stz. injection	100	ng/day	24 h	**100**	**ng/day**		(Montero 2000)
rat	plasma	Sprague-Dawley	wt	male	control	45	pg/mL	n.a.	**45**	**pg/mL**		(Montero 2000)
rat	plasma	Sprague-Dawley	wt	male	stz. injection	145	pg/mL	n.a.	**145**	**pg/mL**		(Montero 2000)

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