

Summary of Evidence of Effects of *IQOS* on Endothelial function

Response to the article entitled “Vascular endothelial function is impaired by aerosol from a single IQOS HeatStick to the same extent as by cigarette smoke”

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TABLE OF CONTENTS

1	Executive Summary	3
2	Introduction.....	4
3	Analysis of methods and findings by Nabavizadeh et al.	5
	Flow Mediated Dilation.....	6
	Exposure	6
4	Effects of nicotine on endothelial function – literature review.....	7
5	Studies conducted by PMI	8
	Pre-clinical studies.....	8
	Effects of THS on adhesion of human monocytic cells <i>in vitro</i>	8
	Effects of THS on chemotaxis and transendothelial migration <i>in vitro</i>	15
	Cardiovascular and respiratory chronic exposure effects of THS in ApoE ^{-/-} mice.....	19
	Clinical studies	25
	Inflammation.....	25
	Endothelial dysfunction	25
	Oxidative stress markers	26
	Cholesterol levels.....	27
	Summary of results from PMI studies.....	27
6	Discussion.....	28
7	Conclusion	30
8	Abbreviations.....	31
9	References and related documents.....	32

1 EXECUTIVE SUMMARY

Nabavizadeh and colleagues from the University of California, San Francisco, USA have recently published a Research Paper in *Tobacco Control* (Nabavizadeh, 2018) claiming that “mainstream IQOS aerosol from a single HeatStick can rapidly and substantially impair endothelial function in rats comparably to smoke from a cigarette”. Findings from this article are not new and were originally published by the authors in a public comment to the FDA PMI MRTP Application on Dec 4th 2017 (FDA-2017-D-3001-0118) and at the [American Heart Association Conference](#).

PMI appreciates independent research and the motivation to perform studies that investigate potential health impact of smoke-free products compared to cigarettes. We conduct a thorough, objective review of the methodologies used, results generated and the conclusions drawn, and compare with other relevant studies conducted by PMI and other research groups, and whether the results agree or disagree with ours we share our findings from such a review in order to increase the scientific knowledge surrounding the assessment for Tobacco Heated System (THS) commercialized as IQOS.

Our analysis of the study by Nabavizadeh et al. can be summarized as follows:

- Endothelial dysfunction is an important prognostic factor in the development of atherosclerosis, hypertension and heart failure.
- Flow mediated dilation (FMD) is a noninvasive technique used to assess endothelial function and considered predictive of cardiovascular risk when executed following a strictly standardized protocol.
- The internationally accepted guidelines for the conduct of the FMD measurement in humans (published by the International Brachial Artery Reactivity Task Force) clearly state that the ingestion of “substances that might affect FMD such as caffeine, high-fat foods and vitamin C or use of tobacco for at least 4 to 6 hours before the study” should be avoided prior to the measurement.
- The acute change in FMD measured immediately after the ingestion of the above mentioned substances are not predictive of cardiovascular risk.
- Therefore the effects observed in the study by Nabavizadeh et al. are actually expected short-term sympathomimetic effects of nicotine that are well known and are not predictive of the development of cardiovascular disease in humans.
- Furthermore, the comparisons between THS and cigarettes in this study are inappropriate because of the methodological issues that led to nicotine exposure from THS being much higher than nicotine exposure from cigarettes.
- In summary, the conclusion stated by the authors that, based on these results, “IQOS use does not necessarily avoid the adverse cardiovascular effects of smoking cigarettes” is incorrect and misleading and the study provides no reliable scientific information about the effects of THS on the risk of cardiovascular disease.

2 INTRODUCTION

The endothelium refers to cells that line the interior surface of blood vessels. The vascular endothelium is an active paracrine organ responsible for the regulation of the vascular tone through the effects of locally synthesized mediators, predominantly nitric oxide (NO) and superoxide, and endothelial NO synthase (eNOS) responsible for the NO generation. NO is abundantly present in normally functioning vasculature where it acts as a vasodilator, inhibits inflammation, and has an anti-aggregation effect on platelets. Its depletion is both a sign and cause of endothelial dysfunction resulting from reduced activity of eNOS and amplified production of nicotinamide adenine dinucleotide oxidase, which, in turn, results in raised levels of reactive oxygen species. This cascade is the basis for reduced vascular compliance through an imbalanced regulation of tone with a predominance of vasoconstrictive elements. Endothelial dysfunction is a major factor in the development of atherosclerosis (Bonetti, 2003; Brunner, 2005; Corretti, 2002; McLenachan, 1991; Vogel, 1997), hypertension (Bleakley, 2015) and heart failure (Drexler, 1992).

Endothelial dysfunction induced by smoking is initiated by reduced NO bioavailability and further by the increased expression of adhesion molecules and subsequent endothelial dysfunction. Over time, smokers develop low grade inflammatory and pro-oxidative features in the blood that can affect normal vascular regulatory functions including vasotone regulation, endothelial permeability, and coagulation. These changes favor and accelerate the appearance of atherosclerotic plaques. After transendothelial migration and activation, macrophages take up oxidized lipoproteins arising from oxidative modifications and transdifferentiate into foam cells. In addition to direct physical damage to endothelial cells, smoking induces tissue remodeling, and prothrombotic processes together with activation of systemic inflammatory signals, all of which contribute to atherogenic vessel wall changes (Favero, 2014; Messner, 2014; Poussin, 2016; Yanbaeva, 2007). A causal factor is that cigarette smoke contains $\sim 10^{17}$ long-lived radicals/g tar (hydrophobic) fraction and $\sim 10^{15}$ radicals/gram of the volatile fraction.

A number of studies were conducted and published by Philip Morris International (PMI) researchers thoroughly assessing the impact of the aerosol from THS and comparing it to that of cigarette smoke. The authors of the recently published article “*Vascular endothelial function is impaired by aerosol from a single IQOS HeatStick to the same extent as by cigarette smoke*” (Nabavizadeh, 2018) pointed out that the cardiovascular health effects of THS and similar products are incompletely understood. More specifically, the authors stated that there is insufficient scientific evidence addressing the impact of THS on vascular endothelial function tested *in vivo* which motivated them to conduct a study to compare the effects of aerosol of THS (using Russian IQOS Parliament branded HEETS - Philip Morris International) to those of mainstream smoke of Marlboro Red combusted cigarettes (Altria, Philip Morris USA) and fresh air.

The authors exposed anaesthetized Sprague-Dawley rats via a nose cone to THS aerosol from a single HeatStick, mainstream smoke from a single Marlboro Red cigarette or clean air for a series of ten consecutive 30 seconds cycles over 5 minutes. Each cycle consisted of 15 or 5 seconds of exposure to either THS aerosol, smoke or air followed by removal of the rat from the nose cone for 15 and 25 seconds, respectively. Additional, shorter exposure of a separate group of animals to THS aerosol and air for 3 cycles of 30 s (5 s exposure + 25 s break) over 1.5 minutes was performed. The main endpoints were pre- and post-exposure flow mediated dilation (FMD) measured in all exposure groups and post-exposure serum nicotine and cotinine measured immediately and 20 min after the 10 cycles of 5 s exposure + 25 s break. Nicotine concentration in THS aerosol, cigarette

mainstream smoke and THS tobacco were measured as well. Regardless of the exposure regime, the authors reported similar levels of reduction of FMD caused by the exposure to THS aerosol and smoke from combustible cigarette compared to the exposure to air (Table 1):

Table 1. Reduction of FMD in rats after the exposure to THS aerosol and smoke

Exposure regime	10 cycles, 15 s exposure + 15 s break, 5 min in total	10 cycles, 5 s + 25 s break, 5 min in total	3 cycles, 5 s + 25 s break, 1.5 min in total
Pre-exposure vs. post-exposure FMD	THS: 10.6±2.9% vs. 4.5±1.9%, p=0.0009	THS: 10.8±1.0% vs 3.8±2.6%, p=0.0001	THS: 11.0±4.2 to 4.5±1.5, p=0.0019
	Smoke: 10.6±2.0% vs 4.6±1.3%, p=0.0004	Smoke: 11.2±2.6% vs 4.2±2.3%, p=0.0006	Smoke: not performed in the study
	Air: 8.3±1.9% vs 8.8±4.5%, p=0.82	Air: 9.5±3.0% vs 8.1±1.8%, p=0.85	Air: not reported in the study

Those findings led the authors to conclude that: “*mainstream IQOS aerosol from a single HeatStick can rapidly and substantially impair endothelial function in rats comparably to smoke from a cigarette*” (Nabavizadeh, 2018).

This document aims to first clarify findings published by Nabavizadeh *et al.* specifically focusing on the methods used, and then to provide additional context by summarizing the scientific data available to date on nicotine and THS with regard to the effects on endothelial function.

Studies at PMI are performed according to international standards of good scientific practice. For example, use of validated Organization for Economic Co-operation and Development (OECD) test methods where they exist and in compliance with the principles of Good Laboratory Practice (GLP) and Good Clinical Practice (GCP), where relevant. PMI has conducted 2 *in vitro*, 1 *in vivo* preclinical studies and 3 clinical studies relevant for the purpose of this document, to compare the effects of THS aerosol with those of cigarette smoke. At comparable concentrations, THS had only minimal effects on cardiovascular disease (CVD) pathways and did not induce the progression of cigarette smoke-induced atherosclerotic changes. THS aerosol had an overall lower biological impact, indicating that THS aerosol is markedly less harmful than smoke. The results from our clinical studies showed positive changes in clinical risk endpoints linked to CVD, such as HDL, sICAM, prostaglandin and white blood cells even under dual use conditions.

3 ANALYSIS OF METHODS AND FINDINGS BY NABAVIZADEH ET AL.

In the following section the methods used and findings obtained by Nabavizadeh *et al.* have been carefully assessed reviewing relevant scientific literature. The analysis was specifically focused on the clinical relevance and the execution of the FMD measurements and the exposure system used in this study. After the analysis, several methodological issues were identified.

Flow Mediated Dilatation

Flow mediated dilation is a noninvasive technique used to assess endothelial function and is considered predictive of cardiovascular risk in human (Raitakari, 2000). It is based on reactive hyperemia of increased arterial blood flow following a period of transient arterial occlusion. This increase in blood flow increases shear stress on the vessel walls which induces the release of nitric oxide by endothelial cells, and therefore causing vasodilatation. This response is termed nitric oxide-mediated FMD and is typically measured in the brachial artery.

Brachial artery median FMD is considered to independently predict long-term adverse cardiovascular events in healthy subjects with no apparent heart disease in addition to those derived from traditional risk factor assessment (Shechter, 2014). It is important that the technique is performed in accordance with strict guidelines that take into account the technical and interpretive limitation of the technique. Numerous factors affect flow-mediated vascular reactivity, including temperature, food, drugs and sympathetic stimuli, such as nicotine, among others. FMD should be measured at resting and carefully controlled conditions and not as an acute response to a stimulus. Subjects should fast for at least 8 to 12 h before the study, and they should be studied in a quiet, temperature-controlled room. All vasoactive medications should be withheld for at least four half-lives, if possible. In addition, subjects should not exercise, should not ingest substances that might affect FMD such as caffeine, high-fat foods and vitamin C or use tobacco for at least 4 to 6 h before the study (Corretti, 2002). An illustrative example of an acute effect on FMD is the intake of coffee. In studies by Papamichael et al. and Buscemi et al. coffee exerts an acute unfavorable effect on the endothelial function in healthy adults (Buscemi, 2010; Papamichael, 2005), although moderate coffee consumption has either no or a protective effect on cardiovascular disease (Ale-Agha, 2018; Ding, 2014; Rodriguez-Artalejo, 2018). This indicates the criticality of the careful experimental design and the interpretation of the results obtained in the studies where FMD is the main endpoint, especially focusing on avoiding misinterpretation of the acute effects of tested substances and translating them into the risk of disease development.

Relevant for this document, measuring FMD as described by Nabavizadeh et al. after the acute exposure to smoke/THS aerosol, that contains the stimulant substance nicotine, is expected to affect the outcome of the measurement, as outlined in the section above. Therefore, the results of this study should be discussed and interpreted as acute effects and not considered to be predictive of the long-term development of CVD. The aspect of the acute effects of nicotine on endothelial dysfunction has been further elaborated on in the section below: Effects of nicotine on endothelial function.

Exposure

A surprising finding that questions the method of exposure in the study was the post-exposure serum nicotine levels, which was ~4.5 to ~7 fold higher in rats exposed to THS aerosol than in rats exposed to cigarette smoke, despite nicotine being measured in the THS aerosol at ~63% of the amount measured in cigarette smoke. The authors suggested an explanation for the difference in serum nicotine levels between the cigarette and THS exposed group: “*One reason for this difference could be particle size difference which determines to what extent the particles reach the respiratory zone*”. The generation of various sizes particles is highly unlikely when THS aerosol is generated in a well-established aerosol generation machine, as the droplet size is in the respirable range similar to the particle size of cigarette smoke (Wong, 2016). The use of a manual syringe-pump-driven system to generate smoke and THS aerosol for the initial experiment reported by the authors, and use of a

commercially available automatic vaping system for subsequent experiments may have caused the generation of the aerosol containing various particle sizes. The authors did not explicitly state which exposure system was used in the experiments presented in the publication and did not state if the exposure systems have been appropriately validated, which is extremely important for the proper conduct of inhalation exposure studies. Because of the much higher levels of harmful and potentially harmful constituents found in cigarette smoke relative to THS, cigarette smoke is much more irritating when inhaled and the rats are able to reduce their rate of breathing and hence the amount of smoke (and nicotine) inhaled during cigarette smoke exposure. THS aerosol is much less irritating and does not cause the same reflex during exposure, consequently the rats take up much more aerosol into the lung, thereby increasing their nicotine uptake.

Another observation is that 10 cycles of 15s exposure + 15s break, 10 cycles of 5s exposure + 25s break and 3 cycles of 5s exposure + 25s break which were selected to deliver different quantities of aerosol/smoke and nicotine, resulted in the nearly the same FMD post-exposure reduction, which suggests the absence of the concentration response of the FMD method. In the article by Heiss et al. describing the development of the rat FMD model used in the study of Nabavizadeh et al. a clear dose-responses for nitroglycerin and acetylcholine were presented (Heiss, 2008), which shows that the method is sensitive to detect different levels of the effect. However, this feature of the FMD method in rats has not been demonstrated in the study by Nabavizadeh et al. which questions the suitability of this method for the evaluation of the exposure effects of THS vs. combustible cigarette on endothelial function in the experimental setup described by the authors.

Finally, to achieve the reduction of FMD by exposure to THS aerosol similar to that of smoke from combustible cigarette regardless of the exposure regime, nicotine uptake had to be up to 7.1 fold higher in THS exposed animals compared to the uptake by animals exposed to combustible cigarette smoke, which is far from being representative of any real-world THS consumption scenario.

4 EFFECTS OF NICOTINE ON ENDOTHELIAL FUNCTION – LITERATURE REVIEW

Changes reported by Nabavizadeh et al. in FMD after a single THS exposure is an expected acute effect and is most likely driven by the exposure to nicotine and not predictive of the long-term development of CVD (a public comment by Dr. Farsalinos on November 24th, 2017 and can be found [here](#)). Dr. Farsalinos stated: *“To the best of my knowledge, no study has ever found FMD to be a prognostic marker of disease when the measurement was made after an acute exposure to a stimulant.”* The acute effects of nicotine on endothelial dysfunction evaluated by FMD in human was reported by Neunteufl et al. Healthy smokers (n=16) were participants of a randomized, observer-blinded crossover study comparing the effects of two sprays of nicotine nasal spray (Nicotrol NS, Pharmacia and Upjohn, Vienna, Austria, 1 mg nicotine) or one smoked cigarette (Camel Filters, R. J. Reynolds Tobacco Co., Winston-Salem, North Carolina, 1mg nicotine, 12 mg tar) on vascular reactivity in the brachial artery. Using high-resolution ultrasound, FMD and endothelium-independent, nitroglycerin-induced dilation were assessed at baseline and 20 min after the administration of nicotine (spray or cigarette). The findings of this study demonstrate that nicotine causes acute endothelial dysfunction in long-term smokers and suggest that there may be other constituents of cigarette smoke that contribute to this adverse effect since nicotine nasal spray caused less impairment of FMD than smoking a cigarette (Neunteufl, 2002).

The acute effect of nicotine on the endothelium can be exerted via two mechanisms leading to the opposite outcomes: nicotine can decrease coronary blood flow by acting on vascular smooth muscle α 1-adrenergic receptors to constrict coronary arteries, but can also increase coronary blood flow by increasing cardiac output, causing subsequent increase in FMD, and directly stimulating coronary artery β 2-receptor for coronary vasodilation. Thus, the net effect of an acute exposure on coronary blood flow is the balance of the two actions, typically a blunting of the expected FMD increase in coronary blood flow that would be expected from the nicotine-induced increase in cardiac output and increased myocardial oxygen demand (Benowitz, 2016).

Further indirect evidence that the exposure effects observed by Nabavizadeh et al. are acute nicotine effects and not predictive of development of CVD could be concluded from 5-year Lung Health Study. In this study 5,887 middle-aged smokers with chronic obstructive pulmonary disease (COPD) were followed up for 5 years. During that study, two-thirds of the subjects were provided with nicotine replacement therapy (NRT), including nicotine gum. Many of these subjects used nicotine gum heavily for several years. A comparison of smokers versus quitters with nicotine gum versus quitters without nicotine gum showed no increase in hospital admissions for cardiovascular events with nicotine gum treatment (Murray, 1996). In fact, the opposite was observed. Study participants who quit smoking and used nicotine gum had a lower hospital admission rate for cardiovascular disease than participants who quit smoking and did not use gum. The study demonstrated no association between sustained NRT use, therefore sustained nicotine exposure, and the occurrence of cardiovascular events. Although nicotine uptake in smokers and in the study by Nabavizadeh et al. is via inhalation, the cumulative dose of nicotine achievable over time with NRT can be as high as or higher than what is achieved with inhaled nicotine products.

In summary, acute sympathomimetic effects of nicotine are well known and not surprisingly affect the assessment of FMD in the short-term. However, the acute effects of an exposure to nicotine on FMD have no prognostic value for cardiovascular disease.

5 STUDIES CONDUCTED BY PMI

PMI conducted several studies to address cardiovascular effects of THS aerosol. Those studies have demonstrated an absence or limited cardiovascular effects of THS aerosol exposure compared to effects of combustible cigarette smoke. We have conducted 2 *in vitro*, 1 *in vivo* pre-clinical studies and 3 clinical studies to compare the effects of THS aerosol with those of cigarette smoke and fresh air. In the following section, results from those studies relevant to the discussion on findings from Nabavizadeh et al. are outlined. However, due to differences in methodological approaches a direct comparison between PMI results from all relevant studies and Nabavizadeh et al. is not possible.

Pre-clinical studies

Effects of THS on adhesion of human monocytic cells *in vitro*

Alterations of endothelial adhesive properties by cigarette smoke can progressively favor the development of atherosclerosis which may cause cardiovascular disorders. A systems biology/toxicology approach combined with a functional *in vitro* adhesion assay was used to assess

the impact of THS aerosol on the adhesion of monocytic cells to human coronary arterial endothelial cells (HCAECs) and compare with a reference cigarette smoke (3R4F, University of Kentucky) (Poussin, 2016). HCAECs were grown in endothelial cell growth medium. Cells were starved for 24 h in endothelial cell starvation medium containing 0.25% fetal calf serum (FCS) and then treated for 4 h with:

- conditioned media from MM6 cells pre-incubated with low or high concentrations of THS or 3R4F aqueous extracts for 2 h (indirect treatment)
- unconditioned media with low or high concentrations of THS or 3R4F aqueous extracts prepared without MM6 cells (direct treatment)
- freshly generated THS or 3R4F aqueous extracts (fresh direct treatment)

Mainstream smoke from reference cigarette 3R4F (University of Kentucky) was generated on a 20-port Borgwaldt smoking machine according to the Health Canada Intense protocol and bubbled through ice cold PBS (6 cigarettes/36 ml PBS, approximately 1.8 puffs/ml) described previously (Muller, 1995; Muller, 1994). Final concentrations of smoke bubbled phosphate-buffered saline (sbPBS) ranged from 0.06 to 0.225 puff/ml. Aerosol from THS was produced using a pre-defined puff count of 12 puffs per stick on a 30-port rotary aerosol generator (type SM 2000 P1) according to the Health Canada Intense protocol. The aerosol was bubbled into ice cold PBS to trap the water soluble fraction (10 HeatSticks/40 ml, stock solution concentration: 3 puffs/ml).

Functional investigations revealed that 4 h exposure to aqueous 3R4F smoke extract promoted the adhesion of MM6 cells to HCAECs. 3R4F aqueous extract promoted maximum MM6 cell-HCAEC adhesion at low concentrations of 0.045–0.06 puffs/ml in the indirect treatment, and at a high concentration of 0.225 puffs/ml in the fresh direct treatment. However, no significant increase in MM6 cell-HCAEC adhesion was observed when THS aqueous extract was used for 4 h at the same concentrations as 3R4F in an indirect and fresh direct treatments. To achieve similar increases of MM6 cell-HCAEC adhesion with THS aqueous extract as observed for 3R4F, 10-fold higher concentrations (2.25 puffs/ml) were necessary for fresh direct treatment, and 20–25-fold higher concentrations (1.125 puffs/ml) were required for indirect treatment. The differences observed between direct and fresh direct treatment could be explained by possible decay of unstable components of smoke/aerosol constituents during the preparation of unconditioned media used in direct treatment (Figure 1)(Poussin, 2015).

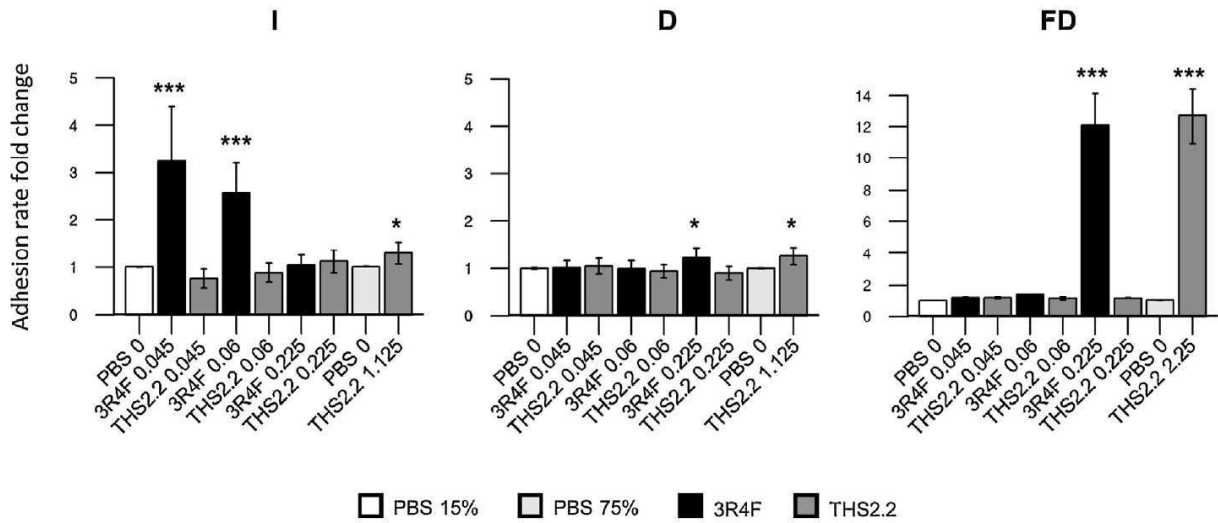


Figure 1. Effects of THS and 3R4F aqueous extracts on the adhesion of MM6 cells to HCAECs following indirect (I), direct (D), and fresh direct (FD) treatments of HCAECs. Bar charts represent fold changes of the adhesion rate relative to respective vehicle controls. The adhesion rate reflects the number of adherent MM6 cells relative to the total number of HCAECs counted in the same well multiplied by 100. Data are presented as the mean \pm SEM; N = 2–3 independent experiments (n = 3–6 replicates). * $p \leq 0.05$, *** $p \leq 0.001$ vs. 0 puffs/ml (PBS 15% or 75%). THS2.2 or 3R4F aqueous extract concentrations are expressed in puffs/ml. (Poussin, 2016).

Transcriptomics profiling was performed on HCAECs following treatment for 4 h with conditioned media, unconditioned media or fresh aqueous extracts generated with THS aerosol or 3R4F smoke. Pairwise comparisons of gene expression levels measured for each concentration-treatment and respective vehicle controls were computed to generate HCAEC system response profiles (SRPs). The graphical representation of SRPs on volcano plots reveals a concentration-dependent increase in the magnitude and statistical significance of gene expression fold-changes in all 3R4F treatments, leading to an increased numbers of differentially expressed genes (DEGs) (Figure 2). At the same concentrations, only a few gene expression changes reached statistical significance following THS exposure. When the concentrations of THS aqueous extract were increased by 10 and 20 fold for indirect and fresh direct treatments, respectively, similar numbers of DEGs, were obtained. Maximum numbers of DEGs were obtained with 3R4F and THS fresh direct treatments.

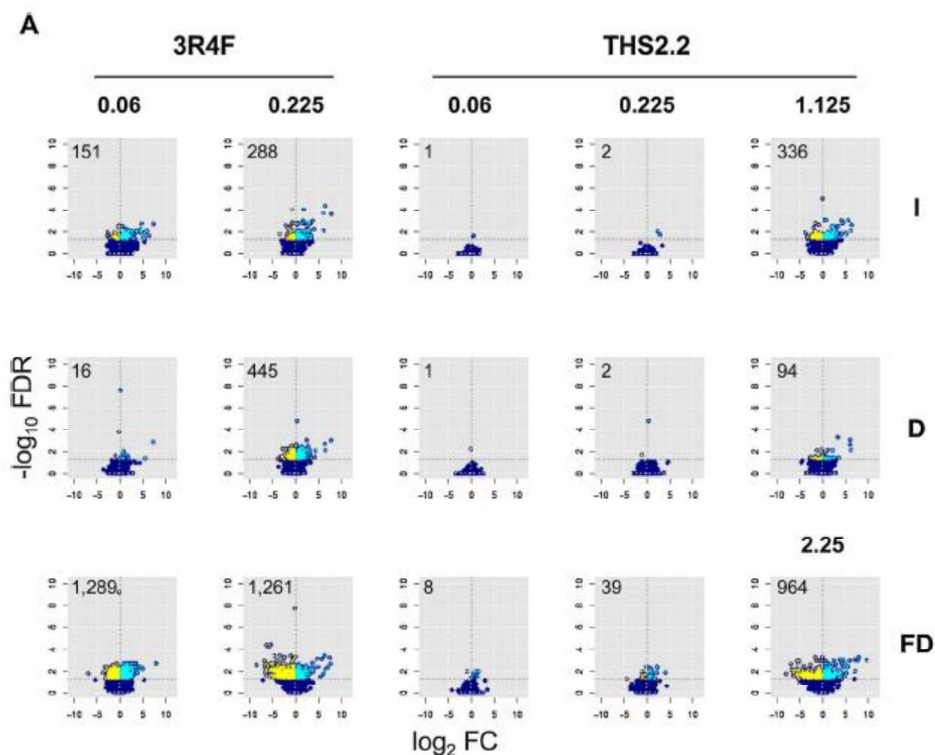


Figure 2. Systems response profiles of HCAECs following indirect (I), direct (D) and fresh direct (FD) treatments with THS or 3R4F. Volcano plots display gene expression change amplitude (as \log_2 fold-change) versus significance (as $-\log_{10}$ of adjusted p-value or false discovery rate) on the x- and y-axis, respectively. Significantly up (right)- and down (left)-regulated genes ($FDR < 0.05$) are highlighted in cyan and yellow, respectively. Total number of significantly regulated genes is indicated in the top-left corner of volcano plots. (Poussin, 2016)

A computational systems biology approach based on transcriptomics data with cause-and-effect network models was used to conduct network perturbation amplitude (NPA) analysis and quantify the overall biological impact factor (BIF) of THS compared with 3R4F after indirect, direct and fresh direct exposure of HCAECs. The NPA scores calculated for each SRP and network/subnetwork were then aggregated as a single BIF value that quantifies the overall perturbation of the system. A comparable pattern of networks was perturbed at a higher concentration of 3R4F aqueous extract (0.225 puffs/ml) with similar underlying perturbed biology ($\delta = 0.91$), but with a lower amplitude (relative biological impact factor (RBIF) = 60%). At the same concentrations (0.06 and 0.225 puffs/ml), the overall magnitude of perturbation promoted by THS aqueous extract represented only 1% and 3% of the total perturbation of the reference, respectively, while this was 78% at the highest concentration with highly similar perturbed biology ($\delta = 0.96$). The pattern and magnitude of perturbed networks for the direct and fresh direct treatments differed from those of the indirect treatment. However, similar to the indirect treatment, the biological impacts of THS in HCAECs represented 1% and 3% for the direct treatment as well as 1% and 8% for the fresh direct treatment at low and intermediate concentrations, respectively. At high concentrations of THS aqueous extract, the RBIF corresponded to 31% and 36% of the total perturbation of the reference for direct and fresh direct treatments, respectively (Figure 3).

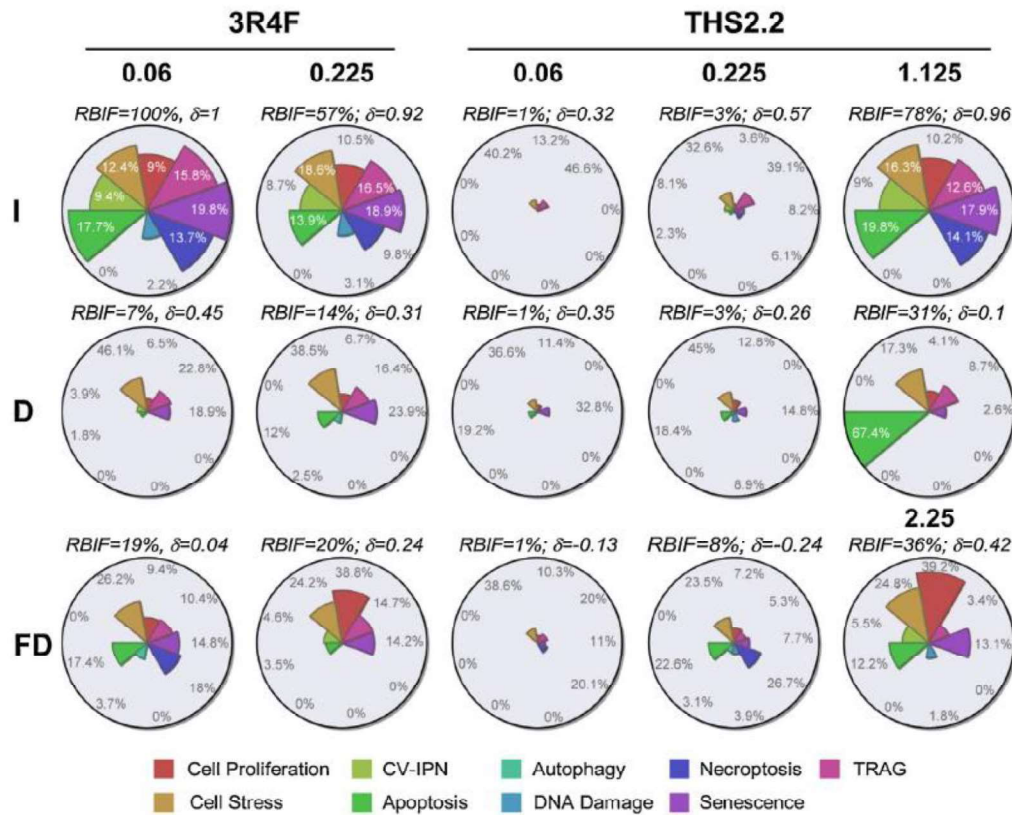


Figure 3. Computed biological impact factors and network perturbation amplitudes in HCAECs following indirect (I), direct (D) and fresh direct (FD) treatments with THS or 3R4F. The NPA computed for each network leveraging transcriptomics data was aggregated as a single value termed the biological impact factor (BIF) that quantifies the overall perturbation of the system modeled in causal networks. For each systems response profile (SRP), the contribution of significantly perturbed biological networks to the overall BIF is represented by colored surfaces proportionally covering circular plots and indicated as a percentage. These colored surfaces are also comparable across SRPs. The BIF associated with the SRP that induces the maximum network perturbation was automatically set to 100% as the reference (relative (R)BIF). RBIF related to other SRPs was expressed as a percentage of the RBIF. The delta (δ , [-1,1]) value reflects the degree to which the underlying biology modeled in the networks is similarly perturbed compared with the reference (Poussin, 2016).

The activated Vascular-Inflammatory Processes network (V-IPN) and the Cell Stress network were investigated in more detail. Significant perturbations of the V-IPN/endothelial cell–monocyte interaction subnetwork were only observed in the indirect treatment, at a high concentration for THS aqueous extract (1.125 puffs/ml), and at low (0.06 puffs/ml) and high (0.225 puffs/ml) concentrations for 3R4F aqueous extract.

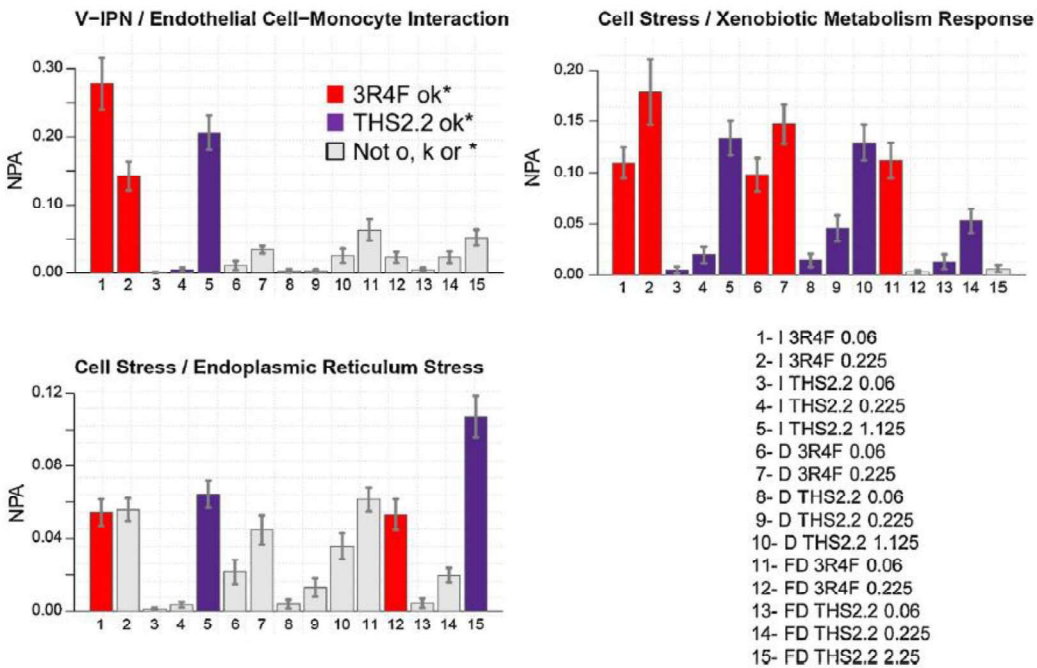


Figure 4. Individual network perturbation amplitudes after the exposure HCAECs to THS and 3R4F. Treatments: indirect (I), direct (D) and fresh direct (FD); OK*-indication of the relevance of the network downstream transcripts and backbone topology (Poussin, 2016).

The Cell Stress network, and the Xenobiotic Metabolism Response subnetwork were perturbed in a concentration-dependent manner with considerably lower amplitudes at low (0.06 puffs/ml) and intermediate (0.225 puffs/ml) concentrations of THS aqueous extracts when compared with 3R4F. At the highest concentrations of the fresh direct treatment, the nature of the stress responses differed compared with direct and indirect treatments. The Endoplasmic Reticulum Stress subnetwork and the Oxidative Stress subnetwork were significantly and similarly perturbed in both indirect and fresh direct treatments (at high concentrations only); however they displayed different pattern of leading nodes, indicative of different underlying perturbed biology (Figure 4).

A concentration-dependent increase of DEGs in MM6 cells was observed in response to both THS and 3R4F aqueous extracts after 2 h exposure (Figure 5A). At low (0.06 puffs/ml) and intermediate (0.225 puffs/ml) concentrations of THS aqueous extract, no and 54 DEGs were counted, respectively, while those numbers increased to 796 and 2874, respectively for 3R4F aqueous extract. A 20-fold higher concentration of THS aqueous extract (1.125 puffs/ml) led to similar number of DEGs compared with that observed for the low 3R4F aqueous extract concentration.

The levels of inflammatory marker proteins measured in conditioned media revealed a concentration-dependent release of soluble mediators, with increased release of TNF α , TNF receptor 2, and interleukin (IL)-8 at low 3R4F aqueous extract concentrations only, but a decreased production for the other mediators. At low (0.06 puffs/ml) and intermediate (0.225 puffs/ml) concentrations, THS aqueous extract had restricted effects, while at a high concentration, the pattern of mediator release was intermediate between that observed for 3R4F at low and high concentrations (Figure 5B and C).

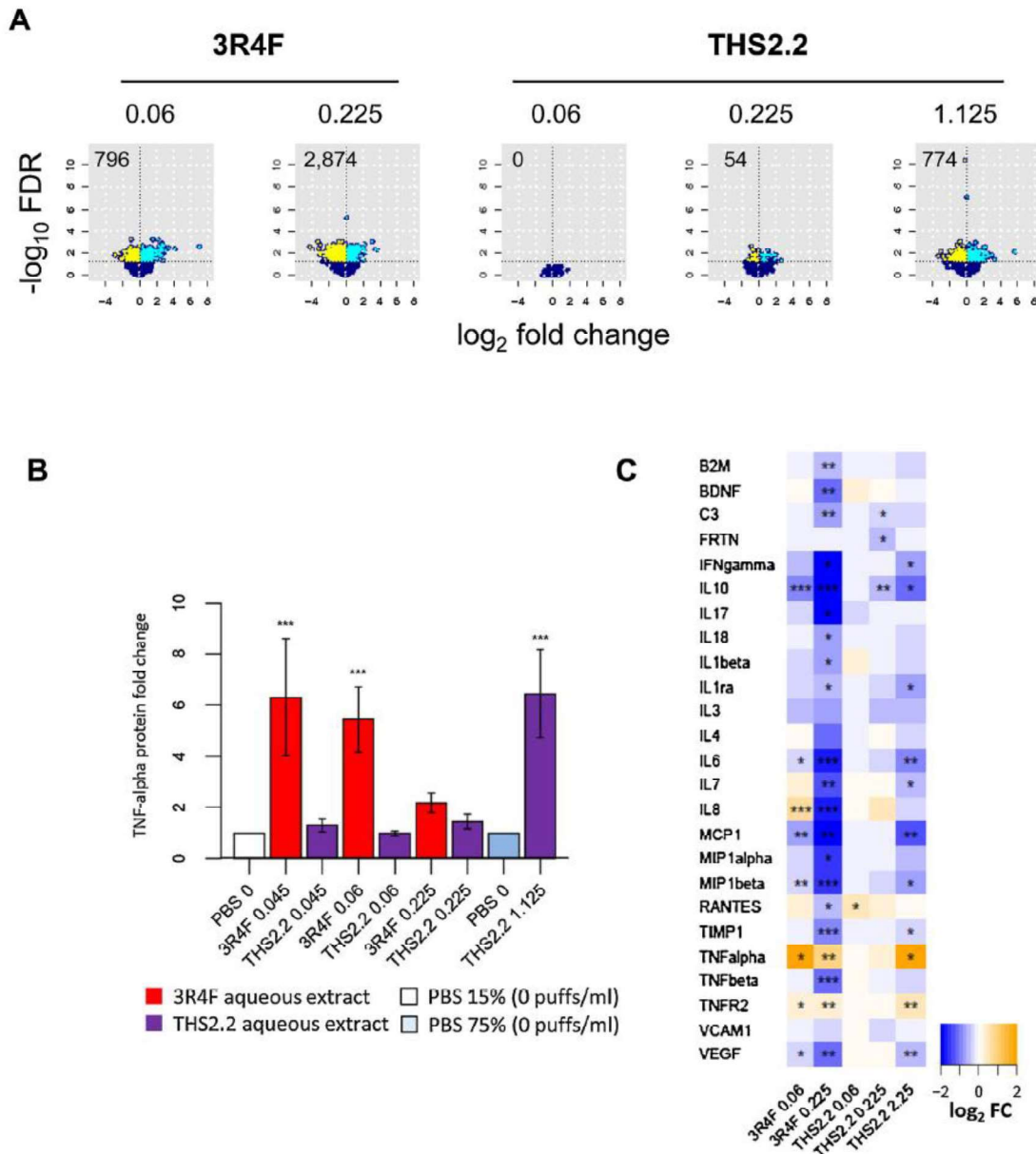


Figure 5. Systems response profiles of MM6 cells and inflammatory protein markers released in conditioned media following exposure to freshly generated THS or 3R4F aqueous extracts. (A) Volcano plots display gene expression change amplitudes (as \log_2 fold change) versus significance (as $-\log_{10}$ of adjusted p-value or false discovery rate) on the x- and y-axis, respectively. Significantly up- (right) and down (left)-regulated genes (false discovery rate < 0.05) are highlighted in cyan and yellow, respectively. (B) Relative TNF α levels measured in conditioned media (MM6 cell supernatants) by ELISA, and expressed as fold change relative to the vehicle controls. (C) Heatmap of inflammatory markers quantified in conditioned media (fold change values of treatment group versus their respective vehicle control). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (unadjusted p-values are reported). THS or 3R4F aqueous extract concentrations are expressed in puffs/ml (Poussin, 2016).

Biological network perturbations and the overall impact factor were evaluated for MM6 cells (Figure 6). As observed with HCAECs, the largest biological impact was promoted by a low concentration of 3R4F aqueous extract (0.06 puffs/ml). At the highest concentration of 3R4F aqueous extract (0.225 puffs/ml) the RBIF was 17% and the delta value was 0.1, indicating a low similarity

regarding underlying perturbed biology compared with the low 3R4F aqueous extract concentration. The network perturbations observed with THS showed consistent concentration-dependent inflation leading to a RBIF of 34% and a δ value of 0.52.

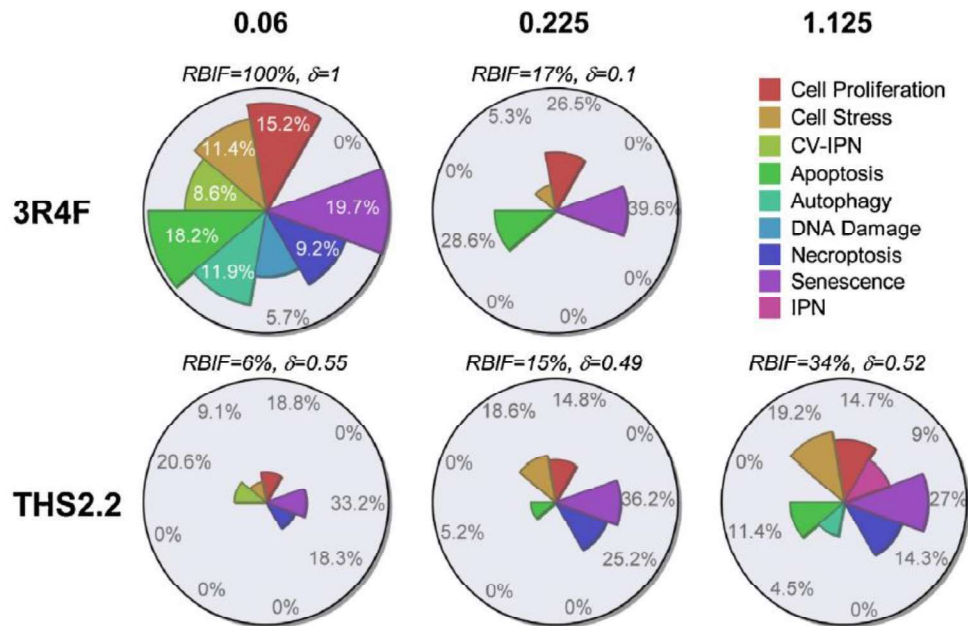


Figure 6. Computed biological impact factors and network perturbation amplitudes in MM6 cells following exposure to THS or 3R4F aqueous extracts. THS or 3R4F aqueous extract concentrations are expressed in puffs/ml (Poussin, 2016).

The results show that 3R4F smoke in the form of an aqueous extract promoted the adhesion of monocytic cells to endothelial cells via distinct indirect and direct smoke-induced concentration-dependent mechanisms. At matching concentrations with equivalent nicotine levels, the THS aqueous extract did not trigger MM6 cell-HCAEC adhesion, and clearly showed reduced impact at molecular levels in HCAECs and MM6 cells. MM6 cell-HCAEC adhesion accompanied by molecular changes in both cell types only reached similar levels of increase to those induced by 3R4F aqueous smoke extract when concentrations of THS aqueous extract were raised by 10- and 20-fold for fresh direct and indirect exposure modalities, respectively. Comparisons at gene and biological network/pathway levels demonstrated strong similarities in molecular changes induced by high THS aqueous extract concentrations compared with low 3R4F aqueous extract concentrations in both cell types indicating that the THS aqueous extract promoted adhesion of monocytic cells to endothelial cells via similar indirect and direct mechanisms to those described for 3R4F aqueous extract. These data, along with ones cited, indicate the potential of a heat-not-burn tobacco product to reduce the risk for cardiovascular disease compared to combustible cigarettes.

Effects of THS on chemotaxis and transendothelial migration *in vitro*

Monocytes play a key role in the pathogenesis of atherosclerosis by adhering to and crossing the endothelial cell layer to the subintimal space, where they take up large amounts of oxidized low-

density lipoproteins and acquire a foam cell phenotype (Libby, 2011). Increases in extravasation of monocytes from the vasculature or reductions in the intravasation of these cells from vascular lesions contribute to both the formation and progression of atherosclerotic lesions (Randolph, 2008). We investigated the effect of THS aerosol on the migratory behavior of monocytes in comparison with smoke from 3R4F reference cigarettes. The monocytic cell line (THP-1) and human coronary arterial endothelial cells (HCAECs) were used to analyze chemotaxis and transendothelial migration (TEM). To assess the influence of aerosol from THS and smoke from 3R4F on toxicity and inflammation, flow cytometry and ELISA assays were performed (van der Toorn, 2015a).

Extracts of THS aerosol and 3R4F smoke were generated by bubbling aerosol or smoke through RPMI 1640 cell culture medium containing 2 mM l-glutamine and 1% penicillin–streptomycin (3R4F: six items/36 mL RPMI 1640; THS: 10 items/40 mL RPMI 1640) on ice. The 3R4F extract stock solution was further diluted in serum-free medium to obtain final concentrations ranging from 0.01 to 0.5 puffs/mL. The THS stock extract solution was diluted in serum-free medium to obtain final concentrations ranging from 0.01 to 3 puffs/mL. To monitor the concentration of nicotine and eight key aldehydes, chemical analyses were conducted directly after the extract generation (Table 2).

Table 2. Selective chemical analysis of 3R4F and THS extracts (van der Toorn, 2015a).

	3R4F(μ g/cig)	THS2.2(μ g/stick)	3R4F/THS2.2
Nicotine	80.2 \pm 7.3	76.8 \pm 15.9	1
Formaldehyde	37.9 \pm 8.6	3.0 \pm 0.6*	12
Acetaldehyde	1089 \pm 150	174 \pm 44*	6.2
Acetone	408 \pm 80	24.1 \pm 4.8*	17
Acrolein	100 \pm 13	5.5 \pm 1.0*	18
Propionaldehyde	65 \pm 10	11.5 \pm 1.6*	5.6
Crotonaldehyde	48 \pm 6	2.3 \pm 0.4*	21
Methyl ethyl ketone	87 \pm 21	4.6 \pm 0.9*	19
Butyraldehyde	19 \pm 3	9.9 \pm 2.0*	1.9

Serum-starved THP-1 cells and HCAECs were stimulated for 4 h with increasing concentrations of freshly prepared extracts from 3R4F and THS. Then, the cells were washed with fresh medium and seeded in chemotaxis and TEM inserts.

The results show that treatment of THP-1 cells with extract from 3R4F or THS induced concentration-dependent increases in cytotoxicity and inflammation (Figure 7). Cytotoxicity was determined by flow cytometric analysis of 7 amino actinomycin D (7-AAD) stained cells. The effective concentration of THS-induced cytotoxicity was more than ~26 times higher than that required for the 3R4F extract to induce cell death. Similarly, the 3R4F extract was ~10 times more potent than the THS extract in the induction of IL-8 and TNF- α secretion by THP-1 cells, measured using ELISA assay.

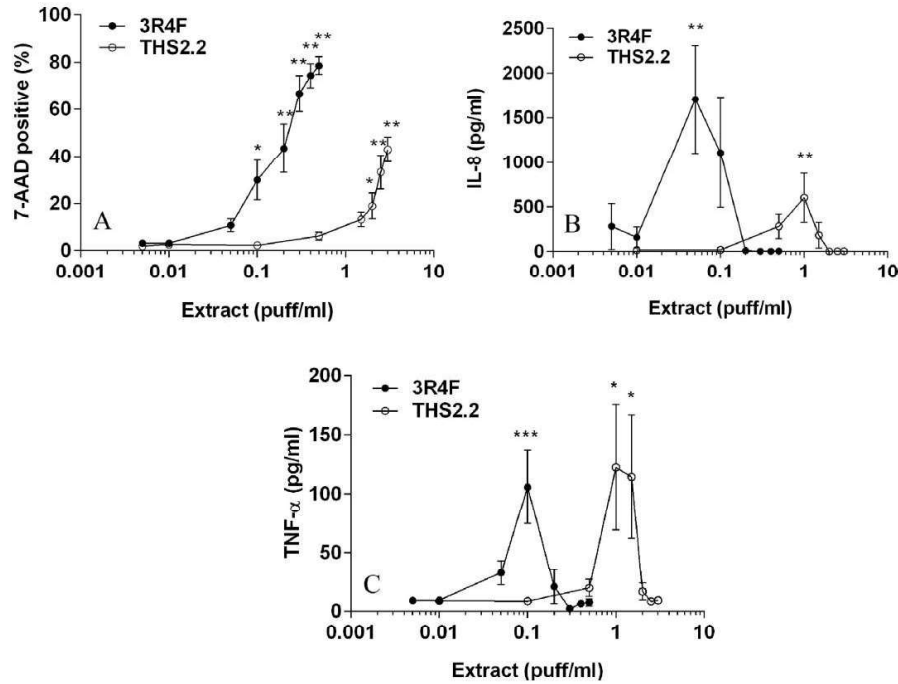


Figure 7. Effects of 3R4F and THS extracts on THP-1 cell cytotoxicity and inflammation. A. Percentage of THP-1 cells positive for 7-AAD determined by flow cytometric analysis after stimulation with 3R4F (filled circles) or THS (hollow circles) extract. B and C. Mean concentrations of IL-8 and TNF- α in the culture supernatants of THP-1 cells treated with 3R4F (filled circles) or THS2.2 (hollow circles) extract. Data are expressed as the mean \pm SEM of four independent experiments. *P < 0.05 vs control; **P < 0.01 and ***P < 0.001 vs control by Dunnett's multiple comparison test. THS2.2: tobacco heating system (van der Toorn, 2015a).

The inhibitory effects of THS extract for chemotaxis and TEM were \sim 18 times less effective compared to 3R4F extract (Figure 8). Chemotaxis analysis was performed using Boyden chambers and TEM assays using CIM plates and a RTCA xCELLigence instrument as described before (van der Toorn, 2015b).

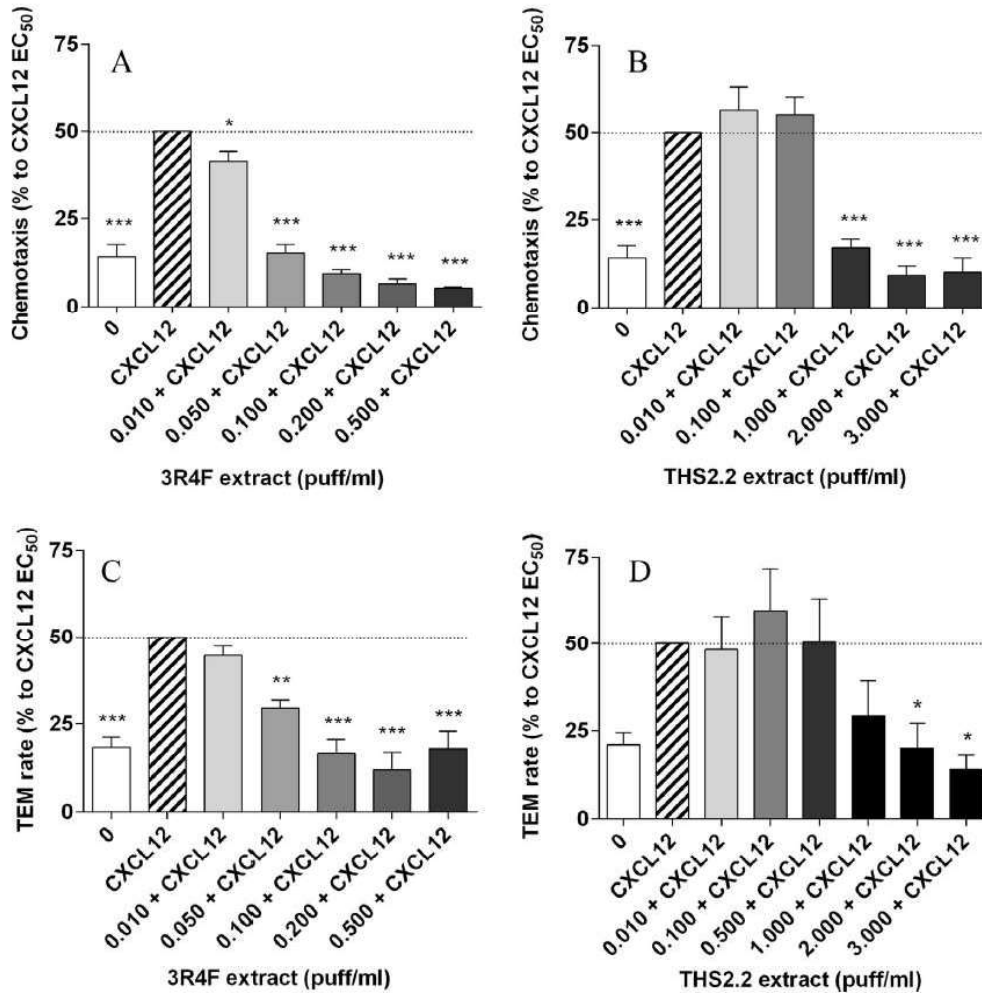


Figure 8. Effect of extracts from 3R4F and THS on monocyte migration and real-time impedance-based TEM. A and B. Migration of THP-1 cells exposed to increasing concentrations of extract from 3R4F (A) or THS (B) in conventional Boyden chambers. C and D. Real-time impedance-based TEM of THP-1 cells exposed to increasing concentrations of extract from 3R4F (C) or THS (D) in CIM chambers. The CXCL12 concentration that induced 50% migration was used in these assays (EC₅₀). Data are expressed as the mean \pm SEM of four independent experiments. *P < 0.05 vs control; **P < 0.01 vs control; ***P < 0.001 vs control by Dunnett's multiple comparison test. CIM: cell invasion/migration, THS2.2: tobacco-heating system 2.2, TEM: transendothelial migration (van der Toorn, 2015a).

Endothelial barrier dysfunction can be an initiating factor that contributes to lesion development and clinical complications. The barrier function of a confluent monolayer of HCAECs was challenged with increasing concentrations of extract from 3R4F or THS using electronic plates with microelectrode sensors in the bottom of the wells. Extract from 3R4F or THS induced concentration-dependent decreases in the integrity of HCAEC monolayer. A rapid concentration-dependent disruption of endothelial barrier integrity as indicated by lower impedance values immediately after extract application. The 3R4F extract was 16 times more potent than the THS extract in the reduction of impedance (3R4F; IC₅₀ = 0.047; THS; IC₅₀ = 0.790 puff/mL) (Figure 9).

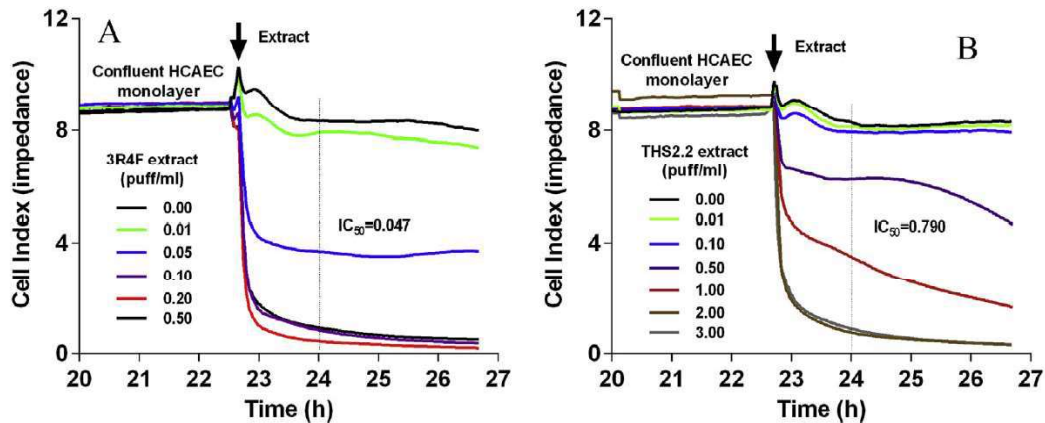


Figure 9. Effects of extracts from 3R4F and THS on the integrity of a HCAEC monolayer. HCAECs seeded in electrical impedance plates were grown to confluency for ~24 h and then stimulated with increasing concentrations of extract from 3R4F (A) or THS (B). The concentrations required to inhibit transendothelial integrity by 50% (IC₅₀) were determined at 1 h after addition of the extracts. Representative recordings from three independent experiments are shown. *P < 0.0001 vs control by Dunnett's multiple comparison test (van der Toorn, 2015a).

For all examined endpoints, the extract from 3R4F showed more than one order of magnitude stronger effects than that from THS extract. These data indicate the potential of a heat not burn tobacco product THS to reduce the risk for cardiovascular disease compared to combustible cigarettes.

Cardiovascular and respiratory chronic exposure effects of THS in ApoE^{-/-} mice

The development and progression of CVD and chronic obstructive pulmonary disease (COPD) were investigated in ApoE^{-/-} mice following chronic exposure to mainstream cigarette smoke or to an aerosol from THS (Phillips, 2016). In this study female ApoE^{-/-} mice were exposed to cigarette smoke from 3R4F reference cigarette at a target nicotine concentration of 29.9 µg/L, to aerosol from THS at a matched nicotine concentration, or to filtered fresh air for 3 hours per day, 5 days per week, for up to 8 months. After 2 months exposure to 3R4F reference cigarette smoke, designated animals were switched to THS aerosol exposure (switching group) or to filtered air (cessation group) for up to 6 months, following the same daily exposure regimen. At designated time points (months 1, 2, 3, 6 and 8 after commencing exposure), animals were examined for multiple parameters to comprehensively assess the development and progression of COPD or CVD indicators. These parameters included hematology and clinical chemistry assessments, 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 by transcriptomics, proteomics and lipidomics in various tissues. In the following sections results relevant for this document are presented.

Clinical chemistry

Nonfasted plasma samples were evaluated using a panel of clinical chemistry metabolic parameters, revealing a statistically significant increase in cholesterol in the smoke-exposed group at months 1 and 3, with a decrease to sham levels after 1 month of either cessation or switching (Figure 10A). Accompanying the increase in total cholesterol (TC), a similar increase in high-density lipoprotein (HDL) cholesterol was observed (3R4F group increased from months 1–3, though merging to the sham level by month 8). There were no obvious differences to the sham controls in low density lipoprotein (LDL) levels among any of the groups at any dissection time-point. Although triglycerides were higher in 3R4F exposed mice during the first 3 months of the study ($P < .05$ at months 1 and 3), by months 6 and 8 there were no differences among any of the groups.

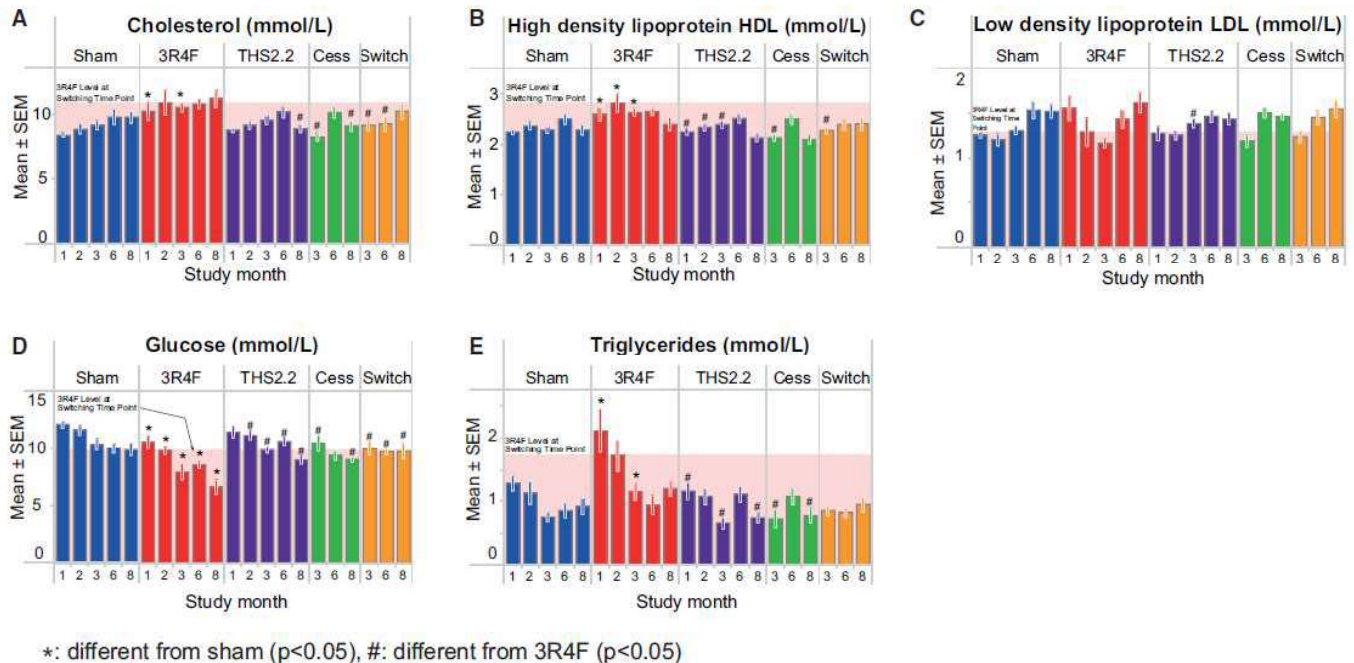


Figure 10. Clinical chemistry. Measurements by autoanalyzer. A, Total cholesterol (enzymatic/biochemical detection). B, High-density lipoprotein (enzymatic/biochemical detection). C, Low density lipoprotein (calculated according to the Friedewald formula). D, Glucose (enzymatic/biochemical detection). E, Triglycerides (enzymatic/biochemical detection). Data are means \pm SEM, $n = 8-10$. Statistics describe comparison with sham-exposed animals. $*P < .05$ or with 3R4F-exposed animals $\#P < .05$. The shaded area indicates the 3R4F value at the switching/cessation time-point (month 2) (Phillips, 2016).

Plasma glucose levels were decreased with age in all groups including sham-exposed mice (Figure 10D). Significantly lower plasma glucose concentrations compared with sham were observed in 3R4F-exposed mice across all time-points, corresponding with the body weight reduction seen in smoke-exposed mice that may have potentially affected these metabolic parameters, an observation that is common to smoke-exposed mice (Chen, 2007; Lietz, 2013; Phillips, 2015; Stinn, 2010; von Holt, 2009). Cessation and switching resulted in the return of glucose concentrations to sham levels within 1 month, again recapitulating the increased body weight gain. There were no statistically significant changes in THS exposed group compared to sham.

Aortic Arch Plaque Formation

Image analysis measurements of the plaque area revealed an expected and progressive increase in plaque burden in all groups over the 8-month experimental time frame (Figure 11). From month 2 onwards, a significantly increased aortic plaque area was observed in mice exposed to 3R4F smoke compared with either sham or THS aerosol-exposed animals. Exposure to THS aerosols resulted in plaque area measurements that did not significantly differ from values for sham-exposed mice ($P > .05$ across all time-points, except month 6 where the plaque area was increased [$P < .05$] relative to sham, though less than in smoke exposed mice). Following either cessation or switching to THS aerosol, the plaque area was significantly smaller than in the 3R4F exposure group at the months 3 and 8 time-points, though the plaques remained slightly, but not significantly, larger than those of sham or chronic THS aerosol-exposed mice.

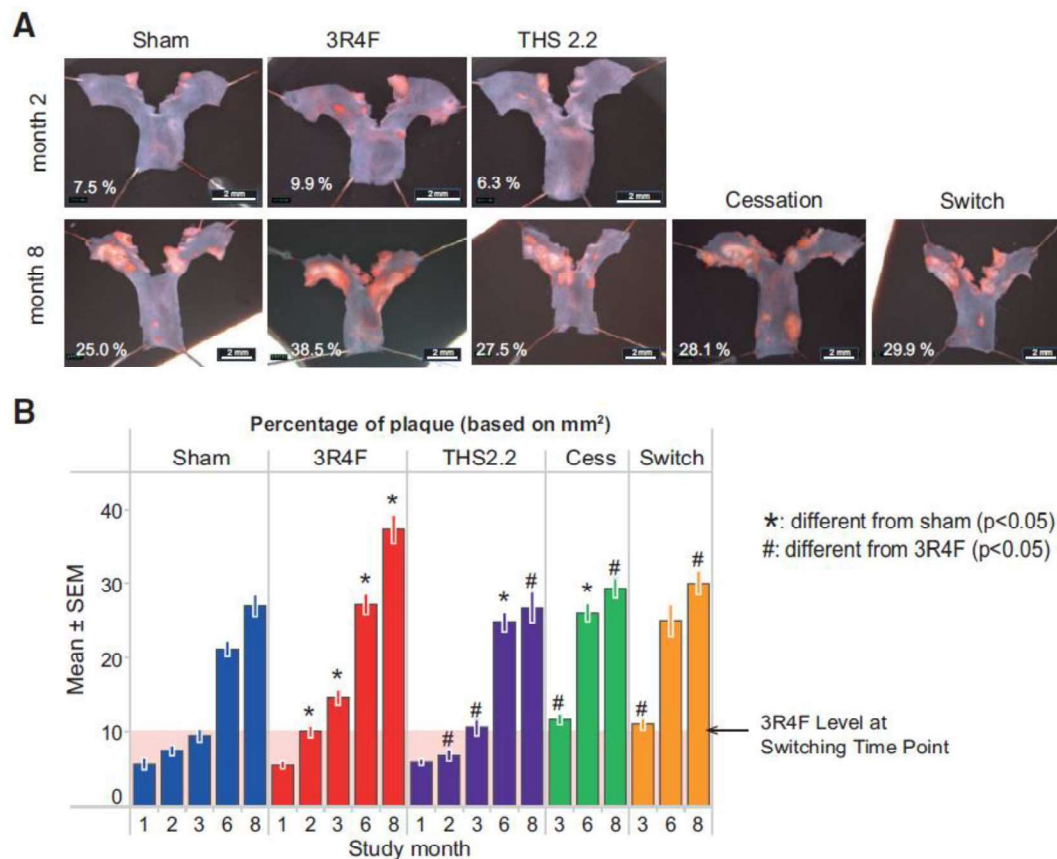


Figure 11. Aortic arch plaque area measurements. Aortic arches were dissected, longitudinally opened, pinned down, and stained with OilRedO for planimetry. A, Representative images from the dissection microscope. B, Plaque size determined by planimetry in the dissected aortic arch. Data are means \pm SEM. The shaded area indicates the 3R4F value at the switching/cessation time-point (month 2) (Phillips, 2016).

The additional quantitative micro-CT investigation of the aortic arch plaque formation *in situ* at the 7-month time-point confirmed the morphometric results from the plaque surface assessment: for 3R4F-exposed mice, all 3 parameters (plaque volume, plaque area, and aortic occlusion) were significantly higher compared with sham-exposed mice, but the THS, cessation, and switching groups

were not different from sham. The aorta plaque surface area (the micro-CT parameter most closely resembling the morphometric plaque area) was 78% higher for the 3R4F group versus sham, while manual quantification of plaque area in the isolated aortas showed a 39% higher value following 3R4F smoke exposure (Figure 12).

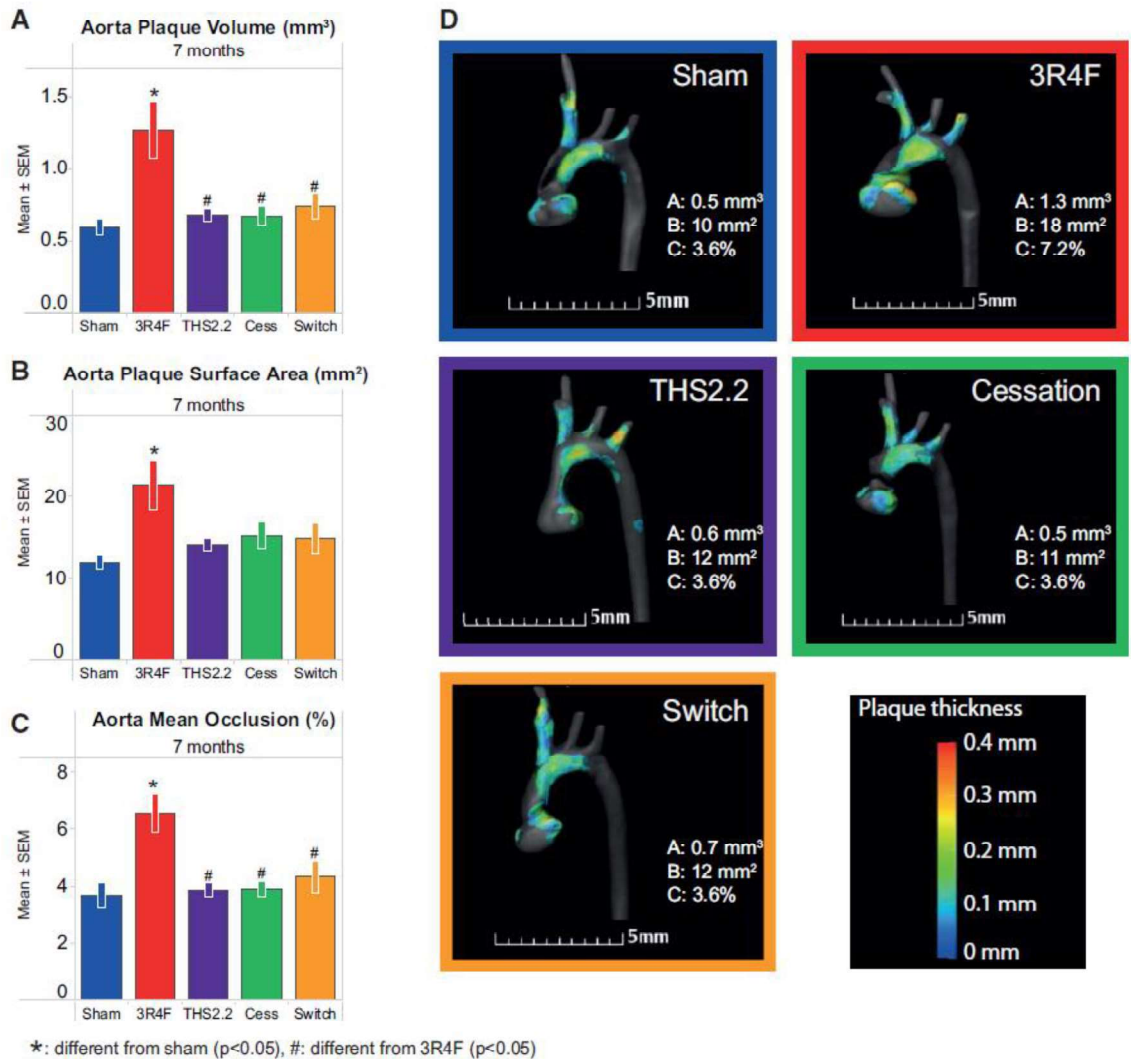


Figure 12. Micro computed tomography (micro-CT)-based aortic arch (in situ) plaque measurements. A, Plaque volume. B, Plaque surface area. C, Aortic occlusion (mean ± SEM). D, Representative micro-CT images (Phillips, 2016).

Plasma and Aortic Arch Lipidomics

Lipidomics in these 2 tissues was performed at the 8-month time-point (Figure 13). In plasma, a few glycerolipids were significantly more abundant in the 3R4F group, and a similar trend was also observed in the THS group (although not statistically significant), whereas the cessation group showed no changes in their glycerolipid levels. From the other lipid classes, some sphingolipids showed significant decreases in the 3R4F, THS, and switch groups, although these were not always the same lipid molecules. Interestingly, 2 individual ceramides (LacCer(d18:1/18:0) and Gb3(d18:1/24:0)) were lower in both the 3R4F and switch group, while a lower level of Glc/GalCer(d18:1/16:0) was only

observed in the THS group. It is noteworthy that, in general, the THS and switch groups did not show as strong changes in these lipids as seen the 3R4F group, while no changes were seen in the cessation group. In the aortic arch, the relative differences for all groups compared with sham are shown in the lower panel of Figure 13. In the 3R4F group, glycerophospholipids, sphingolipids, and sterol lipids showed significantly higher levels than in sham, while only small differences in the glycerophospholipids PE P-18:0/22:6 and PC 18:1/20:3 were seen in the THS group relative to sham. Small increases of a few other lipids, mostly sphingolipids and glycerophospholipids, were detected in the cessation and switch groups.

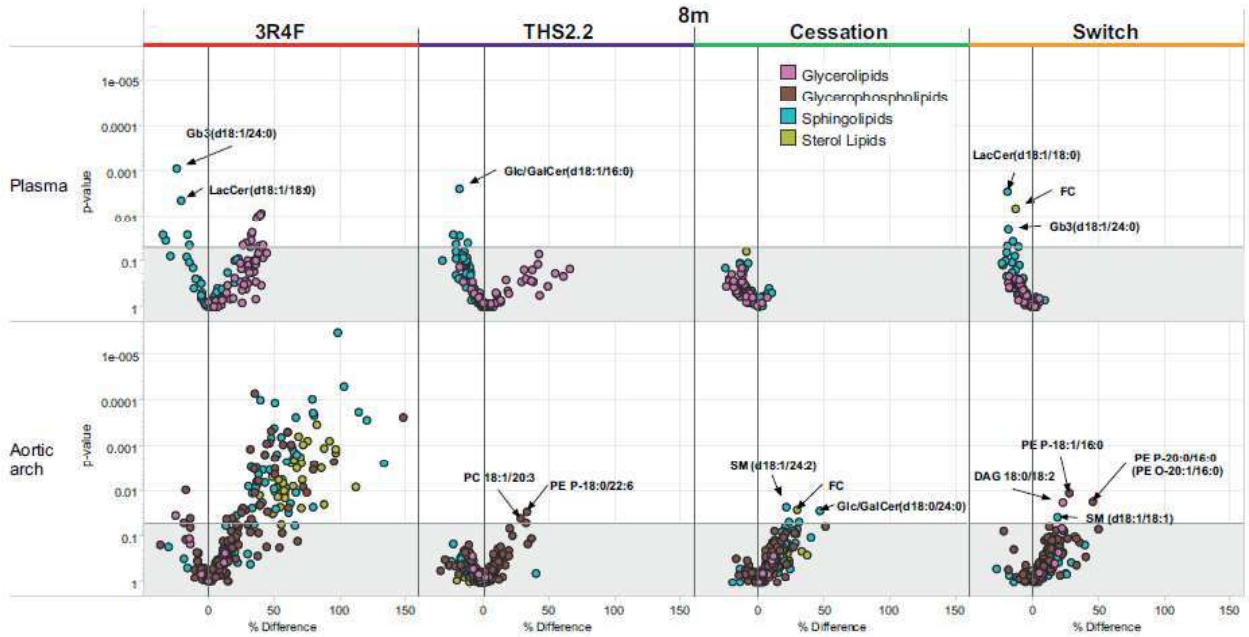


Figure 13. Lipidomics profiles of aortic arch and plasma in *Apoe*^{-/-} mice exposed for 8 months to cigarette smoke (3R4F), THS, or to a cessation or switch protocol (2 months 3R4F followed by 6 months fresh air or THS, respectively) compared with sham exposure for 8 months. Each dot in the volcano plot represents a measured lipid. Lipid classes, as defined by the Lipid Maps consortium, are color coded and indicated in the legend (Phillips, 2016).

BALF Analysis

Relative to sham, the absolute number of total free lung cells (FLCs) in bronchoalveolar lavage fluid (BALF) was much higher after 1 month of 3R4F exposure and continued to be elevated throughout the study, indicating inflammation. In contrast to this smoke effect on FLCs, even prolonged exposure to the THS aerosol had no effect relative to sham-exposed animals. Both cessation and switching resulted in a rapid decline in total FLC counts, almost reaching the level of sham- or THS aerosol-exposed mice after just 1 month of cessation, and completely returning to the FLC counts of sham- and THS aerosol-exposed mice by month 8 (Figure 14).

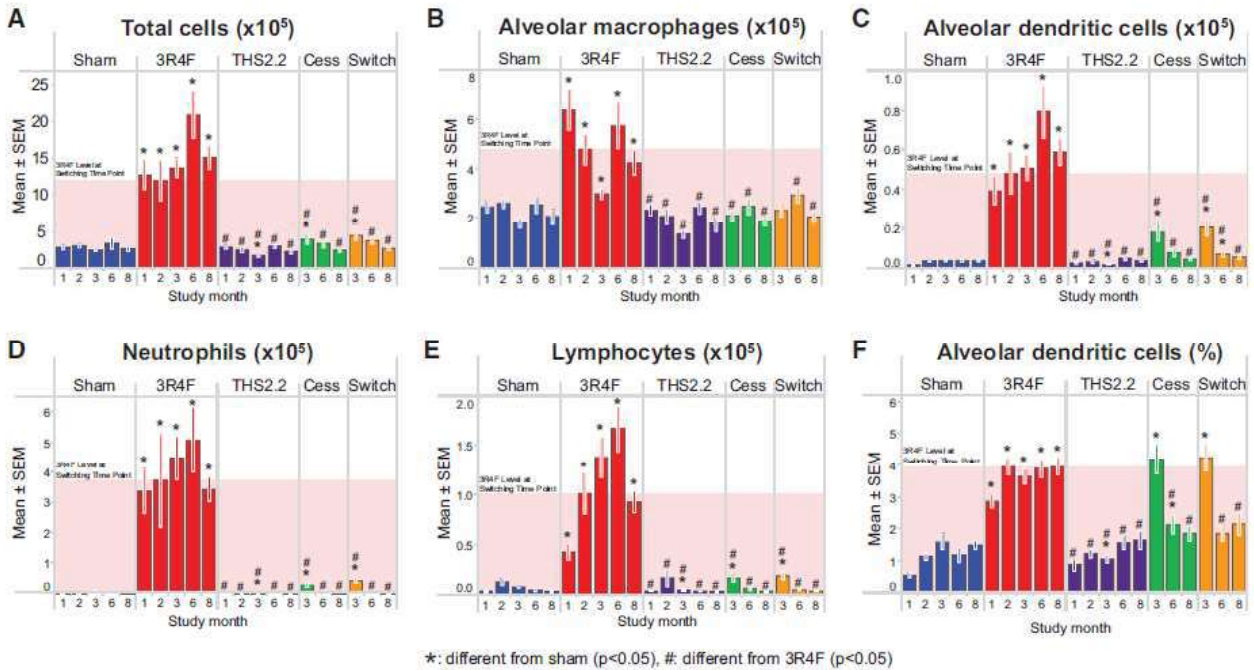


Figure 14. Free lung cells in BALF. Light scatter and relative immunofluorescence were measured in BALF cells by flow cytometry. A, Total cell number. B, Macrophage count. C, Dendritic cells, absolute counts. D, Neutrophil count. E, Total number of lymphocytes. F, Dendritic cells, relative numbers. Data are means ± SEM and were analyzed by t test, potentially accounting for variance heterogeneity. The shaded area indicates the 3R4F value at the switching/cessation time-point (month 2) (Phillips, 2016).

Similar changes and kinetics were also seen in the FLC subpopulations in 3R4F-exposed mice, indicating that the increased FLC count reflected increases in the numbers of neutrophils, macrophages, lymphocytes, and dendritic cells (Figure 14B-F). The strongest 3R4F exposure-related change was observed for neutrophils, followed by lymphocytes, dendritic cells, and macrophages. The numbers of all cell types returned to the sham/THS group level upon cessation or switching: macrophages within 1 month, neutrophils and lymphocytes within 4 months, and dendritic cells within 6 months. Macrophage activation markers (CD54, CD86, and CD11b) indicated that the activated subpopulations returned to sham levels within 4 months, with the slowest kinetics observed for CD86⁺ cells. No significant effect on the numbers and differentiation of FLCs was observed for THS aerosol exposure.

Other results from this study can be reviewed in Phillips et al. (Phillips, 2016).

Conclusions of ApoE^{-/-} study

These results collectively show that 3R4F smoke exposure of ApoE^{-/-} mice accelerated the development of atherosclerotic plaques and emphysema, as demonstrated by a number of anatomical and molecular disease indicators. By contrast, the chronic exposure of mice to a THS aerosol (with nicotine concentration matched to 3R4F) had minimal biological impact on disease endpoints and weak effects on molecular endpoints. Although the 2-month exposure of mice to smoke resulted in early manifestations of emphysema and atherosclerosis endpoints, both switching and cessation

resulted in a partial (lung function, plaque area, and lung morphometry) or even complete (pulmonary inflammation) recovery to sham-exposed levels.

Clinical studies

PMI conducted 3 clinical studies to evaluate the effects of long term exposure to THS aerosol.

Two of PMI's Reduced Exposure, three-arm, parallel-group studies, on 160 subjects with an exposure period of 3 months, 5 days in confinement and prolonged by 86 days in an ambulatory setting were conducted in Japan and in the US. The studies were primarily designed to assess exposure to harmful toxicants but included clinical risk markers as secondary endpoints ([Ludicke, 2018a, b](#)) and [PMIs Modified Risk Tobacco Product \[MRTP\] Application for IQOS, section 7.3.1](#). The studies compared the effects of switching to THS with those of smoking abstinence. The Exposure Response study was a randomized, controlled, two arm parallel group, multi-center, open-label study with 984 participants to evaluate biological and functional changes in healthy adult smokers who switch to THS (the non-menthol product variant) as compared to those who continue smoking for 6 months in an ambulatory setting. Further details about the results of those studies can be found in the Response to the article entitled "PMI's own *in vivo* clinical data on biomarkers of potential harm in Americans show that IQOS is not detectably different from conventional cigarettes," by Stanton Glantz, 2018 at pmiscience.com ([here](#)).

Inflammation

One of the clinical risk markers included was White Blood Cell Count (WBC) as a marker of inflammation. WBC count was assessed in the 2 ambulatory Reduced Exposure studies conducted in Japan and the US. In both of the ambulatory Reduced Exposure clinical studies, there was a reduction in WBC count over the course of the study in the THS and Smoking Abstinence (SA) arms ([see PMIs Modified Risk Tobacco Product \[MRTP\] Application for IQOS, section 7.3.1](#)). The reductions were generally largest in the smoking abstinence group, but there were consistent reductions approaching these levels in the groups of smokers who had switched to THS. In the Japanese study, THS exposure resulted in reduced WBC count, as early as Day 30, to levels similar to those seen with smoking abstinence. In the US study, THS exposure resulted in WBC counts that were lower than for continued smoking, at each time point except for at the end of study (Day 90). There was a lower evaluable sample size in the US study, due to non-adherence with product allocation (as compared with the Japanese study) and the timeframe for these studies was shorter than optimal (WBC reductions following smoking cessation are optimally detected after 6–12 months). Nonetheless, both studies showed that, in smokers who switched to THS, there was a decline in WBC count, in the same direction as with smoking cessation, over the course of the study. The WBC results from the 6 months Exposure Response study, designed to assess changes in clinical risk endpoints in healthy adult smokers, corroborated these results. The WBC counts at months 6 were lower in THS group compared to the results obtained in the group of smokers by 0.420 GI/L.

Endothelial dysfunction

Soluble ICAM (sICAM-1), a clinical marker of endothelial dysfunction and therefore associated with CVD was also included as a clinical risk endpoint in the ambulatory reduced exposure

clinical studies and in the exposure response study. Reports in the literature strongly supported a role for sICAM as a clinical risk marker for inflammation (Lawson, 2009). Levels of sICAM-1 were strongly associated with smoking and showed reversibility upon smoking cessation, usually within 3 months. In both studies, smokers who switched to THS had lower sICAM-1 levels than did subjects who continued to smoke, after adjusting for baseline sICAM-1 levels, sex and baseline CC consumption. sICAM-1 levels (approximately 8.5–10.5% reduced) aligned with those in subjects who abstained from smoking. These changes were generally seen within the first 30 days of exposure and were maintained throughout the ambulatory period. The levels of sICAM measured in subjects who switched to THS in the Exposure Response study were lower than those measured in participants who continued to smoke (reduction of 2.86% at 6 month), which is in line with the results of two Reduced Exposure studies. Lower levels of reduction of sICAM-1 levels in the Exposure Response study compared to the results from 2 Reduced Exposure studies may be explained by the fact that given the ambulatory nature and duration of the study, some subjects randomized to THS concomitantly used THS and cigarettes. A post-hoc analysis using an objective biochemical verification of cigarette smoke exposure was performed and showed that the magnitude of the favorable change upon switching to predominant THS use is dependent on the amount of concomitant cigarette smoke exposure (see [PMIs Modified Risk Tobacco Product \[MRTP\] Application, June 8, 2018 Amendment: Additional Information and Data from a Recently Completed Clinical Study, added November 29, 2018, FDA MRTP application](#))

Oxidative stress markers

Based on literature reviews for oxidative stress, both 8-epi-PGF2 α and 11-DTX-B2 are regarded as clinical risk endpoints potentially informative about changes in oxidative stress within the timeframe of our ambulatory reduced exposure clinical studies (Chehne, 2002). The 8-epi-PGF2 α levels were consistent across the two Reduced Exposure studies, with the 8-epi-PGF2 α levels in smokers that switched to THS showing consistent reductions in the same direction as those observed with smoking abstinence. The smokers who switched to THS showed a more than 12% reduction in 8-epi-PGF2 α levels compared with smokers who continued to smoke. Similarly, in the Exposure Response study the levels of 8-epi-PGF2 α at 6 month measurement were lower compared to the levels determined in group of smokers by 6.8 %. This effect was consistent with results reported in the literature review in subjects following smoking cessation (Chehne, 2001).

Though shifts in the 11-DTX-B2 levels for smokers who switched to THS were in the same direction as those seen with smoking abstinence in both reduced exposure studies, the magnitude of the change was smaller than expected, especially in the US study. Variability of results were not surprising and reported in the literature for 11-DTX-B2, with the most relevant and consistent results seen in heavier smokers. In the Exposure Response study, levels of 11-DTX-B2 were slightly lower compared to the results obtained in the group of smokers (reduced by 4.8%). Similarly to the lower reduction of levels of marker of the endothelial function sICAM-1 in the Exposure Response study compared to two Reduced Exposure studies (see [Endothelial dysfunction section](#)), lower reduction levels for both 8-epi-PGF2 α and 11-DTX-B2 may be explained by the fact that some subjects randomized to THS group concomitantly used THS and cigarettes.

Overall the 8-epi-PGF2 α data supported the existence of changes in oxidative stress for smokers who switch to either THS or to smoking abstinence. The 11-DTX-B2 data were encouraging because

they consistently trended in the right direction (similar to the data for cessation). However, the reductions were not large enough to be conclusive, especially with the high variability seen in the Reduced Exposure studies.

Cholesterol levels

In the ambulatory Reduced Exposure clinical studies, smokers who switched to THS had higher HDL-C levels as compared to smokers who continued to smoke. In the Japanese study, the HDL-C levels in smokers following the switch to THS were similar to those following smoking abstinence. Though the levels of HDL-C in smokers switching to THS were greater than those in smokers that continued to smoke, the results for the smokers switching to smoking abstinence were not replicated in the US study; the HDL-C levels in smokers continuing to smoke were very similar to those in smokers who switched to smoking abstinence. In the US ambulatory reduced exposure clinical study, only 9 of the 40 subjects randomized to the smoking abstinence group reported adherence with smoking abstinence during the 3-month follow-up period, thereby limiting the ability to interpret the smoking abstinence findings in that. In the Exposure Response study, HDL-C levels measured in the THS group was significantly higher compared to smokers who continued to smoke at 6 month measurement by 3.09 mg/dL.

The other clinical risk endpoints measured in the ambulatory reduced exposure studies (LDL-C, total cholesterol, triglycerides, ApoA1 and ApoB) did not change and results were inconsistent within and between product exposures (even with smoking abstinence) and within or between the two studies. This was not surprising given the healthy nature of the study populations, the small sample size, the level of variability among measurements of each marker and the limited exposure follow-up in these studies.

Summary of results from PMI studies

These results collectively show that smoke exposure contributes to endothelial injury and dysfunction, a pro-atherogenic lipid profile, chronic inflammation and an abnormally increased tendency toward coagulation. Evidence from our own *in vitro*, *in vivo* and clinical data available to date with regards to the assessment of the effects of exposure to THS on development of CVD indicate a beneficial profile of THS compared to cigarette smoking with the effects achieved being close to what was observed on smoking abstinence (exposure to air). Given the fact that THS delivers nicotine on the same level as a cigarette, the effects of THS exposure could be attributed to nicotine.

6 DISCUSSION

In the study by Nabavizadeh et al., anaesthetized Sprague-Dawley rats were acutely exposed via a nose cone to THS aerosol from either a single HeatStick (Russian IQOS Parliament brand), mainstream smoke from a single Marlboro Red cigarette or clean air for a series of consecutive 30 seconds cycles over 1.5 to 5 minutes, each cycle consisted of 15 or 5 seconds of exposure followed by removal of the rat from the nose cone. The primary endpoint of this study was pre-exposure and post-exposure FMD of the iliac artery engaging a previously developed method (Heiss, 2008). Based on the results of the FMD measurement assay the authors concluded that “*Acute exposures to IQOS aerosol impairs FMD in rats. IQOS use does not necessarily avoid the adverse cardiovascular effects of smoking cigarettes.*”

After careful analysis of the methods and the results of this study, some methodological issues were identified and should be considered for the interpretation of the results and the conclusions drawn.

The guidelines published by the International Brachial Artery Reactivity Task Force clearly mention that numerous factors (such as temperature, food, drugs and sympathetic stimuli) affect flow-mediated vascular reactivity (Corretti, 2002). For this reason, “*subjects should not exercise, should not ingest substances that might affect FMD such as caffeine, high-fat foods and vitamin C or use tobacco for at least 4 to 6 hours before the study*”. Measuring FMD after acute intake of a stimulant substance (such as nicotine) is expected to affect the outcome. Hence, changes in FMD after a single THS stick exposure is an expected acute effect, which is most likely driven by the exposure to nicotine and not predictive of the long-term development for CVD.

Nicotine is not listed as a cardiovascular toxicant (2012 FDA list of HPHCs) but has been reported in the literature to impact angiogenesis (Cooke, 2004; Lee, 2012; Pillai, 2012). However, in our study on ApoE^{-/-} mice (see section Cardiovascular and respiratory chronic exposure effects of THS in ApoE^{-/-} mice) molecular perturbations of angiogenesis network at the level of transcriptomics in lung tissue of THS exposed mice were minimal compared to those observed in cigarette smoke exposed animals (Phillips, 2016).

The role of nicotine compared to the effects of exposure to all other HPHCs in smoke is considered limited and nicotine is not considered a primary causative factor for CVD. The Royal College of physicians in their 2016 report on “Nicotine without smoke, Tobacco harm reduction” (RCOP, 2016) state: “*Nicotine is not, however, in itself, a highly hazardous drug (see Chapters 4 and 5 of the report). It increases heart rate and blood pressure, and has a range of local irritant effects, but is not a carcinogen (IARC, 2015). Of the three main causes of mortality from smoking, lung cancer arises primarily from direct exposure of the lungs to carcinogens in tobacco smoke, COPD from the irritant and proinflammatory effects of smoke, and cardiovascular disease from the effects of smoke on vascular coagulation and blood vessel walls. None is caused primarily by nicotine.*”

With regards to the effect of nicotine, the Surgeon General Report 2014 (USDHHS, 2014) states that “*it is likely that the sympathomimetic effects of nicotine increase heart rate and myocardial contractility, increase coronary vascular resistance, and reduce insulin sensitivity, contributing to some extent to increasing cardiovascular risk in smokers.*” In the detailed review about the cardiovascular safety of nicotine by Neal Benowitz, which is an important question in the current debate on the benefits vs. risks of electronic cigarettes and related public health policy, he states: “*Short-term nicotine use, such as nicotine medication to aid smoking cessation, appears to pose little cardiovascular risk, even to patients with known CVD*” (Benowitz, 2016). Long-term use of NRT

including nicotine gum in 5-year Lung Health Study revealed no association between sustained NRT use, (and therefore sustained nicotine exposure), and the occurrence of cardiovascular events (Murray, 1996).

A surprising finding that questions the method of exposure in the study by Nabavizadeh et al., were the post-exposure nicotine levels, which were ~4.5 to ~7 fold higher in rats exposed to IQOS than to cigarettes (immediately and 20 minutes after the exposure, respectively), despite nicotine being measured in the IQOS aerosol at ~63% of the amount measured in smoke. There was no correlation between the blood biomarkers of nicotine exposure and nicotine levels in the test atmosphere, which is an essential characteristic for well controlled experimental setup of the inhalation exposure study. The authors proposed following explanation: “*One reason for this difference could be particle size difference which determines to what extent the particles reach the respiratory zone*”. When well-established and controlled aerosol generation system is used, the droplet size for THS aerosol is in the respirable range similar to the particle size of cigarette smoke. Nabavizadeh et al. did not provide the particle size distribution data. Therefore, the explanation they proposed remains at the level of hypothesis. Based on the results of our studies, this phenomenon can be explained by higher levels of harmful and potentially harmful constituents found in cigarette smoke relative to THS. Cigarette smoke is much more irritating when inhaled and the rats are able to reduce their inhalation during cigarette smoke exposure. Another possible explanation for this exposure issue could be a dual use of a manual syringe-pump-driven system to generate smoke and THS aerosol for the initial experiment, and a commercially available automatic vaping system for subsequent experiments. It is unclear which results were presented, those obtained with manually constructed or purchased system for smoke/aerosol generation? A proper validation of the exposure system is of utmost importance for the conduct of studies where the route of administration of tested substance(s) is inhalation. Nabavizadeh et al. did not mention this critical aspect.

In the hypothetical situation where the authors would design their experiment aiming to understand at which THS concentrations the same or similar effects to those of smoke on endothelial dysfunction could be achieved, and that was possible only with THS aerosol concentrations ~7 fold higher than smoke from combustible cigarette, such results would be in line with results of our studies where similar effects caused by the exposure to smoke could be achieved only by up to 10 fold higher THS aerosol concentrations (Poussin, 2016; van der Toorn, 2015a). However, the results of the aerosol characterization of Nabavizadeh et al. are suggesting that the experiment was not originally designed with such an intention. Even though the authors reflected on the exposure conditions used in the study and how relevant they are to real-world smoking, it can be stated that exposure conditions used by Nabavizadeh et al. are far from representing real life THS consumption scenarios.

Regardless of the exposure regime used, even with only 3 exposure cycles of 5 s exposure + 25 s break, a very similar FMD post-exposure reduction was observed for THS and smoke exposed animals. In the article by Heiss et al. describing the development of the rat FMD model used in the study of Nabavizadeh et al. a clear dose-responses for nitroglycerin and acetylcholine were presented (Heiss, 2008), which shows that the method is sensitive to detect different levels of effect. However, this feature of the FMD method in rats has not been demonstrated in the study by Nabavizadeh et al. which questions the suitability of this method for the evaluation of the exposure effects of THS vs. combustible cigarette. The authors proposed “*an extremely rapid saturation of the response to mainstream levels of smoke and IQOS aerosol*”, as a possible explanation. This is an experimental system in which the use of specific dosimetry parameters would be recommended. Such an approach would provide a concentration-response data and allow the comparison of the effects of exposure to

different products. A nicotine only control exposure group with different concentrations would have been helpful to interpret the results and to better understand the nicotine only effects.

PMI *in vitro*, *in vivo* and clinical studies relevant for the scope of this document were conducted according to International Standards, GLP, GCP and good toxicological practices and fulfilling statistical requirements to achieve robustness and relevance of the results. These results collectively show that smoke exposure contributes to endothelial injury and dysfunction, a pro-atherogenic lipid profile, chronic inflammation and an abnormally increased tendency toward coagulation. Evidence from our data available to date with regards to the assessment of the effects of exposure to THS on development of CVD indicate a beneficial profile of THS compared to cigarette smoking with the effects achieved being close to what was observed on smoking abstinence (exposure to air). Given the fact that THS delivers nicotine on the same level as a cigarette, while other HPHCs are significantly reduced compared to combustible cigarette, the effects of THS exposure on reduction of FMD could be attributed to nicotine (as previously reported) and residual HPHCs.

7 CONCLUSION

The authors of the recently published article “*Vascular endothelial function is impaired by aerosol from a single IQOS HeatStick to the same extent as by cigarette smoke*” pointed out that the cardiovascular health effects of THS and similar products are incompletely understood. More specifically, the authors stated that there is insufficient scientific evidence addressing the impact of THS on vascular endothelial function tested *in vivo* which motivated them to conduct a study to compare the effects of aerosol of THS. Their motivation to conduct such a study to compare THS to cigarettes is well intended and appreciated as more independent verification on the harm reduction potential of different smoke-free products is needed. We welcome independent research, an open dialogue about the results and the knowledge exchange in a form of collaborative work resulting in scientifically substantiated findings. We conduct a thorough, objective review of the methodology used and results generated, compare where possible with PMI data and other relevant studies available in public domain whether the results agree or disagree and share our findings from such a review in order to increase the scientific knowledge surrounding the assessment for THS. If an independent research reaches different conclusions from ours, we need to understand why.

The conclusion that are drawn from study by Nabavizadeh et al. are misleading due to methodological issues described above. The acute effects of an exposure on FMD can be attributed to the acute sympathomimetic effects of nicotine and have no prognostic value for cardiovascular disease. In addition, the comparisons between THS and cigarettes in this study are inappropriate because the nicotine exposure from THS was much higher than the exposure from cigarettes, which in itself demonstrates a methodological problem with the way cigarette smoke or THS aerosol was delivered to the test animals

The totality of scientific evidence gathered to date demonstrates that THS presents less risk of harm compared to continuing to smoke. Although not risk free, THS presents less risks of harm and commercialized IQOS is a much better choice for current adult smokers compared to continuing to smoke cigarettes.

8 ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
BIF	Biological impact factor
COPD	Chronic obstructive pulmonary disease
CVD	Cardio vascular diseases
DEGs	Differentially expressed genes
EC	Effective concentration
eNOS	Endothelial NO synthase
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FCS	Fetal calf serum
FLC	Free lung cells
FMD	Flow mediated dilation
GCP	Good clinical practice
GLP	Good laboratory practice
GVP	Gas vapor phase
HDL	High density lipoprotein
HPHC	Harmful and potentially harmful constituents
HCAEC	Human coronary arterial endothelial cells
IC50	Inhibitory concentration 50
IL-8	Interleukin 8
LDL	Low density lipoprotein
MM6	Human monocytic Mono Mac 6 cells
NO	Nitric oxide

NPA	Network perturbation amplitude
NRT	Nicotine replacement therapy
OECD	Organisation for Economic Co-operation and Development
PMI	Philip Morris International
RBIF	Relative biological impact factor
sbPBS	Smoke bubbled phosphate buffered saline
SEM	Standard error of the mean
sICAM-1	Soluble intercellular adhesion molecule 1
SRP	Systems response profile
TC	Total cholesterol
TEM	Transendothelial migration
THS	Tobacco heating system
TNF α	Tumor necrosis factor alpha
TPM	Total particulate matter
THP-1	Monocytic cell line
V-IPN	Vascular inflammatory processes network
3R4F	University of Kentucky Reference cigarette
7-AAD	7 amino actinomycin D
11-DTX-B2	11-Dehydrothromboxane B2
8-epi-PGF2 α	8-epi prostaglandin F2 α

9 REFERENCES AND RELATED DOCUMENTS

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