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# Short-term e-cigarette vapour exposure causes vascular oxidative stress and dysfunction: evidence for a close connection to brain damage and a key role of the phagocytic NADPH oxidase (NOX-2)

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## Aims

Electronic (e)-cigarettes have been marketed as a ‘healthy’ alternative to traditional combustible cigarettes and as an effective method of smoking cessation. There are, however, a paucity of data to support these claims. In fact, e-cigarettes are implicated in endothelial dysfunction and oxidative stress in the vasculature and the lungs. The mechanisms underlying these side effects remain unclear. Here, we investigated the effects of e-cigarette vapour on vascular function in smokers and experimental animals to determine the underlying mechanisms.

## Methods and results

Acute e-cigarette smoking produced a marked impairment of endothelial function in chronic smokers determined by flow-mediated dilation. In mice, e-cigarette vapour without nicotine had more detrimental effects on endothelial function, markers of oxidative stress, inflammation, and lipid peroxidation than vapour containing nicotine. These effects of e-cigarette vapour were largely absent in mice lacking phagocytic NADPH oxidase (NOX-2) or upon treatment with the endothelin receptor blocker macitentan or the FOXO3 activator bepridil. We also established that the e-cigarette product acrolein, a reactive aldehyde, recapitulated many of the NOX-2-dependent effects of e-cigarette vapour using *in vitro* blood vessel incubation.

## Conclusions

E-cigarette vapour exposure increases vascular, cerebral, and pulmonary oxidative stress via a NOX-2-dependent mechanism. Our study identifies the toxic aldehyde acrolein as a key mediator of the observed adverse vascular consequences. Thus, e-cigarettes have the potential to induce marked adverse cardiovascular, pulmonary, and cerebrovascular consequences. Since e-cigarette use is increasing, particularly amongst youth, our data suggest that aggressive steps are warranted to limit their health risks.

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**Keywords**

Behavioural risk factor • Lifestyle drug • E-cigarette vapour • Endothelial dysfunction • Oxidative stress

**Translational perspective**

With the present studies, we demonstrate that acute e-cigarette smoking produced a marked impairment endothelial function in chronic smokers determined by flow-mediated dilation. Translational experiments in mice revealed that e-cigarette vapour causes oxidative stress in the brain, the lung and in vessels, induces inflammation, and lipid peroxidation. These adverse health effects were largely absent in mice lacking phagocytic NADPH oxidase (NOX-2). The e-cigarette product acrolein, a reactive aldehyde, recapitulated many of the NOX-2-dependent effects of e-cigarette vapour using *in vitro* blood vessel incubation. These results indicate that e-cigarettes use has the potential to induce marked adverse cardiovascular, pulmonary, and cerebrovascular consequences. Since e-cigarette use is increasing, particularly amongst youth, our data suggest aggressive steps are warranted to limit the cardiovascular and cerebrovascular health risks of e-cigarettes.

**Introduction**

According to the results of the Global Burden of Disease (GBD) Study, tobacco smoking ranked second of all risk factors for global deaths, just after high blood pressure.<sup>1</sup> Electronic (e)-cigarettes have been marketed as a 'healthy' alternative to traditional cigarettes, and they are reported as an effective method of smoking cessation.<sup>2</sup> As a consequence, there has been a rapid growth of e-cigarette use, particularly in young people, such that by 2014 e-cigarettes were the most commonly used tobacco product in the USA.<sup>3</sup> The US Center for Disease Control and Prevention (CDC) reports that among high school students the e-cigarette use markedly increased from 1.5% to 20.8% (~3.05 million students) between 2011 and 2018. This rapid raise in e-cigarettes has raised concern about adverse health impacts to middle and high school students.<sup>4</sup> This concern is compounded by the recent release of high nicotine content vaping devices without complete understanding of the health consequences.<sup>5</sup> Significant concern comes also from very recent reports by the CDC, Food & Drug Agency (FDA), and State Health Departments on ongoing investigations on severe pulmonary disease and deaths among people using e-cigarettes<sup>6</sup> and a preliminary report of a coordinated public health investigation.<sup>7</sup>

There is considerable lack of clarity as to the overall population health consequences of e-cigarette use. A majority of available studies provide evidence that e-cigarette vaping is somewhat less detrimental than tobacco cigarette smoking,<sup>8,9</sup> however, the number of studies and the amount of mechanistic insight are limited. If one considers that e-cigarette vaping is associated with a decrease in the average age of first-time (e)-cigarette users, the 'healthier' e-cigarette profile might easily be abrogated (or even reversed) by the higher portion of adolescent users. Thus, it is possible e-cigarette vaping could adversely impact overall population disability-adjusted life years thereby bestowing a higher disease burden.

A deeper understanding of the health consequences of e-cigarettes is warranted. There are several reports describing that e-cigarette vaping can stimulate vascular and cardiac dysfunction, or prompt oxidative stress and inflammation in the vasculature.<sup>8,10</sup> Similarly,

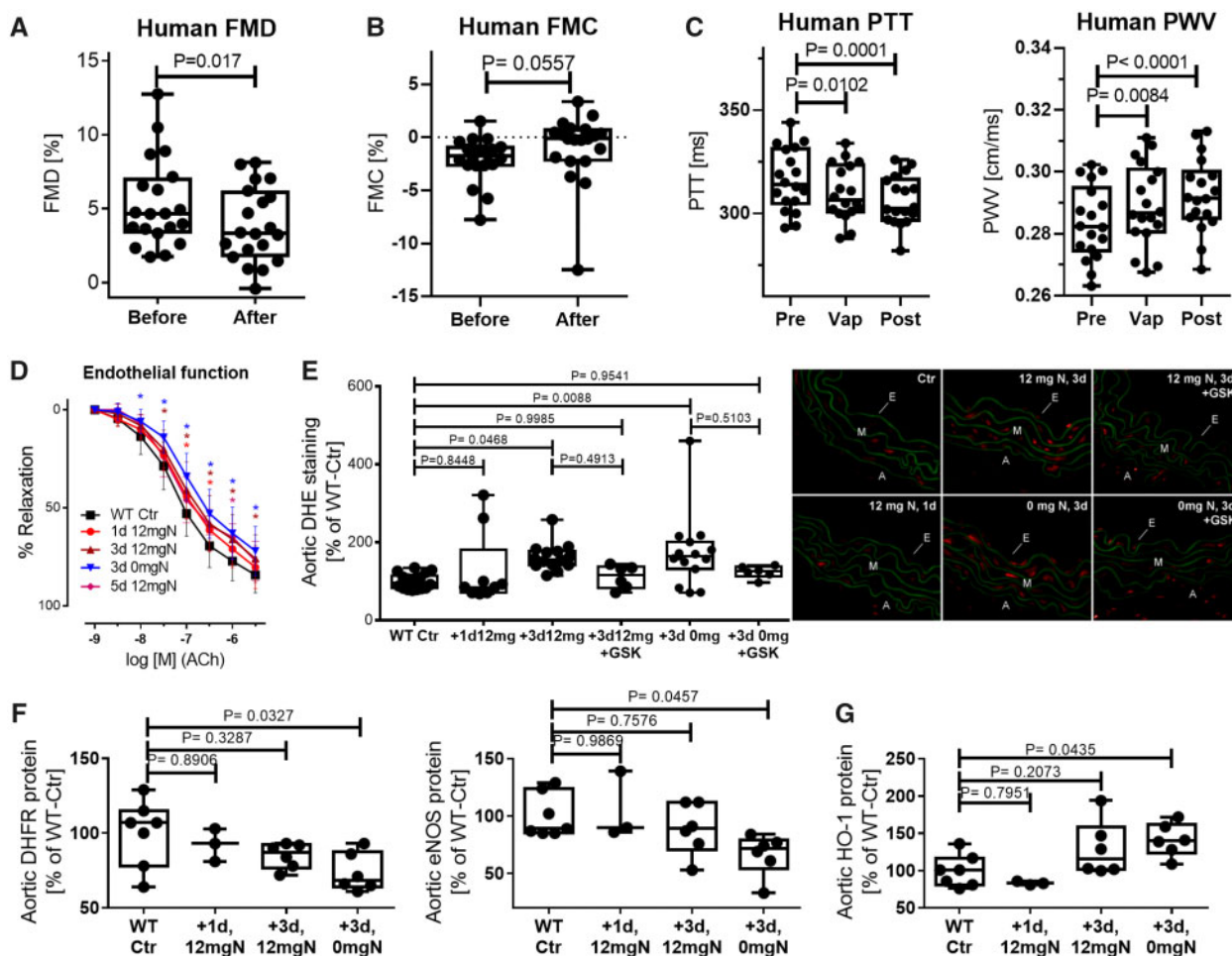
vaping has been linked to increased blood pressure and enhanced thrombogenesis.<sup>10-13</sup> Collectively, these consequences of vaping would be expected to initiate and/or exacerbate the process of atherosclerosis.<sup>9,14</sup> Thus, to garner further insight into the cardiovascular consequences of e-cigarette vaping, we conducted a study in mice using unflavoured e-cigarette liquids with and without nicotine to determine the impact on vascular (endothelial) function. In addition, we characterized the mechanisms regarding oxidative stress and inflammation, and validated our findings in human endothelial cells and in healthy smokers.<sup>15</sup>

**Materials and methods****E-cigarette exposure of human healthy subjects and measurement of flow-mediated dilation**

All human data were collected in accordance with the declaration of Helsinki and ethical approval was granted by the Landesärztekammer Rheinland-Pfalz (Mainz, Germany; permit number: 837.412.14(9651)). Written consent was received from all included individuals. Participants were exposed to nicotine containing vapour from a commercially available e-cigarette device (Joyetech e-Go C). Endothelial function was assessed in smokers by measuring flow-mediated dilation (FMD) and low flow-mediated constriction (FMC) of the brachial artery prior to and 15 min after the e-cigarette vaping using our established methods.<sup>15</sup> Arterial tonometry was used to measure shear rate and skin perfusion as well as arterial stiffness was measured by pulse transit time (PTT) and pulse wave velocity (PWV). Measurements were performed in smokers since compliance of non-smokers (e.g. inhalation frequency and depth) was very low based on a pilot study in  $n = 3$  non-smokers vs. smokers. For detailed protocol, characteristics of the included subjects and inclusion/exclusion criteria see [Supplementary material online \(Supplementary material online, Table S1\)](#).

**E-cigarette exposure of mice**

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the US National Institutes of



**Figure 1** Effects of short-term e-cigarette vapour exposure on vascular function and oxidative stress in wild-type mice—validation of human vascular function data. Endothelial function in healthy subjects was measured by FMD (A), FMC (B), and arterial stiffness by PTT/PWV (C) after single use of e-cigarette in a cross-over fashion. Data are mean  $\pm$  SD from  $n = 20$  healthy subjects. (D) E-cigarette vaping for 6 h/day for 1, 3, and 5 days led to a significant impairment of the endothelial function in wild-type mice. (E) E-cigarette vaping increased aortic reactive oxygen species (ROS) formation as assessed by DHE staining, which was inhibited by NOX-2 inhibition by GSK2795039 (compound of GlaxoSmithKline). Representative microscopy images are shown besides the densitometric quantification. (F) E-cigarette vaping significantly decreased endothelial •NO synthase (type 3) (eNOS) and DHFR protein expression and (G) increased HO-1 protein expression. Representative blots are shown in [Supplementary material online, Figure S3D](#), all normalized to  $\alpha$ -actinin. Data are presented as box (first and third quartiles, line = median) and whiskers (min, max) with jitter plot of single values from  $n = 20$  healthy subjects (A–C) or  $n = 6–18$  (E) animals/group or 3–7 (F), 3–7 (G), or mean  $\pm$  SD from  $n = 8–38$  (D) samples/group (pooled from 2–3 mice/sample). *P*-values as indicated except for (D) with \*,  $P < 0.05$  vs. unexposed controls.

Health and approval was granted by the Ethical Committee of the University Medical Center Mainz and the Landesuntersuchungsamt Rheinland-Pfalz (Koblenz, Germany; permit number: 23 177-07/G 16-1-055 and extension E2). We used a total number of 124 C57BL/6 (male, age  $12 \pm 3$  weeks) and 27 *Nox2* null mice (C57BL/6 background; male, age  $13 \pm 3$  weeks). The e-cigarette exposure system (inExpose, emka Technologies, France) was equipped with an e-cigarette module ([Supplementary material online, Figure S1](#)). Mice were exposed to e-cigarette vapour over 1, 3, or 5 days for 2 h/day (6 times 20 min). E-cigarette liquid used for exposures (GermanFlavours, Germany) consisted of 50% propylene glycol, 50% vegetable glycerol, and contained no additional flavouring. For detailed protocol, see [Supplementary material online](#).

The protocols for all other determined parameters and treatments are described in the [Supplementary material online](#).

## Results

### E-cigarette smoking causes endothelial dysfunction in smokers

In otherwise healthy smokers ( $n = 20$ ), e-cigarette vapour exposure reduced FMD, increased FMC (by clear trend), decreased PTT, and increased PWV ([Figure 1A–C](#)), clearly indicating profound induction of endothelial dysfunction, arterial stiffness, impaired shear rate [ $561.2 \pm 267.7$  vs.  $647.0 \pm 264.6$  (mean  $\pm$  SD);  $P = 0.016$ ] and skin perfusion ( $75.67 \pm 47.73$  vs.  $41.90 \pm 50.31$ ;  $P = 0.009$ ) by e-cigarette vaping. Smoking habits were characterized by an actual daily tobacco cigarette consume of  $14 \pm 5.03$  and long-term use of  $11.6 \pm 8.0$  pack-years. Sixty-five percent of the volunteers had at least 1 and 15% >4

attempts in the past to quit smoking. More parameters (e.g. increased heart frequency and sympathovagal balance) and subject characteristics are summarized in [Supplementary material online, Figure S2 and Table S1](#).

## Effects of e-cigarette vapour exposure on vascular function and oxidative stress

E-cigarette vapour exposure (with nicotine) for 1, 3, and 5 days caused endothelial dysfunction determined by acetylcholine-dependent relaxation in wild-type mice upon all exposure protocols ([Figure 1D](#)). Based on these initial findings on the time-dependent effects, we decided to use the 3-day exposure protocol for comparison of liquids without and with nicotine as well as for other measurements. Exposure to e-cigarette vapour without nicotine for 3 days induced the most pronounced impairment of endothelial function, followed by exposure to e-cigarette vapour with nicotine for 3 days ([Figure 1D](#)). The 3-day exposure to e-cigarette vapour without nicotine also caused supersensitivity to norepinephrine-dependent vasoconstriction ([Supplementary material online, Figure S3A](#)). Endothelium-independent vasodilation (nitroglycerin-dependent relaxation) was not impaired by e-cigarette vapour ([Supplementary material online, Figure S3B](#)). Endothelial dysfunction was coincident with increased aortic oxidative stress determined as dihydroethidium (DHE) fluorescence, with the most pronounced signal in the 3-day exposure group without nicotine, which could be blocked by the NOX-2 inhibitor GSK2795039 ([Figure 1E](#)) and was accompanied by increased immune cell marker CD68 in the aorta ([Supplementary material online, Figure S3C](#)). We also observed increased aortic and cardiac 4-hydroxynonenal (4-HNE) immunostaining, despite no significant down-regulation of its detoxifying enzyme, mitochondrial aldehyde dehydrogenase (ALDH-2) ([Supplementary material online, Figure S4](#)). eNOS and dihydrofolate reductase (DHFR) protein expression was significantly decreased ([Figure 1F](#)). Aortic oxidative stress condition was also reflected by up-regulated stress response protein heme oxygenase-1 (HO-1) in the mice exposed for 3 days ([Figure 1G](#)).

## NOX-2 (gp91phox) is responsible for e-cigarette vaping-induced vascular damage

Oxidative burst was augmented in e-cigarette vapour-exposed wild-type mice but not in *Nox2*<sup>-/-</sup> mice ([Figure 2A](#)). In addition, ROS formation in unexposed *Nox2*<sup>-/-</sup> mice was significantly lower compared to unexposed wild-type mice ([Figure 2A](#)). *Nox2*<sup>-/-</sup> mice had preserved endothelial function upon 3 days e-cigarette vapour (without nicotine) exposure ([Figure 2B](#)), whereas wild-type mice exhibited profound endothelial dysfunction ([Figure 2B](#), dashed lines). Likewise, *Nox2*<sup>-/-</sup> mice were protected from the increased blood pressure observed in wild-type mice ([Figure 2C](#)). We observed increased aortic expression of eNOS and no change in DHFR or stress response protein HO-1 upon e-cigarette vapour-exposure in *Nox2*<sup>-/-</sup> mice ([Figure 2D, E](#)). HO-1 activity and plasma bilirubin levels were increased in exposed wild-type but not *Nox2*<sup>-/-</sup> mice ([Figure 2F](#)). The lack of *Nox2* also prevented any increase in aortic ROS/superoxide formation upon e-cigarette vapour exposure as measured by three different methods ([Figure 2G-I](#)), which was also confirmed for

cardiac mitochondrial superoxide formation ([Supplementary material online, Figure S5A](#)). NOX-2 induced oxidative stress also contributes to apoptotic cell death in e-cigarette vapour exposed wild-type mice as revealed by cell death detection assay, whereas staining was absent in *Nox2*<sup>-/-</sup> mice ([Supplementary material online, Figure S5B](#)).

## Effects of e-cigarette vaping on vascular/pulmonary phenotypic changes revealed by immunohistochemistry and immunoblot analysis

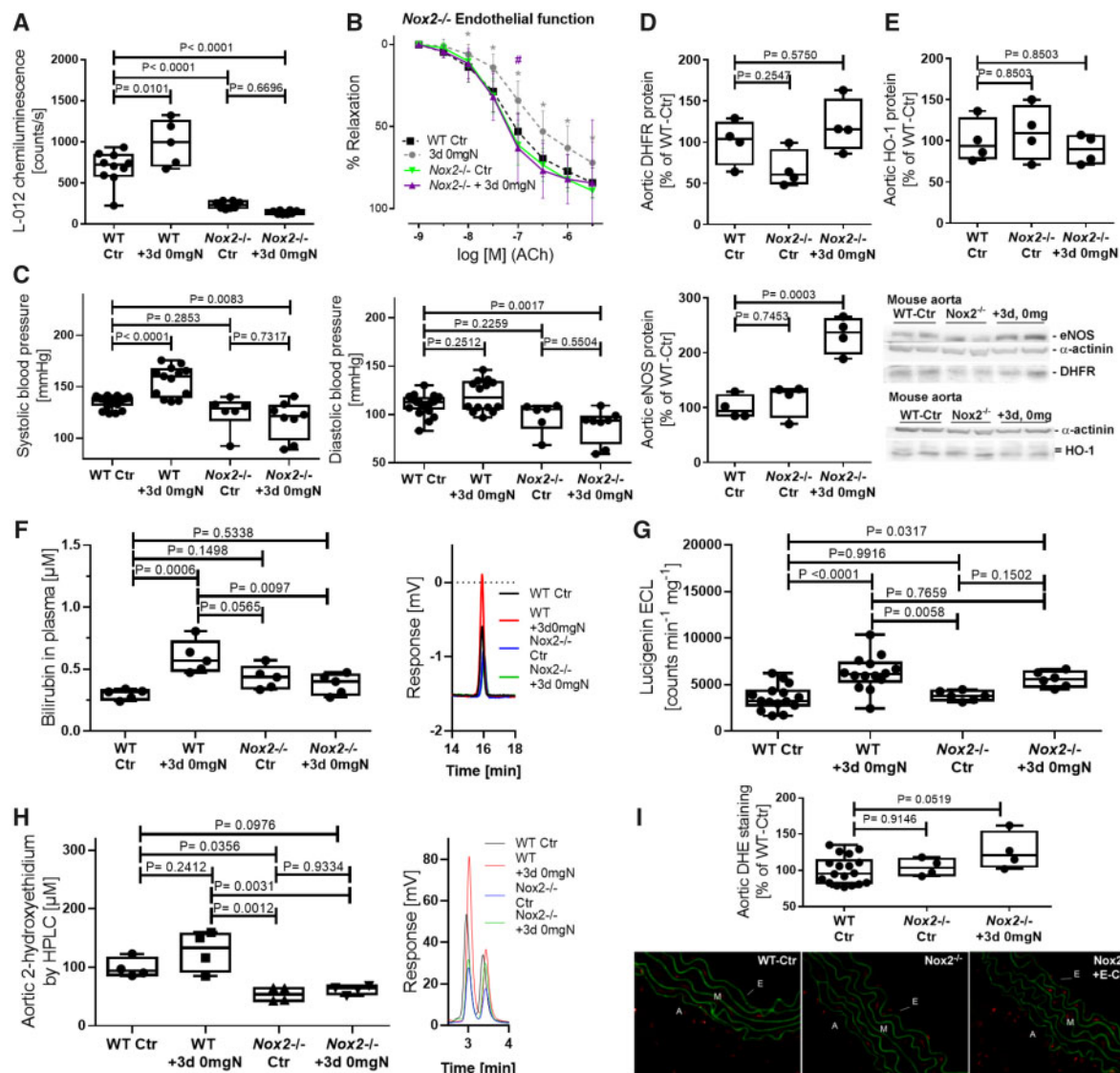
Immunohistochemical analysis revealed that e-cigarette smoking for 3 days increased significantly the staining for the oxidative stress marker, 3-nitrotyrosine (3-NT), and the vasoconstrictor peptide, ET-1 ([Figure 3A](#)). Similarly, e-cigarette smoking increased the inflammation marker, inducible nitric oxide synthase (NOS-2, iNOS), and the ROS-producing enzyme NOX-2 ([Figure 3B](#)). In lung sections and homogenate, the immunostaining for the ROS producing enzyme NOX-2 was augmented in the 3-day exposure groups ([Supplementary material online, Figure S6](#)). Overall, *Nox2*<sup>-/-</sup> mice exhibited reduced e-cigarette vapour-induced lung staining for oxidative stress (3-NT and 4-HNE) and inflammation (interleukin-6 and CD68) compared to wild-type mice ([Supplementary material online, Figure S7](#)). Collectively, these data indicate that e-cigarette vapour induces a NOX-2-dependent pro-oxidative and inflammatory state in the lungs and vasculature.

## Effects of e-cigarette vaping on cerebral oxidative stress and antioxidant defence

Exposure to e-cigarette vapour for 3 days without nicotine increased ROS formation (DHE cryo staining) in the frontal cortex of the brain, an effect that was severely blunted by pharmacological inhibition with the NOX-2 inhibitor GSK2795039 ([Figure 4A](#)). Likewise, the specific neuronal •NO synthase (type 1) (nNOS) inhibitor ARL-17477 and *Nox2* deficiency suppressed the DHE signal identifying uncoupled nNOS and NOX-2 as the sources of oxidative stress in brain of exposed mice ([Figure 4B](#)). The fluorescence staining results were also mirrored by specific superoxide quantification using an HPLC-dependent method ([Figure 4D](#)). Exposure to e-cigarette vapour for 3 days without nicotine caused a down-regulation of nNOS in whole brain homogenate at the mRNA and protein levels ([Figure 4C, E](#)), which was prevented by genetic *Nox2* deletion ([Figure 4F](#)). We found that cerebral Forkhead box protein 3 (*Foxo3*) mRNA, an antioxidant transcription factor, was significantly down-regulated in brain tissue of mice in the 3-day exposure groups, whereas *Nox1* was up-regulated at the mRNA level ([Figure 4E](#)). These effects were not observed in *Nox2*<sup>-/-</sup> mice ([Figure 4F](#)). Thus, e-cigarette vapour also induces a NOX-2-dependent pro-oxidative and inflammatory milieu in the cerebral circulation.

## Effects of e-cigarette vapour condensate vs. e-cigarette liquid on viability of cultured human endothelial cells and phenotypic changes

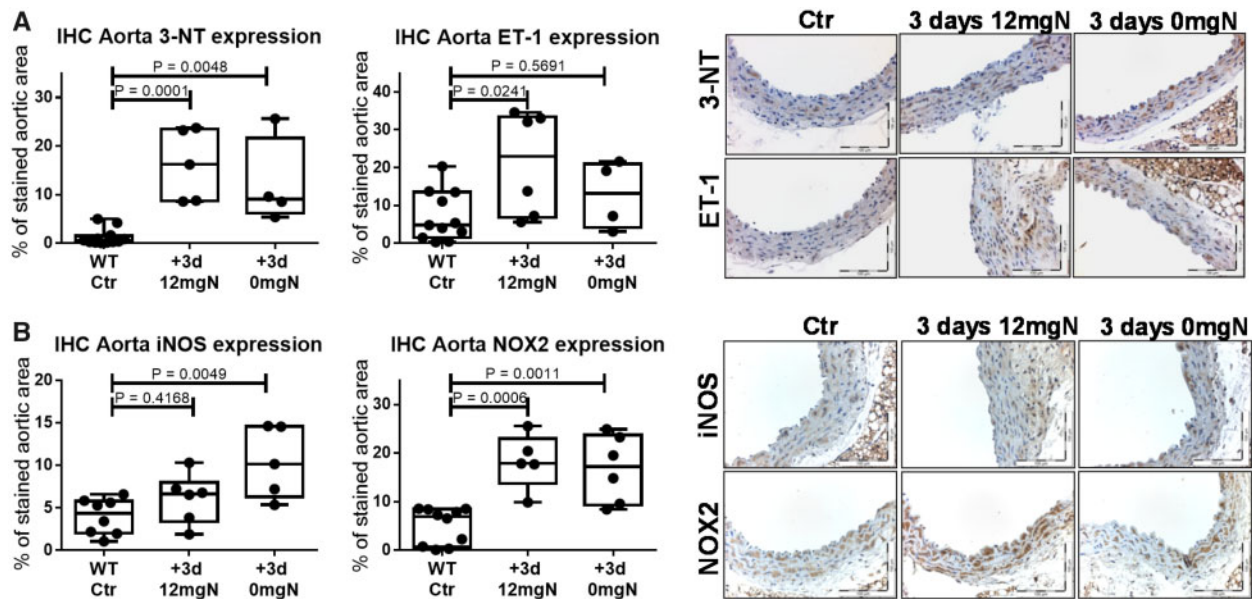
Treatment of cultured human endothelial cells with e-cigarette vapour condensate caused a concentration- and time-dependent loss



**Figure 2** Effects of short-term e-cigarette vapour exposure on vascular function and oxidative stress in  $Nox2^{-/-}$  ( $gp91phox^{-/-}$ ) vs. wild-type mice. (A) E-cigarette vaping caused a stronger oxidative burst in whole blood of wild-type mice, whereas no significant change was observed in whole blood of  $Nox2^{-/-}$  mice. In addition, the overall stimulated signal was much lower in  $Nox2^{-/-}$  mice. (B) E-cigarette vaping caused endothelial dysfunction in wild-type mice, which was prevented by  $Nox2^{-/-}$ . (C) E-cigarette vaping increased systolic and diastolic blood pressure in wild-type but not in  $Nox2^{-/-}$  mice. (D, E) E-cigarette vaping paradoxically increased protein expression of eNOS while no changes were observed in the regulation of expression of DHFR and HO-1. Representative blots are shown below the densitometric quantification. (F) Bilirubin levels (product of HO-1 activity) in plasma were increased in wild-type but not in  $Nox2^{-/-}$  mice. Representative chromatograms are shown along with the quantification.  $Nox2$  knock-out prevented e-cigarette vaping-induced increase in aortic ROS/superoxide formation as assessed by lucigenin enhanced chemiluminescence (ECL) (G), high performance liquid chromatography (HPLC)-based quantification of DHE oxidation products (H) and DHE staining (I). Representative chromatograms and microscopy images are shown along with the quantifications. Data are presented as box (first and third quartiles, line = median) and whiskers (min, max) with jitter plot of single values from  $n = 5-10$  (A), 6-22 (C), 4 (D, E) and at least 5 (F), 6-15 (G), 4 (H), or 4-18 (I) or mean  $\pm$  SD from 6-38 (B) animals/group.  $P$ -values as indicated except for (B) with \*,  $P < 0.05$  vs. unexposed controls.

of cellular viability (Supplementary material online, Figure S8A, B). The e-cigarette liquid itself had a qualitatively similar impact on the cells as the condensate at the highest concentration (Supplementary material online, Figure S8A, C). However, lower concentrations of the condensate exhibited greater toxicity than the liquid (compare Supplementary material online, Figure S8B, C), suggesting that

vaporization creates additional toxicity. There was no significant effect of nicotine on cell viability, in either condensate or liquid. The condensate also induced a more pronounced inflammatory phenotype (IL-6 levels) in the endothelial cells than the liquid after 72 h of treatment (Supplementary material online, Figure S8D). The antioxidant enzyme PEG-catalase and the NOX-2 inhibitors gp91 ds-tat or



**Figure 3** Effects of short-term e-cigarette vaping on vascular nitrotyrosine (3-NT), endothelin-1 (ET-1), inducible nitric oxide synthase, and NADPH oxidase subunit (NOX-2) expression. (A) E-cigarette vaping increased vascular 3-NT and ET-1 as well as (B) iNOS and NOX-2 expression. Representative microscopy images are shown below the densitometric quantification. Data are presented as box (first and third quartiles, line = median) and whiskers (min, max) with jitter plot of single values from  $n = 4-8$  (A) and  $5-8$  (B) animals/group.  $P$ -values as indicated.

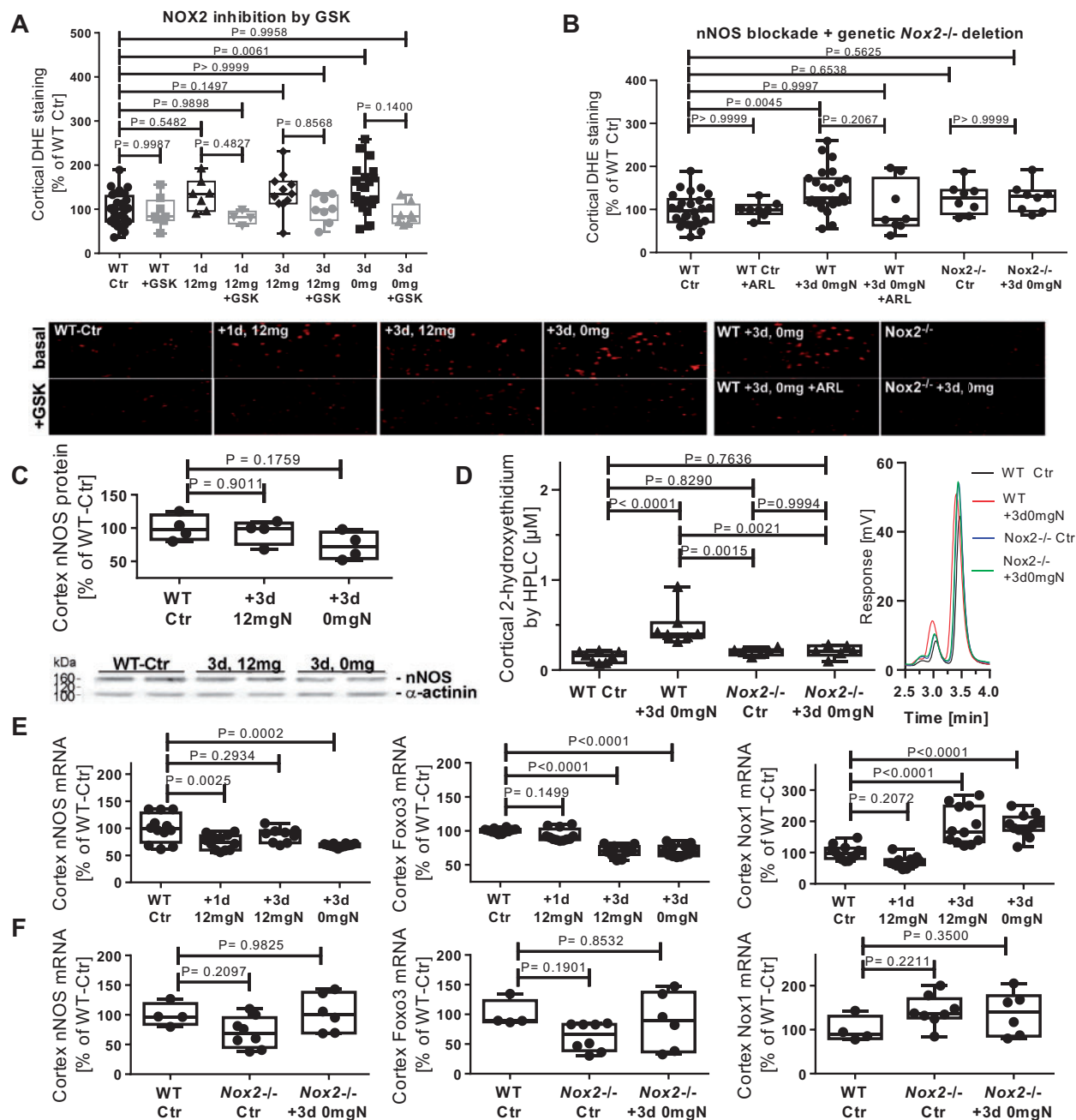
GSK2795039 increased cell viability after incubation with condensate pointing to a significant role of NOX-2 dependent ROS formation in causing cell death (Supplementary material online, Figure S9). Although the used concentration of the condensate in the cell culture medium is supra-physiological and clearly represents a model of cellular toxicity in order to study adverse effects in a short-time scale, these data suggest that e-cigarette vaporization adds additional toxicity to its components via NOX-2 induced oxidative stress, prompting us to investigate this process.

### E-cigarette liquid and vapour condensate contain toxic aldehydes exerting adverse effects on cells and tissue ex vivo by activation of NOX-2

Using HPLC analysis of 2,4-dinitrophenylhydrazine (DNPH)-derivatization products, we found  $\mu\text{M}$  concentrations of formaldehyde, acetaldehyde, butyraldehyde, and acrolein in E-cigarette liquid and to much larger extend in vapour condensate:  $16 \pm 2$  formaldehyde,  $0.19 \pm 0.04$  acetaldehyde,  $1.6 \pm 0.1$  butyraldehyde and  $0.49 \pm 0.09$  acrolein (Figure 5A, for representative chromatograms see Supplementary material online, Figure S10). These values were in the range of or slightly below previously reported values in other e-cigarette studies.<sup>16,17</sup> Moreover, we could confirm the presence of formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, and acrolein in the E-cigarette liquid by liquid chromatography/mass spectrometry (LC/MS) analysis and their signal intensities were at least 9-fold higher in vapour condensate (Figure 5B and Supplementary

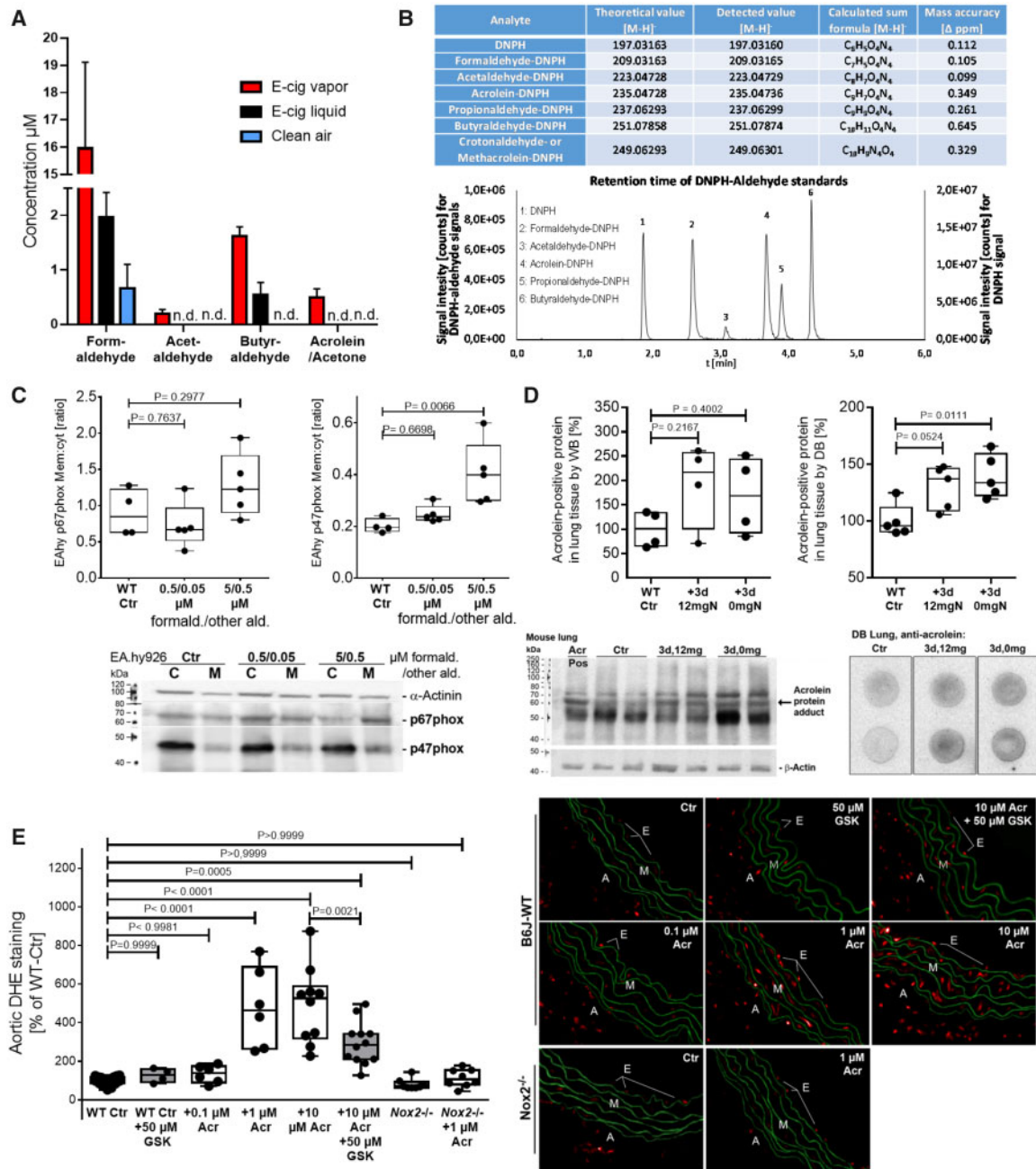
material online, Table S2). In addition, we observed a [M-H]<sup>-</sup> C10H9N4O4 signal, providing evidence for the presence of DNPH adducts of either crotonaldehyde and/or methacrolein, both toxic aldehydes with known harmful effects on the cardiovascular system.<sup>18-21</sup>

To estimate the toxicity of these vapour-enhanced products, we incubated cultured human endothelial cells with mixtures of aldehydes at relevant concentrations. We found they produced concentration-dependent cell death (Supplementary material online, Figure S11A) and increased expression by trend of the inflammatory marker COX-2 (Supplementary material online, Figure S11B). This was accompanied by aldehyde-dependent NOX-2 activation as measured by higher p47phox protein content in membranous fractions of endothelial cell pellets as determined by ratio of membrane/cytosol p47phox content (Figure 5C). Since acrolein can cause NOX-2 activation,<sup>22</sup> we tested lung tissue for acrolein protein-adducts and found more pronounced staining in lungs of in e-cigarette vapour-exposed mice (Figure 5D). Finally, we observed that vascular ROS formation was significantly increased by acrolein in aorta of wild-type but not *Nox2*<sup>-/-</sup> mice and was blocked by GSK2795039 (Figure 5E). Mixtures of aldehydes also caused phenotypic changes in cultured RAW macrophages and increased expression by trend of p47phox, p67phox, and COX-2 (Supplementary material online, Figure S12). Although the used concentrations of the aldehydes in the cell culture medium were based on those found in the e-cigarette condensate and liquid, these experiments are supra-physiological and represent a model of cellular toxicity in order to study adverse effects in a short-time scale.

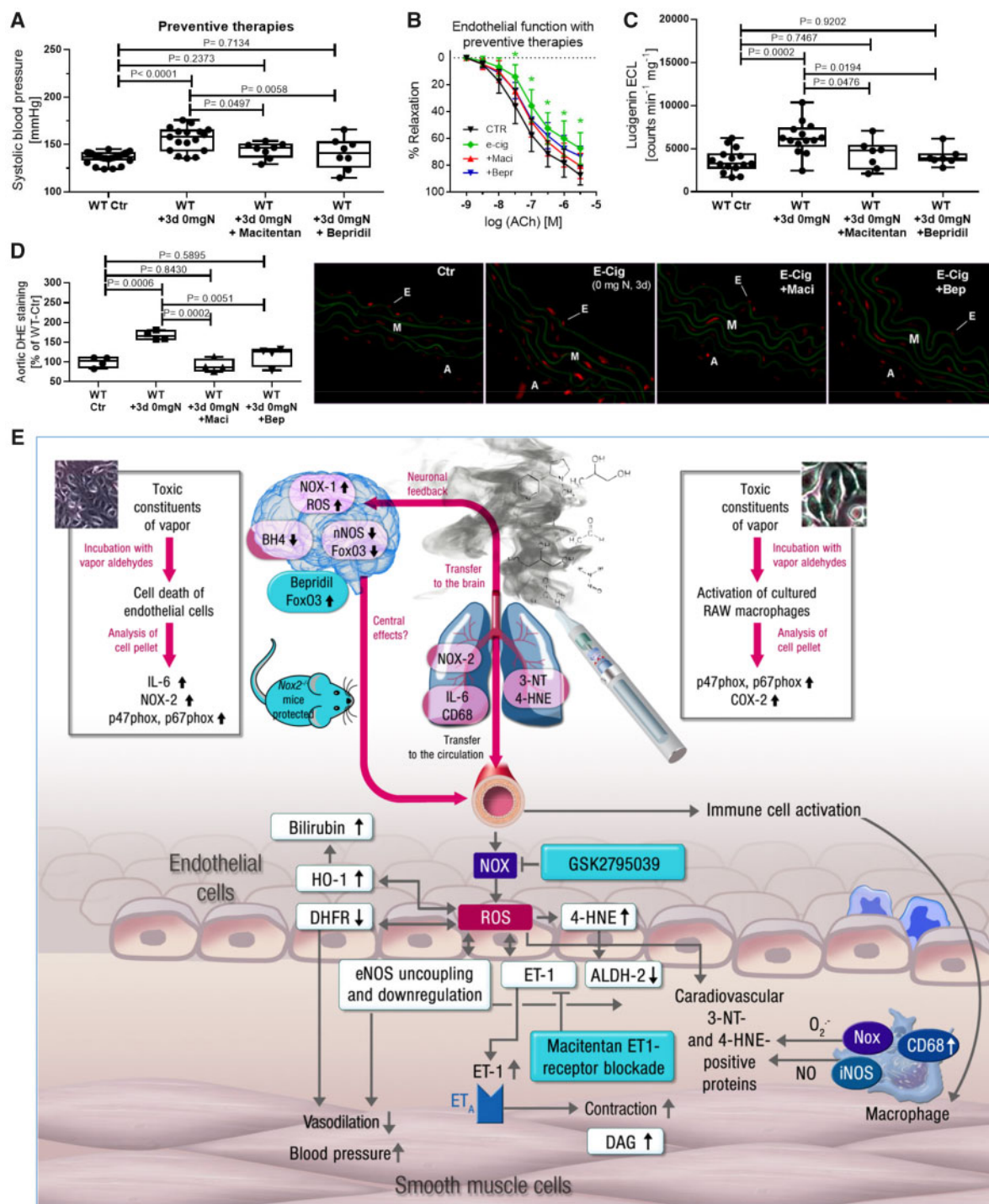


**Figure 4** Effects of short-term e-cigarette vaping on cerebral oxidative stress. (A) E-cigarette vaping increased cerebral ROS formation (assessed by DHE) which was inhibited by the specific NOX-2 inhibitor GSK2795039. (B) Cerebral ROS formation (assessed by DHE) was also inhibited by the specific nNOS inhibitor ARL-17477 and genetic *Nox2* deletion. (C) nNOS in the frontal cortex tended to be decreased in response to E-cigarette vaping. Representative blots are shown next to the densitometric quantification. (D) E-cigarette vaping increased cerebral superoxide formation (assessed by HPLC-based quantification of DHE oxidation products) which was inhibited by genetic *Nox2* deletion. Representative chromatograms are shown along with the quantification. (E) E-cigarette vaping decreased mRNA expression of *Foxo3* and *nNOS* and increased expression of *Nox1* mRNA levels. (F) These genes were differentially or not significantly regulated in the frontal cortex of e-cigarette vapour-exposed *Nox2*<sup>-/-</sup> mice. Data are presented as box (first and third quartiles, line = median) and whiskers (min, max) with jitter plot of single values from  $n = 4$  (GSK-group) and at least 4 (Ctr and exposures) animals/group (A, B) and 4 samples/group (pooled from 3 mice/sample) (C), 6–8 (D), 9–12 (E), and 4–8 (F) animals/group. *P*-values as indicated.





**Figure 5** E-cigarette liquid and vapour condensate contain toxic aldehydes as revealed by DNPH derivatization with HPLC and LC-MS analysis exerting toxic effects on cultured endothelial cells. (A) Quantification of identified aldehydes by HPLC analysis and known DNPH-aldehyde standards. Representative chromatograms are shown in [Supplementary material online, Figure S10](#). (B) List of identified compounds (DNPH or aldehyde-DNPH) adducts in e-cigarette vapour condensate by LC-MS analysis with a representative chromatogram. The relative abundance of the aldehyde-DNPH adducts is provided in [Supplementary material online, Table S2](#). (C) Protein expression of p67<sup>phox</sup> and p47<sup>phox</sup> was determined by western blot analysis in membranous and cytosolic protein-fractions from pellets of EA.hy926 cells upon incubation with toxic aldehyde mixtures of formaldehyde (0.5–5 μM) and acetaldehyde/butyraldehyde/acrolein (each 0.05–0.5 μM). The ratio of membranous/cytosolic p67<sup>phox</sup> and p47<sup>phox</sup> reflects NOX-2 activation state. Representative western blots are shown besides the densitometric quantification. (D) Protein adducts of acrolein were determined by western and dot blot analysis in lung tissues of control or E-cigarette vapour-exposed mice. Representative original western and dot blots are shown besides the densitometric quantifications. (E) Vascular ROS formation by acrolein was determined using dihydroethidium (DHE, 1 μM)-dependent fluorescence microtopography in aortic cryo-sections. The contribution of NOX-2 to this ROS signal was assessed by comparison of aortic sections of wild-type vs. *Nox2* deficient mice and pharmacological inhibition by GSK2795039. Representative microscopy images are shown below the densitometric quantification. Data are presented as box (first and third quartiles, line = median) and whiskers (min, max) with jitter plot of single values from  $n = 4–5$  (C, D) and 4–16 (E) or mean  $\pm$  SD from  $n = 3$  (A, B) experiments or animals per group. *P*-values as indicated.



**Figure 6** E-cigarette vaping induced cardiovascular complications are prevented by pharmacological ET-1 receptor blockade and FOXO-3 activation. ET-1 receptor blockade by macitentan and FOXO-3 activation by bepridil prevent e-cigarette vaping induced blood pressure increase (A), endothelial dysfunction (B), aortic superoxide formation by lucigenin ECL (C), and aortic ROS formation by DHE staining (D). Representative microscopy images are shown besides the densitometric quantification. Data are presented as box (first and third quartiles, line = median) and whiskers (min, max) with jitter plot of single values from at least  $n = 8$  (A), 7 (C), and 4 (D) or mean  $\pm$  SD from at least  $n = 8$  (B) animals per group. Significance as indicated except for (B) with \*,  $P < 0.05$  vs. unexposed controls. (E) Central scheme: proposed mechanisms of E-cigarette mediated cerebro/cardiovascular complications from studies in mice. The described adverse effects are also operative in the disease development and progression of classical cerebro/cardiovascular disease such as stroke, atherosclerosis, hypertension, and coronary artery disease. Light blue colour items represent pharmacological/genetic interventions. NOX and Nox refers to protein and mRNA of NOX-2 isoform.

## Mechanistic insights into e-cigarette vapour induced cardiovascular complications in mice by pharmacological interventions

In order to further elucidate the detrimental role of ET-1 and FOXO-3 signalling, mice were exposed to e-cigarette vapour and treated simultaneously with the ET-1 receptor blocker macitentan and the FOXO-3 activator bepridil. Both therapies prevented blood pressure increase, endothelial dysfunction and vascular ROS/superoxide formation (Figure 6A–D), suggesting a central role for ET-1 and FOXO-3 in aggravating or preventing e-cigarette vapour induced cardiovascular complications.

## Discussion

With the present study, we found that a single episode of e-cigarette smoking induced endothelial dysfunction even in chronic smokers. To gain mechanistic insight, we examined the vascular and cerebral consequences of short-term e-cigarette vapour exposure in mice and found impaired endothelial NO signalling and endothelial dysfunction with e-cigarettes. We also determined that e-cigarette vapour exposure increased oxidative stress and inflammation in aorta, lung, and brain. Moreover, this phenotype was dependent upon the NADPH oxidase family member, NOX-2, as *Nox2*<sup>-/-</sup> mice were resistant to the consequences of e-cigarette vapour exposure. Since we observed that vaporization enhanced the toxicity of e-cigarette liquid, we examined vaporization products. Using quantitative HPLC and qualitative LC/MS analysis, we identified toxic aldehydes generated during vaporization and validated their deleterious effects in cultured endothelial cells. One model aldehyde, acrolein, was observed to recapitulate the impact of e-cigarette vapour in a NOX-2-dependent manner. Thus, e-cigarette vapour exerts a broad injurious influence on the vasculature due, in part, to vaporization-enhanced aldehyde generation that activates NOX-2 leading to oxidative stress, inflammation, and endothelial dysfunction.

## E-cigarette use in current smokers and adolescents

The preponderance of data regarding e-cigarettes and smoking cessation clearly indicates these devices are not superior to approved nicotine replacement therapy or usual care.<sup>23</sup> Indeed, a recent American College of Preventive Medicines Practice Statement reported that there is limited evidence to support short-term exclusive use of e-cigarettes in adults who quit smoking. Moreover, the report indicates that among youth, there is no known benefit, but there is significant concern for harm.<sup>23</sup> Our data highlight this concern as we observed significant consequences of e-cigarettes on the vascular endothelium with just one episode of vaping. Our observations provide additional support for the FDA concern for an 'epidemic' surge in e-cigarette use by adolescents that prompted regulatory action against more than 1300 US retailers and five major manufacturers for their roles in perpetuating youth access to e-cigarettes.<sup>5</sup> Recent warnings and reports by the FDA, CDC, and other public health authorities on severe pulmonary disease and deaths among people using e-cigarettes further support these concerns.<sup>6,7</sup> Thus, the data

reported here suggest that adolescent e-cigarette use clearly should not be considered as being safe from a vascular, cardiac, or pulmonary point of view.

## Cardiovascular side effects of e-cigarette and tobacco smoking

We examined the effects of e-cigarette smoking on vascular function using the FMD/FMC<sup>15</sup> and fingertip tonometry approach. Acute e-cigarette smoking caused a significant reduction in FMD in smokers, FMC increase by trend, impaired skin perfusion, and increased arterial stiffness parameters (PTT, PWV), indicating that e-cigarette vapour induces endothelial dysfunction with a reduction in vascular nitric oxide bioavailability,<sup>15</sup> most likely due to increased oxidative stress. This observation bears a striking resemblance to the adverse impact of chronic cigarette smoking that causes endothelial dysfunction that is improved by the acute administration of the antioxidant, vitamin C.<sup>24</sup> These observations are also in keeping with other data comparing e-cigarette vapour and cigarette smoke. Both appear to increase oxidative stress, to degrade blood–brain barrier integrity and to induce vascular inflammation in endothelium.<sup>10</sup> Likewise, both tobacco smoke and e-cigarette vapour induce cerebrovascular inflammation and post-stroke ischaemia/reperfusion damage in mice and these effects are attenuated by stimulating the NRF2 antioxidant response.<sup>10</sup> Chronic (8-month) mouse exposure to e-cigarette vapour and cigarette smoke (3R4F reference) revealed qualitatively comparable adverse effects on cardiac function, arterial stiffness, and endothelial function.<sup>14</sup> Thus, abundant data in this report and by other investigators indicate that e-cigarette vapour shares many of the same adverse vascular consequences of authentic tobacco smoke. This notion is also strongly supported by a recent large scale cross-sectional study in more than 30 000 subjects indicating that daily e-cigarette use, adjusted for smoking conventional cigarettes as well as other risk factors, is associated with increased risk of myocardial infarction.<sup>25</sup>

A human interventional study showed a significant increase in serum oxidative stress markers (including soluble NOX2-derived peptide), decrease in circulating nitric oxide products and vitamin E levels, as well as impaired endothelial function upon e-cigarette vapour and tobacco cigarette smoke exposure.<sup>8</sup> Likewise, regular e-cigarette users had higher cardiac sympathetic activity and serum oxidative stress markers,<sup>13</sup> healthy non-smokers showed increased blood pressure and heart rate upon single use from an e-cigarette<sup>11</sup> and smokers responded with increased blood pressure and pulse-wave velocity.<sup>9</sup> Although the majority of available studies provide evidence that e-cigarette vaping is somewhat less detrimental than tobacco cigarette smoking, they are all in good accordance with our present human vascular/hemodynamic findings as well as NOX-2 centred mechanism in exposed mice.

## E-cigarettes stimulate superoxide production via NOX-2 activation

In the study presented here, we established that e-cigarette vapour exposure produced increased markers of oxidative stress in aortic and brain tissue of mice. These observations of increased oxidative stress with e-cigarette vapour may explain its impact on the endothelium. Oxidative stress is known to produce endothelial dysfunction

by increased oxidative breakdown of NO and peroxynitrite formation,<sup>26</sup> as well as limiting the availability of tetrahydrobiopterin, an essential eNOS cofactor.<sup>27</sup> An important contribution of this work is the identification of Nox-2 as a key mediator of e-cigarette vaping-induced oxidative stress. We demonstrated that e-cigarette vapour leads to up-regulation of NOX-2 activity and that mice lacking the *Nox2* gene do not demonstrate oxidative stress and endothelial dysfunction in response to e-cigarette vapour, similar to mice exposed to the environmental risk factor aircraft noise that were also protected when *Nox2* gene was deleted.<sup>28</sup> This finding nicely mirrors previous reports that patients with chronic granulomatous disease (*Nox2* hereditary deficiency) showed better FMD than healthy controls.<sup>29</sup>

E-cigarette vaping is also known to stimulate inflammatory pathways,<sup>10</sup> findings consistent with our observations of NOS-2 induction and IL-6 release with e-cigarette vapour. Our data implicate NOX-2 as a key mediator of the inflammatory response as we observed attenuated e-cigarette vapour-induced inflammation in the *Nox2*-null mice compared with wild-type animals. These data linking NOX-2 to the adverse consequences of e-cigarette vapour are consistent with a recent study involving healthy subjects (smokers and non-smokers) smoking one classical cigarette and nine puffs from an e-cigarette in a cross-over design.<sup>8</sup> That study also revealed that both traditional and e-cigarettes produced comparable effects such as increases in a serum oxidative stress marker (8-isoprostanes), NOX-2 activation, and reduced endothelial function. Thus, e-cigarette vapour appears to mediate many of its adverse vascular consequences through NOX-2 activation, e.g. as shown here by nNOS uncoupling that was absent in *Nox2* deficient mice.

This was also nicely supported by increased ET-1 signalling supporting the previously reported crosstalk between NOX-2 and ET-1,<sup>30</sup> where ET-1 induces expression of NOX-2 and formation of ROS and vice versa NOX-2 derived ROS activate the promoter for ET-1 expression with enhanced ET-1 dependent vasoconstriction,<sup>31</sup> all of which was corrected by pharmacological ET-1 receptor blockade with macitentan. The compensatory, however futile compensatory up-regulation of HO-1 was observed in our exposed mice together with higher bilirubin plasma levels (NRF2-dependent oxidative stress response), all of which was normalized in *Nox2* deficient mice. In contrast, the FOXO-3 dependent oxidative stress response was inactivated by down-regulation of *Foxo3* mRNA in exposed mice, which was overcome by pharmacological FOXO-3 activation by bepridil. Therefore, the here reported pathomechanisms of e-cigarette vapour exposure show striking similarities with the reported molecular pathways of adverse cardio/cerebrovascular effects of exposure of mice to aircraft noise.<sup>28,32</sup>

## E-cigarettes and aldehyde toxicity in vitro and in vivo

Another important contribution of this study is the identification of reactive aldehydes as important mediators of e-cigarette vapour-mediated endothelial dysfunction. The vaporization process creates compounds with high cellular toxicity that we identified as reactive aldehydes in the vapour condensate. Our data that mixtures of the identified aldehydes in the vapour condensate induce endothelial cell death and immune cell activation is consistent with a well-established

literature on the subject (see also [Supplementary material online](#) for extended discussion).<sup>33–35</sup> Of particular note in our data, we found that one model aldehyde, acrolein, induced stimulation of superoxide production in aortic tissue that was NOX-2-dependent as it was almost completely suppressed in vessels from animals lacking NOX-2. These data provide a proof of concept for previous studies showing that acrolein from cigarette smoke extract activates NOX-2 in cultured endothelial cells.<sup>22</sup> Previous studies also reported toxic effects of acrolein on lung endothelial barrier function, which was associated with oxidative stress and brisk inflammation, however with a more pronounced toxicity in the presence of nicotine.<sup>36</sup> Components of liquid or condensate were previously investigated in cultured human lung cells and almost no adverse effects were observed in contrast to detrimental effects by mainstream tobacco cigarette smoke.<sup>37</sup> These observations clearly link vaporization-induced aldehydes to the processes that mediate e-cigarette vapour-induced endothelial dysfunction and inflammation.

## Limitations

The missed recruitment of healthy non-smokers subjects should be recognized as limitation of the present study. Nevertheless, a repeat demonstration of the substantial adverse side effects of e-cigarette smoking on endothelial function of chronic smokers further substantiates the concept that e-cigarettes are not at all a 'healthy' alternative to traditional combustible cigarettes.

## Conclusions and clinical implications

With the present studies we demonstrate that acute e-cigarette vapour exposure causes endothelial dysfunction in chronic smokers, induces inflammation and oxidative stress in vascular and cerebral tissue, and increases blood pressure in experimental animals (central scheme in [Figure 6E](#)). Importantly, we identified NOX-2 as an important source of e-cigarette-driven vascular oxidative stress and inflammation with an important role of ET-1 (shown by macitentan therapy) and FOXO-3 (shown by bepridil therapy) signalling in aggravation or prevention of these adverse effects. Finally, we established that aldehydes produced during vaporization were present *in vivo* and sufficient to produce NOX-2-driven oxidative stress and inflammation. If one considers the ongoing epidemic of e-cigarette use among adolescents, our data provide a strong warning that the perceived 'safety' of these products (compared to tobacco) is not warranted, which is in good accordance with a recent systemic literature review concluding that 'e-cigarette should not be labelled as a safe cardiovascular product'.<sup>38</sup> We also like to state that the results of the present studies have no e-cigarette industry related conflict of interest, an information that seems to be important since recent studies indicate that e-cigarette industry funding is more likely to lead to results that demonstrate that e-cigarettes are harmless.<sup>39</sup> Our data suggest aggressive steps should be taken to protect our children from health risks caused by e-cigarettes, especially e-cigarettes should be mentioned like tobacco cigarettes as an important health risk factor in the European<sup>40</sup> and American Heart Association Guidelines for prevention.<sup>41</sup>

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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## References

- Cohen AJ, Brauer M, Burnett R, Anderson HR, Frostad J, Estep K, Balakrishnan K, Brunekreef B, Dandona L, Dandona R, Feigin V, Freedman G, Hubbell B, Jobling A, Kan H, Knibbs L, Liu Y, Martin R, Morawska L, Pope CA 3rd, Shin H, Straif K, Shadick G, Thomas M, van Dingenen R, van Donkelaar A, Vos T, Murray CJL, Forouzanfar MH. Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015. *Lancet* 2017;**389**:1907–1918.
- Hajek P, Phillips-Waller A, Przulj D, Pesola F, Myers Smith K, Bisal N, Li J, Parrott S, Sasieni P, Dawkins L, Ross L, Goniewicz M, Wu Q, McRobbie HJ. A randomized trial of E-cigarettes versus nicotine-replacement therapy. *N Engl J Med* 2019;**380**:629–637.
- Grana R, Benowitz N, Glantz SA. E-cigarettes: a scientific review. *Circulation* 2014;**129**:1972–1986.
- <https://www.cdc.gov/mmwr/volumes/67/wr/mm6745a5.htm> (27 October 2019).
- <https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm620184.htm> (27 October 2019).
- [https://www.cdc.gov/tobacco/basic\\_information/e-cigarettes/severe-lung-disease.html](https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html) (27 October 2019).
- Layden JE, Ghinai I, Pray I, Kimball A, Layer M, Tenforde M, Navon L, Hoots B, Salvatore PP, Elderbrook M, Haupt T, Kanne J, Patel MT, Saathoff-Huber L, King BA, Schier JG, Mikosz CA, Meiman J. Pulmonary illness related to e-cigarette use in Illinois and Wisconsin—preliminary report. *N Engl J Med* 2019;doi: 10.1056/NEJMoa1911614.
- Carnevale R, Sciarretta S, Violi F, Nocella C, Loffredo L, Perri L, Peruzzi M, Marullo AG, De Falco E, Chimenti I, Valenti V, Biondi-Zoccai G, Frati G. Acute impact of tobacco vs electronic cigarette smoking on oxidative stress and vascular function. *Chest* 2016;**150**:606–612.
- Vlachopoulos C, Ioakeimidis N, Abdelrasoul M, Terentes-Printzios D, Georgakopoulos C, Pietri P, Stefanadis C, Tousoulis D. Electronic cigarette smoking increases aortic stiffness and blood pressure in young smokers. *J Am Coll Cardiol* 2016;**67**:2802–2803.
- Kaisar MA, Villalba H, Prasad S, Liles T, Sifat AE, Sajja RK, Abbruscato TJ, Cucullo L. Offsetting the impact of smoking and e-cigarette vaping on the cerebrovascular system and stroke injury: is Metformin a viable countermeasure? *Redox Biol* 2017;**13**:353–362.
- Cooke WH, Pokhrel A, Dowling C, Fogt DL, Rickards CA. Acute inhalation of vaporized nicotine increases arterial pressure in young non-smokers: a pilot study. *Clin Auton Res* 2015;**25**:267–270.
- Qasim H, Karim ZA, Silva-Espinoza JC, Khasawneh FT, Rivera JO, Ellis CC, Bauer SL, Almeida IC, Alshbool FZ. Short-term e-cigarette exposure increases the risk of thrombogenesis and enhances platelet function in mice. *J Am Heart Assoc* 2018;**7**; doi: 10.1161/JAHA.118.009264.
- Moheimani RS, Bhetraratana M, Yin F, Peters KM, Gornbein J, Araujo JA, Middlekauff HR. Increased cardiac sympathetic activity and oxidative stress in habitual electronic cigarette users: implications for cardiovascular risk. *JAMA Cardiol* 2017;**2**:278–284.
- Olfert JM, DeVallance E, Hoskinson H, Branyan KW, Clayton S, Pitzer CR, Sullivan DP, Breit MJ, Wu Z, Klinkhachorn P, Mandler WK, Erdreich BH, Ducatman BS, Bryner RW, Dasgupta P, Chantler PD. Chronic exposure to electronic cigarettes results in impaired cardiovascular function in mice. *J Appl Physiol* (1985) 2018;**124**:573–582.
- Gori T, Dragoni S, Lisi M, Di Stolfo G, Sonnati S, Fineschi M, Parker JD. Conduit artery constriction mediated by low flow: a novel noninvasive method for the assessment of vascular function. *J Am Coll Cardiol* 2008;**51**:1953–1958.
- Farsalinos KE, Kistler KA, Pennington A, Spyrou A, Kouretas D, Gillman G. Aldehyde levels in e-cigarette aerosol: findings from a replication study and from use of a new-generation device. *Food Chem Toxicol* 2018;**111**:64–70.
- Salamanca JC, Meehan-Atrash J, Vreeke S, Escobedo JO, Peyton DH, Strongin RM. E-cigarettes can emit formaldehyde at high levels under conditions that have been reported to be non-averse to users. *Sci Rep* 2018;**8**:7559.
- Kim M, Han CH, Lee MY. NADPH oxidase and the cardiovascular toxicity associated with smoking. *Toxicol Res* 2014;**30**:149–157.
- Pei Z, Zhuang Z, Sang H, Wu Z, Meng R, He EY, Scott GI, Maris JR, Li R, Ren J. Alpha, beta-unsaturated aldehyde crotonaldehyde triggers cardiomyocyte contractile dysfunction: role of TRPV1 and mitochondrial function. *Pharmacol Res* 2014;**82**:40–50.
- Baldrige KC, Zavala J, Surratt J, Sexton KG, Contreras LM. Cellular RNA is chemically modified by exposure to air pollution mixtures. *Inhal Toxicol* 2015;**27**:74–82.
- Doyle M, Sexton KG, Jeffries H, Bridge K, Jaspers I. Effects of 1, 3-butadiene, isoprene, and their photochemical degradation products on human lung cells. *Environ Health Perspect* 2004;**112**:1488–1495.
- Jaimes EA, DeMaster EG, Tian RX, Raji L. Stable compounds of cigarette smoke induce endothelial superoxide anion production via NADPH oxidase activation. *Arterioscler Thromb Vasc Biol* 2004;**24**:1031–1036.
- Livingston CJ, Freeman RJ, Costales VC, Westhoff JL, Caplan LS, Sherin KM, Niebuhr DW. Electronic nicotine delivery systems or e-cigarettes: American College of Preventive Medicine's Practice Statement. *Am J Prev Med* 2019;**56**:167–178.
- Heitzer T, Just HR, Münzel T. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation* 1996;**94**:6–9.
- Alzahrani T, Pena I, Temesgen N, Glantz SA. Association between electronic cigarette use and myocardial infarction. *Am J Prev Med* 2018;**55**:455–461.
- Crow JP, Beckman JS. Reaction between nitric oxide, superoxide, and peroxynitrite: footprints of peroxynitrite in vivo. *Adv Pharmacol* 1995;**35**:17–43.
- Chalupsky K, Cai H. Endothelial dihydrofolate reductase: critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 2005;**102**:9056–9061.
- Kröller-Schon S, Daiber A, Steven S, Oelze M, Frenis K, Kalinovic S, Heimann A, Schmidt FP, Pinto A, Kvandova M, Vujacic-Mirski K, Filippou K, Dudek M, Bosmann M, Klein M, Bopp T, Lahad O, Wild PS, Frauenknecht K, Methner A, Schmidt ER, Rapp S, Mollnau H, Münzel T. Crucial role for Nox2 and sleep deprivation in aircraft noise-induced vascular and cerebral oxidative stress, inflammation, and gene regulation. *Eur Heart J* 2018;**39**:3528–3539.
- Violi F, Sanguigni V, Carnevale R, Plebani A, Rossi P, Finocchi A, Pignata C, De Mattia D, Martire B, Pietrogrande MC, Martino S, Gambineri E, Soresina AR, Pignatelli P, Martino F, Basili S, Loffredo L. Hereditary deficiency of gp91(phox) is associated with enhanced arterial dilatation: results of a multicenter study. *Circulation* 2009;**120**:1616–1622.
- Daiber A, Di Lisa F, Oelze M, Kröller-Schon S, Steven S, Schulz E, Münzel T. Crosstalk of mitochondria with NADPH oxidase via reactive oxygen and

- nitrogen species signalling and its role for vascular function. *Br J Pharmacol* 2017; **174**:1670–1689.
31. Kahler J, Mendel S, Weckmuller J, Orzechowski HD, Mittmann C, Koster R, Paul M, Meinertz T, Munzel T. Oxidative stress increases synthesis of big endothelin-1 by activation of the endothelin-1 promoter. *J Mol Cell Cardiol* 2000; **32**:1429–1437.
  32. Munzel T, Daiber A, Steven S, Tran LP, Ullmann E, Kossmann S, Schmidt FP, Oelze M, Xia N, Li H, Pinto A, Wild P, Pies K, Schmidt ER, Rapp S, Kröller-Schön S. Effects of noise on vascular function, oxidative stress, and inflammation: mechanistic insight from studies in mice. *Eur Heart J* 2017; **38**:2838–2849.
  33. Luo J, Hill BG, Gu Y, Cai J, Srivastava S, Bhatnagar A, Prabhu SD. Mechanisms of acrolein-induced myocardial dysfunction: implications for environmental and endogenous aldehyde exposure. *Am J Physiol Heart Circ Physiol* 2007; **293**:H3673–H3684.
  34. Li SY, Li Q, Shen JJ, Dong F, Sigmon VK, Liu Y, Ren J. Attenuation of acetaldehyde-induced cell injury by overexpression of aldehyde dehydrogenase-2 (ALDH2) transgene in human cardiac myocytes: role of MAP kinase signaling. *J Mol Cell Cardiol* 2006; **40**:283–294.
  35. Takeshita D, Nakajima-Takenaka C, Shimizu J, Hattori H, Nakashima T, Kikuta A, Matsuyoshi H, Takaki M. Effects of formaldehyde on cardiovascular system in situ rat hearts. *Basic Clin Pharmacol Toxicol* 2009; **105**:271–280.
  36. Schweitzer KS, Chen SX, Law S, Van Demark M, Poirier C, Justice MJ, Hubbard WC, Kim ES, Lai X, Wang M, Kranz WD, Carroll CJ, Ray BD, Bittman R, Goodpaster J, Petrache I. Endothelial disruptive proinflammatory effects of nicotine and e-cigarette vapor exposures. *Am J Physiol Lung Cell Mol Physiol* 2015; **309**:L175–L187.
  37. Scheffler S, Dieken H, Krischenowski O, Forster C, Branscheid D, Aufderheide M. Evaluation of E-cigarette liquid vapor and mainstream cigarette smoke after direct exposure of primary human bronchial epithelial cells. *Int J Environ Res Public Health* 2015; **12**:3915–3925.
  38. Skotsimara G, Antonopoulos AS, Oikonomou E, Siasos G, Ioakeimidis N, Tsalamandris S, Charalambous G, Galiatsatos N, Vlachopoulos C, Tousoulis D. Cardiovascular effects of electronic cigarettes: a systematic review and meta-analysis. *Eur J Prev Cardiol* 2019; **26**:1219–1228.
  39. Pisinger C, Godtfredsen N, Bender AM. A conflict of interest is strongly associated with tobacco industry-favourable results, indicating no harm of e-cigarettes. *Prev Med* 2019; **119**:124–131.
  40. Piepoli MF, Hoes AW, Agewall S, Albus C, Brotons C, Catapano AL, Cooney MT, Corra U, Cosyns B, Deaton C, Graham I, Hall MS, Hobbs FDR, Lochen ML, Lollgen H, Marques-Vidal P, Perk J, Prescott E, Redon J, Richter DJ, Sattar N, Smulders Y, Tiberi M, van der Worp HB, van Dis I, Verschuren WMM, Binno S, Group E. 2016 European Guidelines on cardiovascular disease prevention in clinical practice: the Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts) Developed with the special contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR). *Eur Heart J* 2016; **37**:2315–2381.
  41. Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, Himmelfarb CD, Khera A, Lloyd-Jones D, McEvoy JW, Michos ED, Miedema MD, Munoz D, Smith SC Jr, Virani SS, Williams KA Sr, Yeboah J, Ziaeian B. 2019 ACC/AHA guideline on the primary prevention of cardiovascular disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation* 2019; **140**:e596–e646.