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Toxicity of the main electronic cigarette components, propylene glycol, glycerin, and nicotine, in Sprague-Dawley rats in a 90-day OECD inhalation study complemented by molecular endpoints

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ABSTRACT

While the toxicity of the main constituents of electronic cigarette (ECIG) liquids, nicotine, propylene glycol (PG), and vegetable glycerin (VG), has been assessed individually in separate studies, limited data on the inhalation toxicity of them is available when in mixtures. In this 90-day subchronic inhalation study, Sprague-Dawley rats were nose-only exposed to filtered air, nebulized vehicle (saline), or three concentrations of PG/VG mixtures, with and without nicotine. Standard toxicological endpoints were complemented by molecular analyses using transcriptomics, proteomics, and lipidomics. Compared with vehicle exposure, the PG/VG aerosols showed only very limited biological effects with no signs of toxicity. Addition of nicotine to the PG/VG aerosols resulted in effects in line with nicotine effects observed in previous studies, including up-regulation of xenobiotic enzymes (Cyp1a1/Fmo3) in the lung and metabolic effects, such as reduced serum lipid concentrations and expression changes of hepatic metabolic enzymes. No toxicologically relevant effects of PG/VG aerosols (up to 1.520 mg PG/L + 1.890 mg VG/L) were observed, and no adverse effects for PG/VG/nicotine were observed up to 438/544/6.6 mg/kg/day. This study demonstrates how complementary systems toxicology analyses can reveal, even in the absence of observable adverse effects, subtoxic and adaptive responses to pharmacologically active compounds such as nicotine.

1. Introduction

Nicotine, propylene glycol (PG), and vegetable glycerin (VG), together with distilled water and flavors, are the main constituents of the liquids used to generate aerosols by electronic cigarettes (ECIGs) (Brown and Cheng, 2014; Iskandar et al., 2016). While the *in vivo* toxicity of nicotine, PG, and glycerin has been individually assessed in separate studies (Phillips et al., 2015; Renne et al., 1992; Suber et al., 1989; Werley et al., 2011), only limited published toxicology data are available on mixtures of the main compounds administrated via inhalation (e.g., Werley et al., 2016).

ECIGs provide an alternative to cessation for cigarette smokers who are unable or unwilling to quit (Breland et al., 2017). Several studies have reported potential positive health effects of switching from combustible cigarettes to ECIGs. It has been reported, for

Abbreviations: DEG, differentially expressed gene; DEP, differentially expressed protein; ECIGs, electronic cigarettes; FDR, false discovery rate; Nic, Nicotine; PG, Propylene glycol; VG, vegetable glycerin.

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example, that toxicant levels were much lower in ECIG aerosol than in cigarette smoke (Flora et al., 2016; Goniewicz et al., 2014; Marco and Grimalt, 2015; Margham et al., 2016), and toxicant and carcinogen metabolites have been shown to be significantly reduced in the urine of ECIG users versus that of smokers (Hecht et al., 2015). Public Health England estimated that ECIGs are 95% less harmful than combustible cigarettes (McNeill et al., 2015). However, other studies have highlighted potential health hazards associated with the use of ECIGs (e.g., Hiemstra and Bals, 2016). Formaldehyde can be formed from PG when the liquid is overheated (Jensen et al., 2015), ECIG aerosols have been reported to be contaminated with oxidants and copper ions (Lerner et al., 2015a), and to induce oxidative stress and inflammatory responses in human lung epithelial cells (Lerner et al., 2015b). To provide a comprehensive hazard evaluation of these ECIG liquids, a stepwise approach has been proposed (Iskandar et al., 2016), in which the potential toxicity of the aerosolized basic components of the ECIG liquids is assessed first. Subsequently, the toxicity of added flavor components should be evaluated, and finally the aerosols formed are assessed, including potential effects of aerosolization (e.g., by heating) in a particular ECIG design.

The ECIG liquid component PG is an aliphatic alcohol used as a solvent in many pharmaceuticals and flavors, and in vaporizers for delivery of pharmaceuticals by inhalation. It is generally recognized as a safe food additive (C.F.R., 2014). The reported oral lethal dose for 50% of a tested population (LD_{50}) for PG in rats is 20 g/kg body weight (RTECS, 1985). A sub-chronic 90-day nose-only inhalation study in rats exposed to PG at 0.16, 1.0, or 2.2 mg/L of aerosol showed no treatment-related histological changes in the trachea, larynx, or lung. However, a decrease in body weight in female rats exposed to 2.2 mg/L PG, an increase in mucin secretion, and mild hemorrhages from nasal passages were detected (Suber et al., 1989). Werley et al. assessed PG in a 28-day rat inhalation study; the only biologically relevant findings included clinical signs of ocular and nasal irritation, indicated by minor bleeding around the eyes and nose, and minimal laryngeal squamous metaplasia (Werley et al., 2011).

The ECIG liquid component VG is also a food ingredient recognized as safe by the FDA (C.F.R., 2014), and the present human threshold limit value for inhaled glycerin is 10 mg/m^3 (ACGIH, 1989). A sub-chronic 90-day nose-only inhalation study in Sprague-Dawley (SD) rats exposed to 0.03, 0.16 and 0.66 mg/L glycerin revealed no treatment-related toxicity other than minimal metaplasia of the epithelium lining at the base of the epiglottis in rats exposed to 0.66 mg/L glycerin (Renne et al., 1992).

Compared with cigarette smoke, the inhalation toxicity of nicotine-containing aerosols and vapors has been investigated only sparsely (Phillips et al., 2015; Salturk et al., 2015; Waldum et al., 1996; Werley et al., 2014, 2016). In one study, Chowdhury et al. exposed male SD rats to aerosols of nebulized saline or nicotine dissolved in saline twice daily for 15, 30, 45, and 60 min for 21 days. The authors investigated plasma levels of nicotine and histopathology of the pancreas, whereas effects on the respiratory tract (proximal target tissue and site of nicotine absorption) and liver (site of major metabolism of nicotine) were not investigated (Chowdhury et al., 1992). In another study, the acute toxicity (LC_{50} test) and pharmacokinetics of inhaled nicotine aerosol after a single bolus-like exposure of 20-min duration were determined (LC_{50} [20 min] = 2.3 mg nicotine/L aerosol) (Shao et al., 2013). Recently, we evaluated the toxicity of three equimolar concentrations of nicotine and pyruvic acid (nicotine concentrations of 0.018, 0.025, and 0.05 mg/L) in a 28-day rat inhalation study (Phillips et al., 2015). In this study, rats exposed to nicotine-containing aerosols displayed decreased body weight gains (males only) and concentration-dependent increases in liver weight. Blood neutrophil counts increased in rats exposed to nicotine compared with sham; alkaline phosphatase and alanine aminotransferase activities were higher, and cholesterol and glucose concentrations were lower. The only histopathologic finding in non-respiratory tract organs was increased liver vacuolation and glycogen content. Respiratory tract findings following nicotine exposure (but also some phosphate-buffered saline [PBS] aerosol effects) were observed only in the larynx, and were limited to adaptive changes.

To further study the effects of aerosols from combined components of e-liquids, we characterized the toxicity following a subchronic (90-day) inhalation of an aerosol generated from a liquid mixture containing PG and VG, with and without nicotine, on SD rats. PG and VG are used in variable amounts in e-liquids, and the mixtures were selected to be generally representative of marketed ECIG products (e.g., El-Hellani et al., 2016). The nicotine concentration (23 μg/L) was selected as an intermediate concentration that induces effects but would not be too high to mask potential effects of PG/VG (Table 1). The inhalation protocol was performed according to the Organization of Economic Cooperation and Development (OECD) Test Guideline 413 (OECD, 2009b). To complement the endpoints suggested by the OECD, a systems toxicology analysis was conducted with groups of additional female rats that included transcriptomics, proteomics, and lipidomics analyses.

2. Material and methods

2.1. Experimental design

A 90-day repeated-dose OECD TG413 (OECD, 2009b) inhalation study was conducted using male and female rats to determine potential toxicological effects after exposure to three concentrations of PG and VG mixtures, with or without nicotine. The study comprised two control groups: one sham group exposed to filtered conditioned fresh air, and one vehicle group exposed to aerosolized PBS. For the measurement of endpoints listed in the OECD guideline, 10 male and 10 female rats were allocated to each group, the "OECD" groups. To characterize exposure-related effects at the molecular level (transcriptomics, proteomics, and lipidomics), six female rats per group were allocated to eight additional experimental groups, the Systems Toxicology arm (see Table 1), exposed concomitantly with the "OECD" groups. Male rats were not included in these endpoints because of space limitations in the exposure chambers. Therefore, females were selected, as other studies have suggested that females show a stronger response to test aerosols, possibly due to larger aerosol uptake relative to their body weight (e.g., Kogel et al., 2016; Oviedo et al., 2016; Wong et al., 2016).

The study was conducted in compliance with the OECD Principles on Good Laboratory Practice (GLP) (OECD, 2009a), with the exception of bronchoalveolar lavage fluid (BALF) analysis using RodentMAP and the transcriptomics, proteomics, and lipidomics investigations.

2.2. Animals

Six-week-old outbred male and nulliparous, non-pregnant female SD rats [Crl:CD(SD)], bred under specific pathogen-free conditions, were obtained from Charles River Laboratories (breeding area Raleigh R04, NC, USA). The study was performed in American Association for the Accreditation of Laboratory Animal Care-approved and Agri-Food & Veterinary Authority of Singapore-licensed facilities at Philip Morris International Research Laboratories (PMIRL, Singapore). Care and use of animals were in accordance with guidelines set by the National Advisory Committee for Laboratory Animal Research in 2004 (NACLAR, 2004). All protocols

Table 1
Exposure groups and target concentrations in the test atmospheres of 90-day repeated-dose OECD TG413 inhalation toxicity study. For the OECD rats, endpoints suggested in the OECD TG 413 were determined (e.g., body weight, clinical chemistry, organ weight, measurement of inflammatory markers in the bronchoalveolar lavage fluid, histopathological changes). For the rats of the Systems Toxicology arm, transcriptomics, proteomics and lipidomics analyses were performed from selected organs.

Group	Carrier [mg/L]		Nicotine [mg/L]	OECD Arm		Systems Toxicology Arm
	PG	VG		Male	Female	Female
Sham (filtered air)	0	0	0	10	10	6
Vehicle (saline)	0	0	0	10	10	6
Low (PG/VG)	0.174	0.210	0	10	10	6
Med (PG/VG)	0.520	0.630	0	10	10	6
High (PG/VG)	1.520	1.890	0	10	10	6
Nic + Low (PG/VG)	0.174	0.210	0.023	10	10	6
Nic + Med (PG/VG)	0.520	0.630	0.023	10	10	6
Nic + High (PG/VG)	1.520	1.890	0.023	10	10	6

were approved by the Animal Care and Use Committee of PMIRL.

2.3. Aerosol generation and animal exposure

Inhalation was selected as the route of administration in this study. Aerosols were generated as previously described (Phillips et al., 2015) by using commercially available 6-jet Collison nebulizers (BGI, Butler, NJ, USA) that function by applying pressure to force liquid solutions through small apertures, resulting in the production of a fine aerosol. Stock solutions were stored in a cold room (2–10 °C) and used within 2 weeks of preparation. The relative proportions, concentrations, and molarities of the stock solution constituents for the target aerosol specifications are given in Table 2. Stock solutions were equilibrated for up to 30 min in a water bath at 25 °C before nebulization; for the high concentrations, the nebulizer was warmed to 30 °C to generate aerosols of appropriate concentrations and particle sizes (Fig. 1A). The generated aerosols were further diluted to the target concentrations using filtered conditioned air.

The nose-only exposure method using a flow-past chamber (FPC1-132) was used to expose rats to the generated aerosols (Cannon et al., 1983). This ensured daily reproducibility of aerosol uptake, minimizing aerosol deposition on the animal fur (often observed when whole body exposure is used), which could be subsequently taken up by grooming and interfere with the uptake by inhalation. The duration of the exposure was 13 weeks, for 5 days per week, 6 h per day. A time-adaptation phase was included in week 1 when animals were exposed to increasing exposure durations over 7 days (1.5 h for the first and second days, 3 h for the third and fourth days, and 4.5 h for days 5–7). Weekend exposures (7 days per week exposure) were performed prior to scheduled dissection as necessary in order to ensure that all animals were subjected to a minimum of two consecutive exposure days before dissection. The setup used for aerosol generation and exposure is shown in Fig. 1A.

2.4. Analytical characterization of test atmospheres and aerosol uptake

To characterize the test atmosphere and to check the reproducibility of aerosol generation, concentrations of total particulate matter (TPM), nicotine, PG, and VG, temperature, flow rate through the exposure chamber, conductivity (ion concentration of aerosol), relative humidity, and particle size distribution were determined at the animals' breathing zone during exposure (Supplementary Table 1). Nicotine concentrations were determined by capillary gas chromatography after trapping on sulfuric acid-impregnated silica gel (Majeed et al., 2014). TPM was determined by gravimetry after trapping on Cambridge filters. For the determination of PG and VG concentrations in the diluted test atmosphere, samples were collected using a Cambridge filter and analyzed by gas chromatography (GC) with a flame ionization detector (see Supplementary Material and Methods for details).

For the measurement of biomarkers of exposure in blood, blood was collected from all animals, 10 male and 10 female rats per group, from the facial vein under isofluorane anesthesia during study days 39–51 within 6–7 min after the rats were removed from the exposure, to minimize clearance from the plasma. Plasma was isolated, and the nicotine, cotinine, PG, and glycerin levels were determined by Analytisch-biologisches Forschungslabor GmbH (Munich, Germany) using LC-MS/MS.

2.5. Biological parameters

Biological parameters were determined as described previously (Phillips et al., 2015). In brief, respiratory physiology was measured once in the study using head-out plethysmography (EMKA Technologies, Paris, France) from each rat, 10 male and 10 female rats

Table 2
Relative proportions, concentrations, and molarities of the stock solution constituents for the target aerosol specifications.

Stock solutions	Relative proportions of stock solution constituents (% V/V)	Concentrations of the stock solution constituents (g/L)	Molarities of stock solution constituents (M)	Target concentrations of aerosol constituents (mg/L)	Estimated doses of constituents (mg/kg)[a]
	Nic/PG/VG	Nic/PG/VG	Nic/PG/VG	Nic/PG/VG	Nic/PG/VG
Low (PG/VG)	0.00/3.78/3.56	0.00/39.14/44.41	0.00/0.50/0.50	0.000/0.174/0.210	0/50/60
Med (PG/VG)	0.00/11.33/10.67	0.00/117.41/133.23	0.00/1.50/1.50	0.000/0.520/0.630	0/150/181
High (PG/VG)	0.00/34.00/32.00	0.00/352.24/399.68	0.00/4.60/4.30	0.000/1.520/1.890	0/438/544
Nic + Low (PG/VG)	0.50/3.78/3.56	5.05/39.14/44.41	0.03/0.50/0.50	0.023/0.174/0.210	6.6/50/60
Nic + Med (PG/VG)	0.50/11.33/10.67	5.05/117.41/133.23	0.03/1.50/1.50	0.023/0.520/0.630	6.6/150/181
Nic + High (PG/VG)	0.50/34.00/32.00	5.05/352.24/399.68	0.03/4.60/4.30	0.023/1.520/1.890	6.6/438/544

[a] Doses of aerosol constituents were calculated according to the adapted formula (Alexander et al., 2008): $D = (C \times RMV \times d)/BW$), where D is the dose (mg/kg), C is the concentration of constituent in aerosol (mg/L), RMV is the respiratory minute volume (for rats, 0.2 L/min was used), d is the duration of exposure (min), and BW is body weight (kg) (for rats, 0.25 kg was used). PG, propylene glycol; VG, vegetable glycerin; Nic, nicotine.

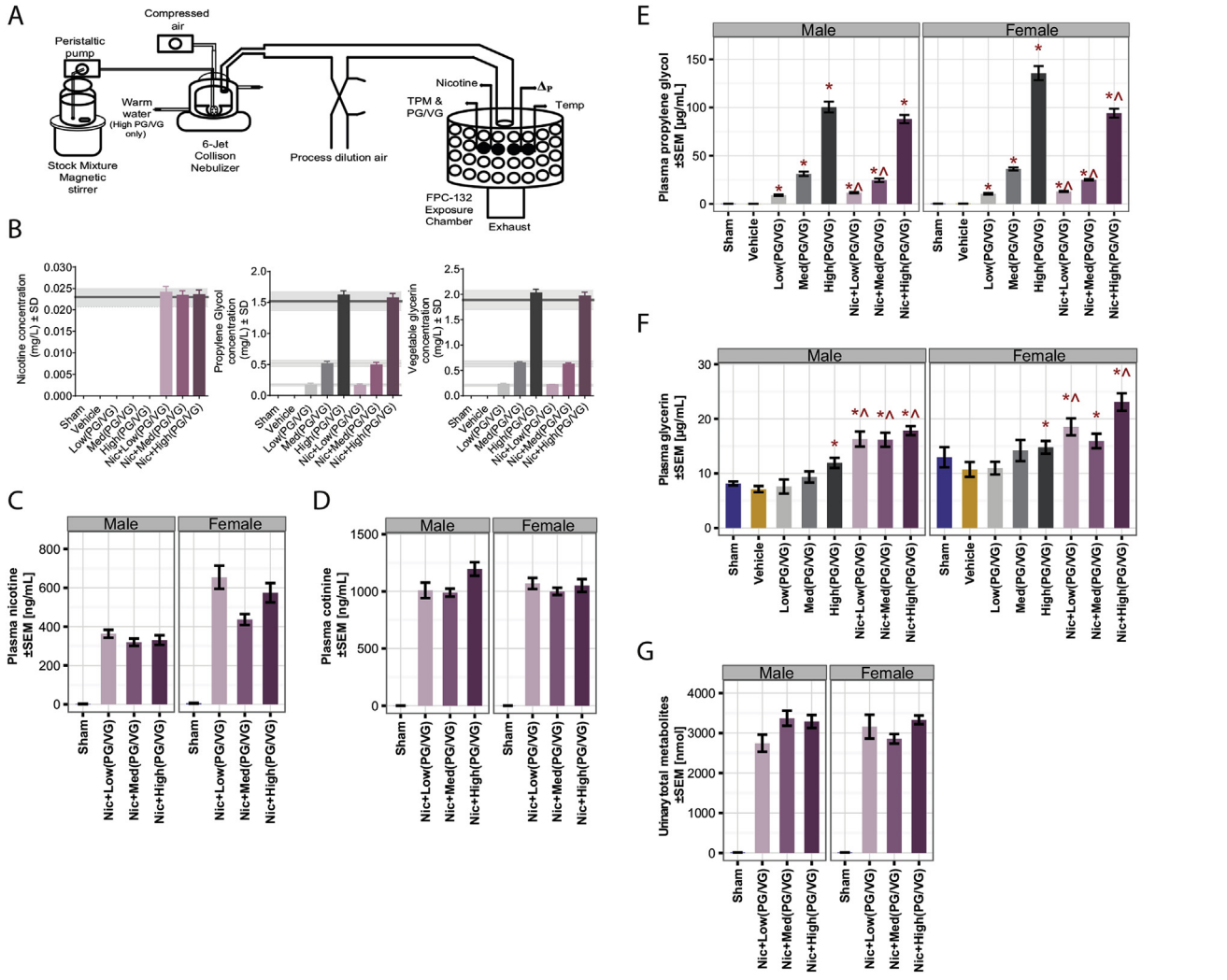

Fig. 1. Exposure setup and characterization. (A) Aerosol generation and exposure setup. For the high concentrations of (PG/VG) ± nicotine, aerosol generation required the nebulizer to be warmed with warm water (at 30 °C). Black circles indicate the positions of the sampling ports. ΔP, change in pressure; TPM, total particulate matter; FPC1-132, flow-past chamber (1 stack of 32 ports). **(B)** Mean concentrations of nicotine, PG, and VG in the aerosols over the 90-days exposure period (mean ± SD). Target concentrations are indicated by the midlines in the gray areas, which represent ±10% of the target concentrations. **(C–F)** Uptake of aerosol constituents in plasma. Mean concentrations of nicotine **(C)**, cotinine **(D)**, PG **(E)**, and glycerin **(F)** from male (left panels) and female (right panels) rats ± SEM (N = 10). Statistically significant differences from the vehicle group are represented by asterisks (*); statistically significant differences between groups with and without nicotine (at the same PG/VG concentration) are represented by carets (^) (p-value < 0.05). **(G)** Total nicotine metabolites in 24-h urine. Mean concentrations ± SEM of absolute (total) nicotine metabolites in urine samples from male (left panel) and female (right panel) rats. PG, propylene glycol; VG, vegetable glycerin; Nic, nicotine. Note that nicotine uptake and its metabolism was only assessed for the sham group (negative control) and the three nicotine-containing aerosol groups, as only for these nicotine uptake could be expected based on the aerosol measurements (Fig. 1B).

per group, for evaluation of breathing frequency, minute volume, tidal volume, and peak inspiratory flow.

All rats in the OECD study were euthanized after 13 weeks of exposure (approximately 16–24 h after the final exposure without diet deprivation) according to OECD TG 413. For hematology analysis, blood samples were collected from the retro-orbital venous plexus at dissection time under pentobarbital anesthesia and analyzed using a Sysmex blood analyzer (Sysmex, Kobe, Japan). For clinical blood chemistry analysis, serum was collected from the abdominal aorta during necropsy, and evaluation was performed using a UniCel® DxC 600i clinical analyzer system (Beckman Coulter, Brea, CA, USA). The weights of the OECD TG 413-specified organs were measured during dissection as absolute values, and the relative weights were then calculated according to body weights after exsanguination.

BALF was collected as described previously (Kogel et al., 2014) and the cellular content was analyzed by flow cytometry. For this assay, the right lung was cannulated and instilled with lavage medium for collection of free lung cells from the first-to-fifth cycle of bronchoalveolar lavage from the right lung of all animals (cycle 1, PBS; cycles 2–5, PBS + 0.325% bovine serum albumin). Cells were collected and analyzed by flow cytometry for viability and differential leukocyte count, as described previously (Kogel et al., 2014; Phillips et al., 2015). Cell-free BALF from cycle 1 (remaining after centrifugation of cells for BALF) was frozen, and submitted to Myriad Rules-Based Medicine (Austin, TX, USA) for exploratory (non-GLP) analyses of a panel of 60 selected proteins (RodentMAP v. 3.0; Life Technologies, Carlsbad, CA, USA) (Phillips et al., 2015).

2.6. Histopathology evaluation

Respiratory tract organs (nose, larynx, trachea, left lung) were fixed in ethanol glycerol acetic acid formaldehyde solution (EGAFS). Histological sections of respiratory tract organs were prepared at

defined levels and stained with hematoxylin and eosin (H&E). Histopathological evaluations of respiratory tract organs were performed on digitalized histological slides. Four nasal cavity levels, including the nasopharyngeal duct, four larynx levels, trachea (transversal and longitudinal section at the bifurcation, including the carina), and three lung sections were evaluated by Dr. Klaus Weber (AnaPath GmbH, Liestal, Switzerland).

Non-respiratory tract organs were collected according to OECD 413 guidelines and fixed in 4% formaldehyde solution except for the sternum and testes, which were fixed in Schaffer's solution and Bouin solution, respectively. The slides of non-respiratory tract organs were prepared by LPT (Hamburg, Germany) and evaluated by Dr. Ansgar Buettner (Histovia GmbH, Overath, Germany). For these non-respiratory endpoints, the histopathological evaluation was performed initially only in the high test and reference item groups, and further analysis of the low and medium were performed only if findings were present.

2.7. Tissue preparation for omics endpoints

Dissection of the animals of the Systems Toxicology arm was also performed 16–24 h after the last exposure. Prior to organ removal, a whole-body perfusion with cold saline was performed. Respiratory nasal epithelium (RNE) for transcriptomics/proteomics analysis was isolated from the left/right side of the nose, snap frozen in liquid nitrogen, and stored at −80 °C. For transcriptomics and proteomics, the left lung lobe was frozen on dry ice and stored at −80 °C. The lung lobe was cryosectioned into 40-μm slices, and the slices were collected alternately for transcriptomics and proteomics analysis. Liver dissections were collected for transcriptomics, proteomics, and lipidomics. The liver sections for transcriptomics were placed in MagNa Lyser tubes (Roche, Basel, Switzerland), and all sections were snap-frozen in N_2(l) and stored at −80 °C. For lipidomics, blood was collected into an EDTA tube, and the plasma was then isolated by centrifugation.

2.8. Transcriptomics analysis

See Supplementary Material and Methods for details. Briefly, RNA from respiratory nasal epithelium (RNE), left lung and liver samples was isolated, processed in randomized order, and hybridized on GeneChip® Rat Genome 230 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Raw data files were processed in the custom Chip Description File environment (Dai et al., 2005) and normalized using frozen robust microarray analysis (fRMA) (McCall et al., 2010). Quality controls were performed with the affyPLM package (Bioconductor suite) (Bolstad et al., 2013). Raw *p*-values were generated for contrasts between vehicle and exposed groups with the *limma* package (Smyth, 2004), and adjusted using the Benjamini-Hochberg false discovery rate (FDR) multiple test correction (Gentleman et al., 2004). In addition, the overall nicotine effect was statistically assessed as the contrast between the average Nicotine + (PG/VG) vs. vehicle and the average (PG/VG) vs. vehicle effect.

2.9. Proteomics analysis

Proteome alterations were assessed by isobaric tag-based quantification using the iTRAQ® approach (Titz et al., 2015a). See Supplementary Material and Methods for details. Briefly, frozen right RNE samples, lung tissue slices, and liver tissue samples were homogenized and processed in randomized order for iTRAQ 8-plex labeling according to the manufacturer's instructions (AB Sciex, Framingham, MA, USA). Mapping of samples to the replicate iTRAQ sets and reporter ion channels was randomized. All labeled samples that belonged to one iTRAQ replicate set were pooled, processed, and analyzed in randomized order using an Easy nanoLC 1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) connected online to a Q Exactive mass-analyzer (Thermo Fisher Scientific). The raw mass spectrometry data were processed using Proteome Discoverer (Thermo Fisher Scientific) and custom processing scripts in the R statistical environment (R Development Core Team, 2007). To detect differentially abundant proteins, a linear model was fitted for each exposure condition and the vehicle group, and *p*-values were calculated from moderated *t*-statistics with the empirical Bayes approach (Gentleman et al., 2004). In addition, the overall nicotine effect was assessed statistically as the contrast between the average Nicotine + (PG/VG) vs. vehicle and the average (PG/VG) vs. vehicle effect. The FDR method was then used to correct for multiple testing effects. Proteins with an adjusted p-value < 0.05 were considered differentially abundant.

2.10. Lipidomics analysis

Lipids were extracted from lung tissue, liver tissue, and plasma and quantified by Zora Biosciences (Espoo, Finland) according to internal Standard Operating Procedures, Lab Method Sheets, and Data Processing Method Sheets (Ansari et al., 2016). Lipid levels were normalized to their respective internal standards and tissue weight or plasma volume.

Statistical analysis of the lipidomics data was similar to the analyses of the transcriptomics and proteomics data. Samples with a total summed lipid concentration below the first quartile −1.5 × the interquartile range or above the third quartile +1.5 × the interquartile range were excluded. For the detection of differentially abundant lipids, only lipid species that were present/quantified in at least 50% of the samples of each compared group were included. A linear model was fitted for each exposure condition and the corresponding vehicle group (and for Nic + High (PG/VG) vs. High (PG/VG)), and *p*-values from a moderated *t*-statistic were calculated with the empirical Bayes approach (Gentleman et al., 2004). The FDR method was used to correct for multiple testing effects. Lipids with an adjusted *p*-value < 0.05 were considered differentially abundant.

2.11. Statistical evaluations and additional data analysis methods

To evaluate group differences in biological and histopathological endpoints, pairwise differences between groups were estimated separately for each sex. Each exposure group was compared with the vehicle group of the same sex and, in addition, the PG/VG groups with and without nicotine were compared for each concentration. Continuous variables were compared using a *t*-test (accounting for variance heterogeneity) or, depending on the non-normality of the data (assessed by a Shapiro-Wilk test at 5% applied on the standardized residuals of both groups being compared), by the mean of a non-parametric Mann-Whitney-Wilcoxon rank-sum test. For incidences, this was done using Fisher's exact test. For ordinal variables, this was done using the Mann-Whitney-Wilcoxon rank-sum test. These statistical analyses were performed using the SAS software package (SAS Institute, Cary, NC, USA).

Gene sets were obtained from the Molecular Signatures Database (mSigDB) (Liberzon et al., 2011). Set analysis was supported by the Piano package in the R statistical environment (Varemo et al., 2013). Set enrichment was assessed by gene-set analysis with the (absolute) log2 fold-change as the gene/protein statistic and the mean as the set statistic. Gene/protein permutation and sample permutation were used to assess statistical significance. *p*-value adjustment was done with the Benjamini-Hochberg procedure.

Functional associations among proteins were obtained from the STRING database (Szklarczyk et al., 2015). Functional clusters were annotated manually, guided by overrepresentation analysis (Varemo et al., 2013). Immune-cell markers were taken from the literature (Kogel et al., 2016).

2.12. Data availability

The mass spectrometry proteomics data are available from the ProteomeXchange Consortium via the PRIDE partner repository (http://www.ebi.ac.uk/pride/archive/) (Vizcaino et al., 2014) (accession numbers: PXD005958, PXD005959, *and PXD006894*). The lipidomics data are available in Supplementary Table 2. The transcriptomics data have been submitted to ArrayExpress (www.ebi.ac.uk/arrayexpress) (accession numbers: E-MTAB-5544, E-MTAB-5545, and E-MTAB-5910). The histopathology data are available in Supplementary Table 3. The data for the other endpoints are available in Supplementary Table 4.

3. Results

3.1. Exposure and aerosol characterization

The aerosols were generated using commercially available 6-jet Collison nebulizers (BGI), diluted to the target concentrations using filtered conditioned air (Table 1), and conveyed to the nose-only exposure chamber (Fig. 1A) (Phillips et al., 2015). The final concentrations of PG, VG, and nicotine in the aerosols were monitored up to four times per day by sampling and analysis, demonstrating that the overall delivery of the constituents was within the target range (target ± 10%) (Fig. 1B, Supplementary Table 1). Particle size distribution was determined using a spectrophotometric aerodynamic particle sizer (Table 3).

Aerosol uptake was confirmed by determining nicotine and cotinine concentrations in the plasma, and by quantification of nicotine metabolites present in 24-h urine samples (Fig. 1C and D,G). In female rats, a higher nicotine concentration was found in plasma; this is likely explained by the smaller body size but not proportionately lower minute volumes (for the nicotine-containing aerosols) (Supplementary Fig. 1). However, levels of the main nicotine metabolite cotinine (Fig. 1D), and the total amount of the recovered nicotine metabolites in urine (see below), did not differ between male and female rats.

PG uptake was demonstrated by measuring plasma concentration, which showed the same pattern as the concentrations of PG measured in the test atmospheres (Fig. 1E). The glycerin concentrations in the plasma from exposed rats did not correlate well with the VG concentrations measured in the aerosols (Fig. 1F), which is likely due to the presence of a significant quantity of endogenous glycerin in plasma, as well as fast-acting glycerin metabolism (Jin et al., 2014). Of note, for both male and female rats, addition of nicotine to the PG/VG aerosols resulted in higher plasma glycerin concentrations, which might be explained by the effects of nicotine on the global metabolic state of these rats. The total amount of the recovered nicotine metabolites in urine samples collected over 24 h (both during and after the exposure) was similar among all nicotine-exposed groups, indicating a similar uptake by the rats (Fig. 1G). Some of the differences in the amounts of urinary nicotine metabolites in the Nicotine + (PG/VG) groups (Low vs. Medium for males, and Medium vs. High for females) are likely related to the technical challenges of urine collection.

Respiratory physiology parameters were measured to assess potential irritation in the upper respiratory tract caused by the aerosols (Supplementary Fig. 1). A few changes for the PG/VG groups were observed, e.g., a lower peak inspiratory flow and breathing frequency for male rats in the High (PG/VG) group vs. vehicle, but overall no clear pattern for PG/VG exposure was shown. More generally, for female rats, addition of nicotine to PG/VG resulted in increases in the peak inspiratory flow, tidal volume, and minute volume – with the resulting minute volumes for the nicotine-containing aerosols, especially for Nicotine + High (PG/VG), in a comparable range for male and female rats (Supplementary Fig. 1). Consequently, a higher relative nicotine uptake per unit of body weight can be expected for female rats, as was indicated by the determinations of nicotine in plasma (Fig. 1C).

Table 3
Particle size distribution (median mass aerodynamic diameter, MMAD). Mean MMAD values were calculated from 14 determinations with the standard deviation (SD). The geometrical standard deviation (GSD) was calculated and the ranges are given.

	Mean MMAD [μm]	SD [μm]	GSD range
Sham	–	–	–
Vehicle	1.4	0.1	1.6–1.7
Low (PG/VG)	1.7	0.1	1.6–1.8
Med (PG/VG)	2.0	0.1	1.7–1.9
High (PG/VG)	2.0	0.1	1.8–2.2
Nic + Low (PG/VG)	1.8	0.1	1.6–1.9
Nic + Med (PG/VG)	2.0	0.1	1.6–1.9
Nic + High (PG/VG)	1.9	0.2	1.6–2.1

3.2. Body weight and food intake

All animals showed increases in body weight over time throughout the 90-day exposure period (Supplementary Fig. 2A). At the end of the exposure period, male rats displayed a slightly lower body weight in exposure groups containing nicotine (Supplementary Fig. 2B). In contrast, female rats had a significantly higher body weight when exposed to PG/VG containing nicotine. While a decrease in body weight in males was also noted upon exposure to nicotine-containing aerosols in our previous 28-day rat exposure study, the body weights of the female rats did not clearly differ in that study (Phillips et al., 2015).

Unexpectedly, food consumption in both sexes was higher in the exposure groups containing nicotine in the PG/VG aerosol (Supplementary Fig. 2C). Nicotine is reported as the appetite-suppressing component of smoke that causes a reduction in food consumption (Grunberg et al., 1986, 1987; Jo et al., 2002). The higher food consumption noted in this study may be explained by differences in the study design and the aerosol compositions, and possibly the complex effects of nicotine on metabolism and stress response systems (see below). Of note, significant differences in food consumption relative to body weight were not observed in any group compared with the sham group in our previous nicotine/pyruvate 28-day rat inhalation study (Phillips et al., 2015). Further studies could determine whether these results are due to nicotine only, or if other constituents of the aerosols may have an effect on food consumption.

3.3. Effects on the respiratory system: standard toxicology

We found higher absolute lung (with larynx and trachea) weights in female rats exposed to the high concentration of PG/VG plus nicotine compared with the vehicle group (Supplementary Fig. 3). However, in female rats the statistically significant effect in absolute values disappeared after normalization to body weight, whereas a slight increase in the normalized weight, compared with the vehicle group, became significant for the Nicotine + High (PG/VG) group in males. Note that a significant decrease in normalized lung weights was also observed for the sham compared with the

vehicle group in male rats, which suggests slight effects of the saline aerosol (vehicle) inhalation on the lung weights.

Histopathological evaluation of the respiratory tract showed only a low incidence of findings (i.e., a low number of animals with a histological response), with minimal severity for a number of parameters which can be considered as adaptive changes, such as induced by dehydration (Burger et al., 1989) (Fig. 2A–D, Supplementary Table 3). These findings were mainly restricted to the larynx, with mild findings in the nicotine-containing PG/VG groups of basal cell hyperplasia and squamous cell metaplasia. Infiltration of unpigmented macrophages in the lung occurred only at low levels, with slightly but significantly lower levels in female rats exposed to low and medium levels of PG/VG (with and without nicotine) (Fig. 2E).

To analyze the degree of inflammation in the lungs caused by the inhalation of the aerosols, the number of free lung cells (cells in BALF) was determined using flow cytometry; only a slightly higher total cell count was observed in female rats exposed to nicotine-containing aerosols (Fig. 2F). This was further supported by the differential cell count (Supplementary Fig. 4), which showed that the macrophage counts were slightly higher for the female Nic + High (PG/VG) group compared with the vehicle group. No clear exposure-related changes were detected by multi-analyte profiling of a cytokine panel (Supplementary Fig. 5).

3.4. Effects on the respiratory system: systems toxicology

We completed the assessment of the respiratory effects with a systems toxicology analysis, which included gene expression (GEX) and protein expression (PEX) profiling of the nasal epithelium and lung tissue of female rats.

Consistent with the low to absent histopathological findings in the nose, GEX profiling of nasal epithelium did not show any differentially expressed genes in the exposure groups compared with the vehicle control (FDR-adjusted p-value < 0.05) (Supplementary Fig. 6A). PEX profiling only showed seven down-regulated proteins in the High (PG/VG) group compared with vehicle, which, however, were not identified for the corresponding Nicotine + High (PG/VG) group (Supplementary Fig. 6B).

In the lung, compared with the vehicle control, all exposure groups displayed only limited effects on the lung transcriptome and proteome, and no gene/protein was differentially expressed between the vehicle and sham controls under the applied threshold of the FDR-adjusted p-value < 0.05 (Supplementary Fig. 6C/D). Only two genes were differentially expressed in the Low PG/PV group: Alas2 (down) and LOC314492 (up) (immunoglobulin heavy chain variable region); four genes were down-regulated in the nicotine-containing Low PG/PV group: Alas2, Hbb, LOC689064 (beta-globin [*Rattus norvegicus*]), and LOC287167 (Hba-a1 hemoglobin alpha, adult chain 1 [*Rattus norvegicus*]) (Fig. 3A and B). Although the expression of these genes was not significantly altered in other groups, they were quantified with similar high fold-changes. These changes do not appear to be treatment-specific, and may, for example, be explained by slight differences in the lung perfusion (residual blood constituents) between the rats.

The comparisons with the vehicle group did not clearly identify genes/proteins associated with nicotine exposure. However, the shape of the volcano plots –with a tendency toward increased

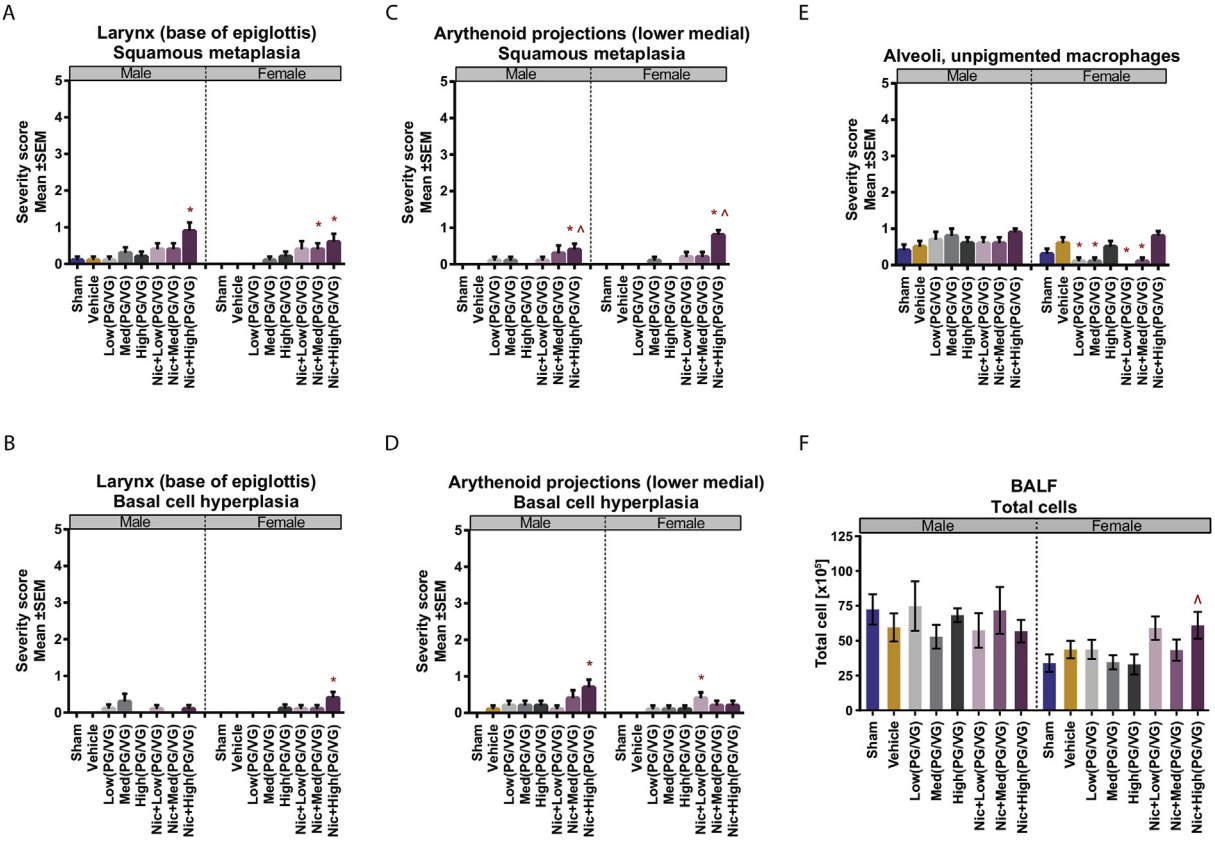

Fig. 2. Effects on respiratory organs. (A–E) Histopathology of the respiratory tract organs (mean scores ± SEM). Plot titles show the evaluated endpoints. Statistically significant differences from the vehicle group are represented by asterisks (*); statistically significant differences between groups with and without nicotine (at the same PG/VG concentration) are represented by carets (^) (p-value < 0.05). **(F)** Mean ± SEM absolute BALF cell counts for male and female rats (N = 10). See Supplementary Fig. 4 for immune-cell type distributions in BALF. PG, propylene glycol; VG, vegetable glycerin; Nic, nicotine; BALF, bronchoalveolar lavage fluid.

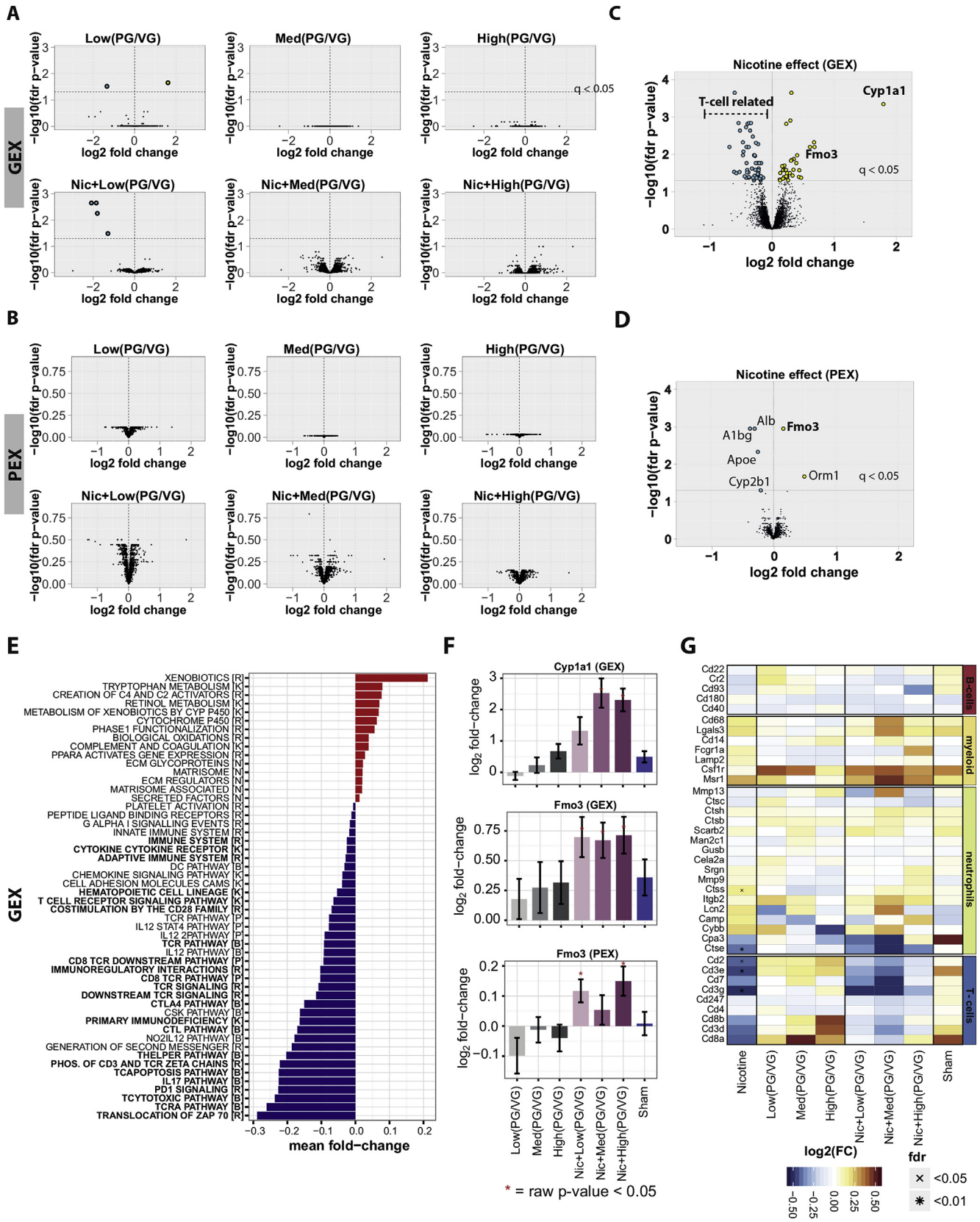

Fig. 3. Evaluation of lung effects by systems toxicology. (A) Lung gene expression response in groups of female rats compared with the vehicle group (N = 6). Volcano plots show the amplitude (log_2 fold-change, x-axis) and significance (−log10 FDR-adjusted *p*-value, y-axis) for each quantified transcript. Transcripts with an FDR-adjusted *p*-value < 0.05 are considered significant and are shown as yellow (up) and cyan (down) dots above the dotted significance threshold line at $-log_{10}$(fdr p-value) = 1.3. **(B)** Lung protein expression response for groups of female rats compared with the vehicle group (N = 6). Volcano plot as in **A**. **(C–D)** Volcano plot showing average nicotine effects for **(C)** gene expression (GEX) and **(D)** protein expression (PEX) data. **(E)** Gene-set analysis (GSA) for GEX data for the average nicotine effect. Set enrichment was assessed by gene-set analysis with the mean of

levels of significance for the nicotine containing PG/VG groups—suggested a low-level effect on the lung transcriptome and proteome from exposure to PG/VG with added nicotine. Thus, to more clearly detect these slight effects of nicotine addition, differentially expressed genes/proteins were identified for the average nicotine effect by contrasting the three nicotine + PG/VG groups vs. vehicle with the three PG/VG (without nicotine) groups vs. vehicle (Fig. 3C and D). This analysis found 97 genes and 6 proteins that were, more generally, significantly associated with the added nicotine in the aerosol (FDR-adjusted p-value < 0.05).

Gene-set but not protein-set analysis identified several bio sets possibly affected by nicotine addition to the aerosol (Fig. 3E): Several up-regulated gene sets reflected changes in the metabolism of xenobiotics (e.g., "Xenobiotics" and "Metabolism of xenobiotics by P450"), and the overwhelming majority of down-regulated gene sets were T-cell-related (e.g., "TCR-a(ctivation) pathway" and "T-helper pathway").

Xenobiotic metabolism enzymes up-regulated by added nicotine included Cyp1a1 and Fmo3 (Fig. 3F). Cyp1a1, which was only quantified by transcriptomics, was previously identified as a nicotine-responsive gene in a 28-day rat inhalation study (Phillips et al., 2015). Flavin-containing monooxygenase 3 (Fmo3) can catalyze the oxidation of nicotine to nicotine N′-oxide (Hukkanen et al., 2005).

To follow up on the observed down-regulation of T-cell-related gene sets, we also evaluated the expression of immune-cell marker genes in the lung tissue (Fig. 3G). Of the cell types evaluated (neutrophils, myeloid cells, B-cells, and T-cells), T-cell markers showed the clearest down-regulation in the nicotine-containing exposed groups, with significant down-regulation of the T-cell receptor components Cd3e and Cd3g for the average nicotine effect (FDR-adjusted p-value < 0.05).

In summary, while PG/VG exposure displayed very limited effects on the lung transcriptome and proteome, a few molecular effects of exposure to the nicotine-containing PG/VG aerosols could be identified; these included the up-regulation of the xenobiotic metabolism enzymes Cyp1a1 and Fmo3 and the down-regulation of T-cell-related transcripts.

3.5. Effects on hematology and blood clinical chemistry

Except for a slightly higher mean corpuscular hemoglobin concentration (MCHC) in the blood of male rats exposed to the highest concentration of PG/VG plus nicotine compared with PG/VG exposure without nicotine, the red blood cell evaluation of male rats revealed no changes in response to exposure compared with the vehicle-exposed group (Supplementary Fig. 7). Female rats had a mild but statistically significant reduction in total erythrocyte counts and a lower mean corpuscular hemoglobin concentration, but a higher mean corpuscular volume after exposure to the highest PG/VG concentration containing nicotine (Supplementary Fig. 7). Similarly, in a 28-day inhalation toxicity study on nicotine and pyruvic acid, no change in red blood cell parameters was detected when male animals were exposed to three concentrations of nicotine compared with a sham group, but female rats displayed higher mean corpuscular volumes (Phillips et al., 2015). Of note, such changes in these red blood cell parameters can occur secondary to acute stress effects (see below) (Everds et al., 2013).

The analysis of other blood chemistry parameters revealed lower total protein concentrations (female rats only), lower total cholesterol, and lower glucose concentrations in rats exposed to all PG/VG concentrations containing nicotine when compared with the vehicle-exposed groups or to the respective PG/VG-exposed groups without nicotine (Fig. 4 A–B, Supplementary Fig. 7D). In female rats, triglyceride levels also tended to be lower in the nicotine-containing PG/VG versus the PG/VG only groups, but significance was only reached for Nicotine + High (PG/VG) versus High (PG/VG), and triglyceride levels displayed variability between the groups in male rats, with significantly increased levels in the vehicle versus sham group (Fig. 4 C). Other changes found only in the female rats exposed to PG/VG plus nicotine included lower creatinine and calcium concentrations (Supplementary Fig. 7). The effects of nicotine on cholesterol and glucose were similar to the findings from a previous study (Phillips et al., 2015), which, in both sexes, also showed lower cholesterol and glucose concentrations upon exposure to nicotine-containing aerosols compared with sham. However, lower calcium concentrations were not observed in the nicotine-exposed female groups within the shorter 28-day time frame of that study.

In line with the global down-regulation of clinical parameters in the PG/VG nicotine-exposed groups, many lipid classes were down-regulated in the plasma of female rats (Fig. 4 D).

3.6. Effects in the liver: standard toxicology

We found higher relative and absolute liver weights in male and female rats exposed to all concentrations of PG/VG with nicotine compared with the vehicle-exposed group (Fig. 5A, Supplementary Table 4). Similar results (higher absolute liver weights in females) were obtained in a previously reported 28-day rat inhalation study on nicotine and pyruvic acid (Phillips et al., 2015).

The exposure to nicotine resulted in increased activities of the two liver enzymes alanine aminotransferase and alkaline phosphatase in female rats, and a tendency for an increased activity of these enzymes in male rats (Fig. 5B and C). However, note that alanine aminotransferase activity also displayed test-item independent changes, with significant differences between the sham and vehicle groups, suggesting that the small relative changes between the groups are to be interpreted with caution. Moreover, ALP is an indicator of cholestasis in rats and humans which, in this study, could be secondary to the diffuse swelling of hepatocytes, causing compression of bile canaliculi and ducts.

A more severe occurrence of hepatocyte vacuolation (maximum severity 1.7 out of 5) was generally observed in liver sections from female and male rats in the nicotine-containing PG/VG groups compared with the vehicle-exposed group (Fig. 5 D–F). To determine the glycogen content of hepatocytes in the liver sections, periodic acid-Schiff (PAS) staining after diastase digestion was performed (Supplementary Fig. 8). With the potential caveat that glycogen staining can be sensitive to the fixation method (Horobin, 2014; Zakout et al., 2010), the results suggested that glycogen storage was not the main reason for cytoplasmic vacuolation, as after diastase digestion only a partial reduction in the intensity of the PAS staining was observed. Moreover, the distribution of the PAS staining was centro-lobular, and not pan-lobular as it would be with cytoplasmic vacuolation. In line with our findings, in a

the absolute log2 fold-change of the average nicotine effect as the gene-set statistic (from mSigDB c2.cp collection). Gene permutation (Q1) and sample permutation (Q2) were used to assess statistical significance. Gene sets with an adjusted p-value < 0.05 for both Q1 and Q2 were considered and represented in a bar chart, with the length of the bars reflecting the mean fold-change of the gene set. **(F)** Gene expression (GEX) and protein expression (PEX) profiles for Cyp1a1 and Fmo3. Differences compared with the vehicle group with a raw p-value < 0.05 are marked (*). Note that the average nicotine effect for these changes is significant (FDR-adjusted p-value < 0.05) and that Cyp1a1 was only detected by GEX. **(G)** Gene expression profiles for a panel of immune-cell markers. Fold-changes of the average nicotine effect and the group comparisons with the vehicle group are color-coded, and statistical significance is marked (adjusted p-value, x = < 0.05, * = < 0.01).

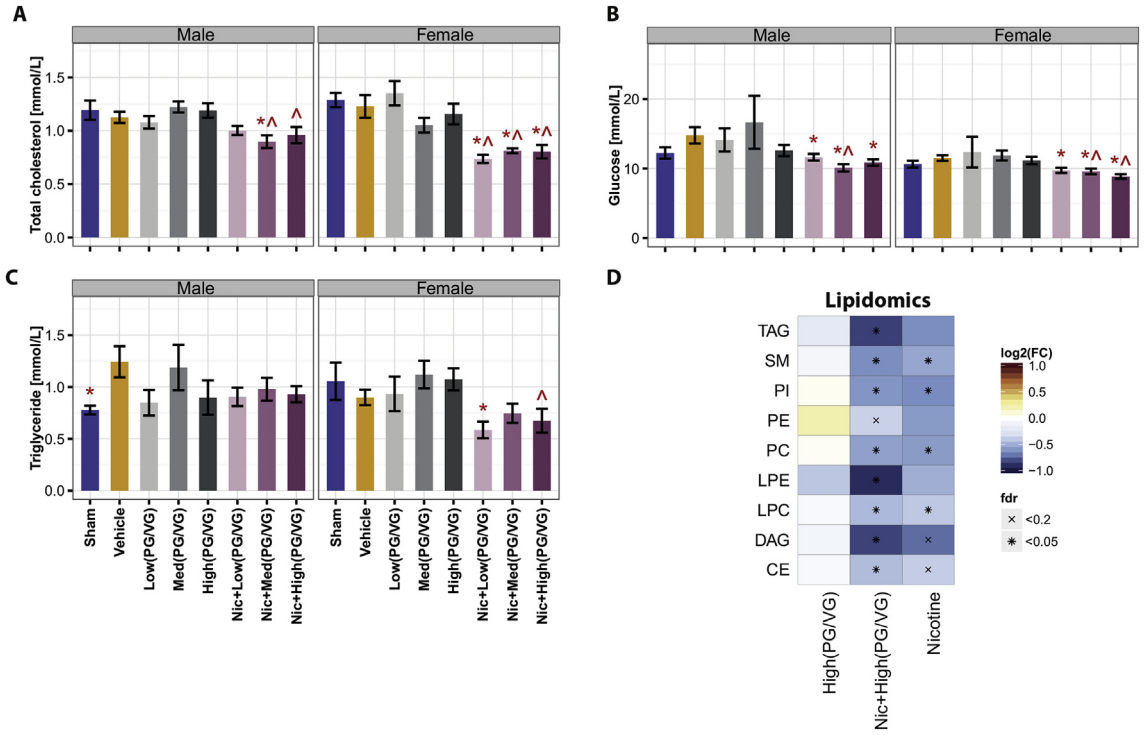

Fig. 4. Blood clinical chemistry and serum lipidomics. (A) Total cholesterol, **(B)** glucose, and **(C)** triglycerides (mean ± SEM). Statistically significant differences from the vehicle group are represented by asterisks (*); statistically significant differences between groups with and without nicotine (at the same PG/VG concentration) are represented by carets (^) (p-value < 0.05). **(D)** Plasma lipid changes measured by lipidomics. Log_2 fold-change of lipid classes compared with vehicle group or for nicotine effect is color-coded, and statistically significant changes are marked. TAG, triacylglycerol; SM, sphingomyelin; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPE; lyso-phosphatidylethanolamine; LPC, lyso-phosphatidylcholine; DAG, diacylglycerol; CE, cholesteryl esters.

previous 28-day rat inhalation study with aerosols of nicotine and pyruvic acid (Phillips et al., 2015), cytoplasmic vacuolation of hepatocytes was observed for most rats of the study with increased levels for rats exposed to nicotine and sodium pyruvate. However, in this study, the increase in vacuolation could be associated with an altered intracytoplasmatic deposition of glycogen.

3.7. *Effects on the liver: systems toxicology*

We leveraged the liver systems toxicology data to characterize the nicotine exposure-related effects in more detail. Gene and protein expression profiling did not show significant effects for the PG/VG exposure groups compared with vehicle exposure; also, no gene or protein was differentially expressed between the sham and vehicle groups (Supplementary Fig. 9). However, a number of genes and proteins demonstrated significant differential expression between the nicotine PG/VG and vehicle exposure groups; there were three, four, and six differentially expressed proteins for the low, medium, and high PG/VG groups with nicotine vs. vehicle groups. Of note, the volcano plots indicated an overall stronger effect for PG/VG groups with nicotine than for the PG/VG groups without nicotine on the group comparison statistics; however, these effects of added nicotine mostly did not reach significance at the 5% FDR level.

To increase the sensitivity of the analysis, the average effect of added nicotine was analyzed across all PG/VG concentrations: 290 genes and 218 proteins were significantly associated with nicotine in the aerosols (Fig. 6A). Gene-set and protein-set analysis revealed several biological bio sets possibly affected by the added nicotine (Fig. 6B/C). Up-regulated categories included cholesterol biosynthesis, xenobiotic metabolism, and lipid metabolism. Down-regulated categories included cytokine- and chemokine-, extracellular matrix-, and nitrogen metabolism-related processes. To complement this analysis, we compared the proteins and genes differentially affected by nicotine in the PG/VG aerosols (Fig. 6D). Of the 218 differentially expressed proteins, 28 (approximately 13%) were also differentially expressed at the gene level. While no biological function was enriched for the 9 shared down-regulated proteins, the 19 shared up-regulated proteins were enriched for biological functions in the metabolism of lipids and xenobiotics. From these gene and protein expression data, nicotine-driven lipid and xenobiotic metabolism emerged as the most clearly affected biological functions.

To further expand this integrative analysis and to evaluate the nicotine addition-related changes in more detail, we applied a joined functional association clustering approach to the proteomics and transcriptomics data (Titz et al., 2015a, 2015b). To do so, we first identified clusters of functionally associated proteins and genes affected by nicotine-containing PG/VG aerosol exposure in the liver; subsequently, identified functional gene and protein clusters that shared a significant number of components were re-clustered and annotated. The main four identified clusters were enriched for “fatty acid metabolism”, “xenobiotic, steroid, retinol, and bile acid metabolism”, “TCA cycle, pyruvate, and gluconeogenesis”, and “cholesterol biosynthesis” functions (Fig. 6E). Thus, the identified clusters were in overall agreement with the gene-set analysis and significant gene/protein overlap results.

Within the “fatty acid metabolism” cluster, the majority of genes/proteins (at least 9 out of 17) were involved in lipid beta-oxidation: Acss2, Eci1, Cpt2, Hadha, Hadhb, Acad11, Acadl, Ech1, and Ehhadh. This included the core enzymes that mediate the lipid oxidation and cleavage cycles; for example, acyl-coA hydrogenases (Acad11 and Acadl) catalyze the initial oxidation step, and Hadha and Hadhb, the components of the mitochondrial “tri-functional

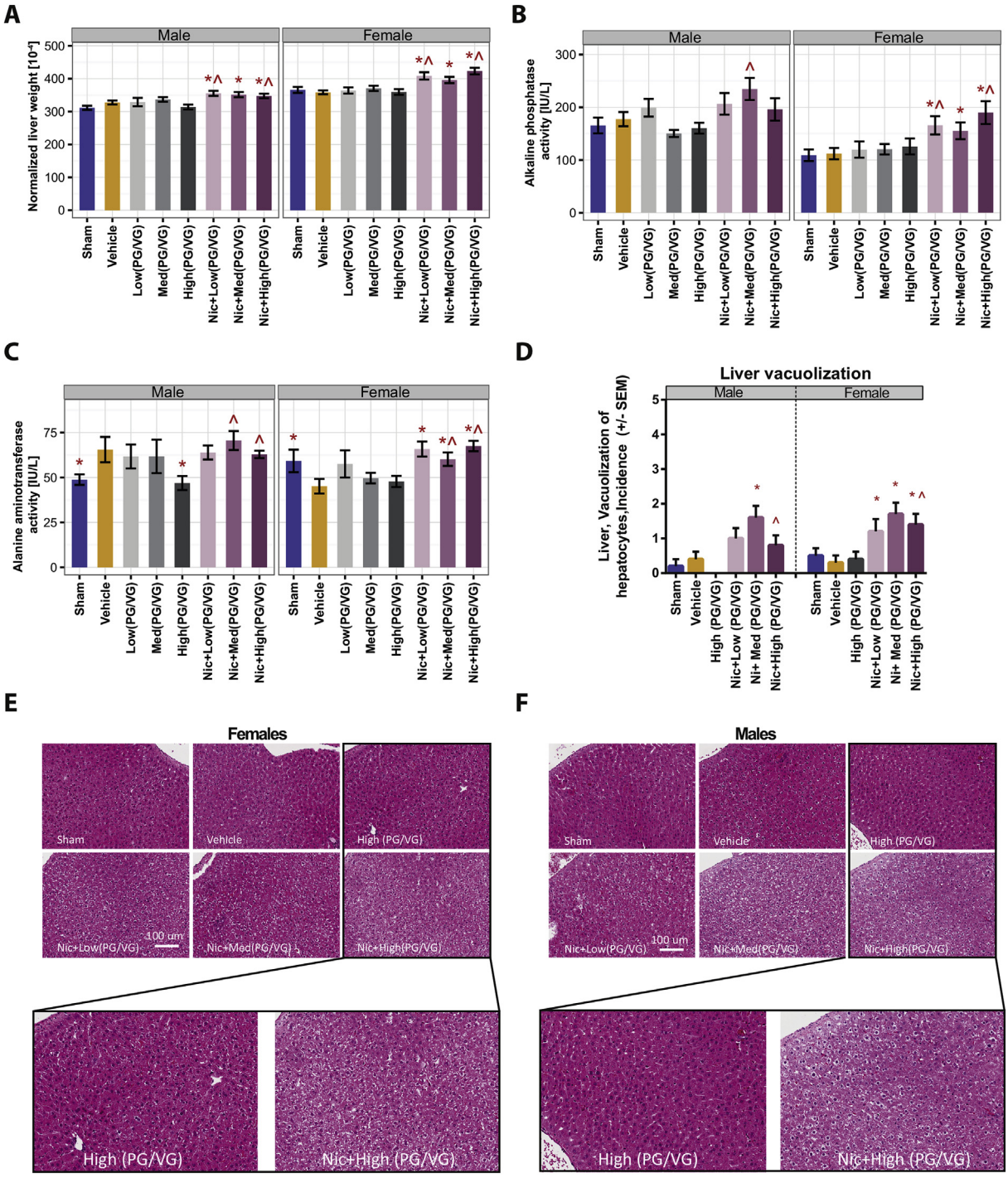

Fig. 5. Effects on the liver. (A) Liver weight normalized to body weight for male and female rats (mean ± SEM, N = 10). Statistically significant differences from the vehicle group are represented by asterisks (*); statistically significant differences between groups with and without nicotine (at the same PG/VG concentration) are represented by carets (^) (p-value < 0.05). **(B–C)** Liver enzyme activities measured in blood (mean ± SEM): **(B)** Alkaline phosphatase and **(C)** alanine aminotransferase. **(D)** Liver vacuolation assessed by histopathology (mean ± SEM). **(E–F)** Microscopy images of hepatocyte vacuolation. Representative examples of H&E staining results are shown for a female **(E)** and a male **(F)** rat. Periodic acid-Schiff (PAS) and PAS-D staining results are shown in Supplementary Fig. 8.

enzyme", catalyze the subsequent hydratase, dehydrogenase, and thiolase steps during the beta-oxidation cycles. This cluster also included two key enzymes involved in ketone body formation: 3-hydroxy-3-methylglutaryl-CoA synthase 2 (Hmgcs2) and type 1 3-hydroxybutyrate dehydrogenase (Bdh1). Ketone bodies include acetoacetate, beta-hydroxybutyrate, and acetone, and are produced from fatty acids in the liver, especially during fasting and extensive prolonged exercise (McGarry and Foster, 1980).

The observed up-regulation of genes/proteins by nicotine exposure in the "TCA cycle, pyruvate, and gluconeogenesis" cluster is likely functionally linked to the shift toward lipid oxidation and ketogenesis (see Discussion). This included protein up-regulation of phosphoenolpyruvate carboxykinase 1 (Pck1), the key enzyme of gluconeogenesis, and serine dehydratase (Sds) and glutamic-

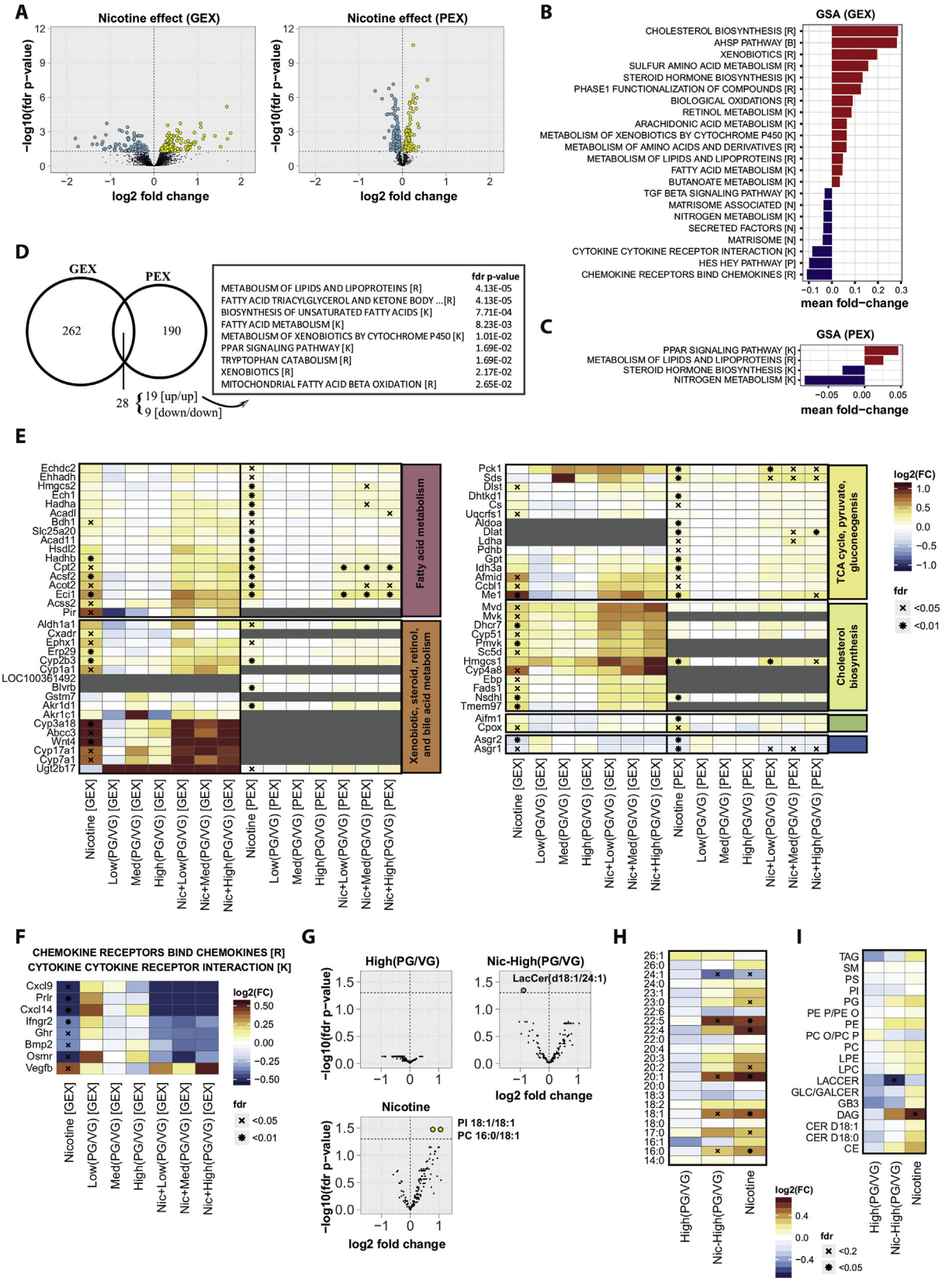

Fig. 6. Evaluation of liver effects using systems toxicology analysis. **(A)** Volcano plot showing average nicotine addition effect for gene expression (GEX, left) and protein expression (PEX, right) data for the liver. **(B)** Gene-set analysis (GSA) for GEX data for the average nicotine effect. Set enrichment was assessed by gene-set analysis with the mean of the absolute log2 fold-change of the average nicotine effect as the gene set statistic (from mSigDB c2.cp collection). Gene permutation (Q1) and sample permutation (Q2) were used to assess statistical significance. Gene sets with an adjusted p-value < 0.05 for both Q1 and Q2 were considered and represented as a bar chart, with the length of the bars reflecting the mean fold-change of the gene set. **(C)** As in **B**, but for PEX data. **(D)** Venn diagram for the unique and shared genes (GEX) and proteins (PEX) affected by nicotine. Gene-set overrepresentation results for the 19 significantly up-regulated proteins/genes shared between PEX and GEX (FDR-adjusted p-value < 0.05). Note that no gene set was

oxaloacetic transaminase 1 (Got1), enzymes that convert amino acids into substrates for gluconeogenesis (Yang et al., 2011). While the aforementioned up-regulated Hmgcs2 enzyme channels Acetyl-CoA into the ketone-body synthesis pathway, its isoform Hmgcs1 channels this molecular building block into the cholesterol biosynthesis pathway (Hegardt, 1999). Added nicotine increased the expression of Hmgcs1, together with other genes/proteins in the "cholesterol biosynthesis" cluster. Finally, the "xenobiotic, steroid, retinol, and bile acid metabolism" cluster contains both enzymes more closely associated with xenobiotic metabolism processes, e.g. Cyp1a1 and Ephx, and enzymes that contribute to the synthesis of endogenous metabolites such as steroids (e.g., Cyp3a18, Cyp17a1) and bile acids (e.g., Cyp7a1).

The most down-regulated gene sets in the liver transcriptomics data included "chemokine receptors bind chemokines" from the Reactome database and "cytokine-cytokine receptor interaction" from the KEGG database (Fig. 6B), but these were not covered by the analyses focused on overlapping effects between the proteomics and transcriptomics data. Thus, to complement the other analyses, we investigated the expression profiles of these gene sets in more detail (Fig. 6F). These down-regulated genes included the chemokines Cxcl9 and Cxcl14 and the interferon gamma receptor 2 (Ifngr2). Of potential interest in our context, Cxcl14 knockout mice have been reported to demonstrate a metabolic phenotype, and Cxcl14 has been proposed as a regulator of glucose metabolism (Hara and Tanegashima, 2012).

Finally, we evaluated whether these nicotine exposure-related gene/protein adaptations resulted in changed lipid profiles in the liver (Fig. 6G–I). As for the other tissues, for lipidomics, we had only included samples from rats exposed to High PG/VG with and without nicotine (and the control groups). Only one lipid, LacCer(d18:1/24:1), was significantly down-regulated in the High Nic + PG/VG vs. vehicle comparison, and two lipids, PI 18:1/18:1 and PC 16:0/18:1, were significantly up-regulated in the comparison of High PG/PV with nicotine vs. PG/PV without nicotine ("Nicotine") (FDR-adjusted p-value < 0.05) (Fig. 6G). Next, we focused on the differential abundance of conjugated different fatty acids (FA) (aggregated over the different lipid classes) (Fig. 6H): FA 22:5, FA 22:4, FA 20:1, FA 18:1, and FA 16:0 (number of carbons: number of double bonds) were significantly more abundant following exposure to nicotine-containing aerosol (FDR-adjusted p-value < 0.05), but no chain-length- or desaturation-dependent pattern could be identified. In addition, for the lipid classes (Fig. 6I), only diacylglycerols (DAGs) showed a significant increase following exposure to nicotine-containing aerosol, and LacCer decreased significantly in the Nic-High PG/PV group vs. vehicle.

Overall, the hepatic response to nicotine exposure suggested a shift toward lipid oxidation, gluconeogenesis, ketone body formation, and cholesterol biosynthesis. However, the effects on the steady-state lipid levels in the liver were limited and did not reveal a clear pattern.

3.8. Systemic stress responses

Previously, systemic effects in toxicology studies have been linked to generalized stress responses (Everds et al., 2013), including in nicotine exposure studies (Phillips et al., 2015). It has been proposed that some of these changes are linked to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which involves corticosterone, adrenocorticotropic hormone (ACTH), and corticotrophin-releasing hormone (CRH). For example, the systemic response of rats to acute and chronic immobilization stress includes metabolic changes such as a decrease in the triacylglycerol concentration in plasma (Ricart-Jan et al., 2002).

A decrease in thymus weight has been proposed as a sensitive indicator of systemic stress (Everds et al., 2013). In the current study, nicotine exposure, especially for female rats, but also for the Nicotine + Low (PG/VG) male group, resulted in a significant decrease in thymus weight (Supplementary Fig. 10A). In addition, an increase in adrenal gland weight is commonly observed with stress. The female rats in particular, but also the male rats, showed a significant increase in adrenal gland weights in our study. The left adrenal weights normalized to body weight are shown in Supplementary Fig. 10B.

In agreement with a previous nicotine inhalation study (Phillips et al., 2015), these observations suggest a nicotine exposure-related stress response that is especially prominent in female rats – which might be due to the higher plasma nicotine concentrations, and thus higher systemic exposure, achieved under these inhalation conditions in the female rats (see 3.1).

4. Discussion

The inhalation toxicology of PG/VG aerosols with and without nicotine was investigated in a 90-day rat inhalation study according to OECD TG 413 (OECD, 2009b), in combination with additional molecular endpoints to allow for a systems toxicology assessment.

Aerosol generation and delivery. The aerosols were administered via the inhalation route using nose-only exposure to ensure reproducibility of aerosol uptake. Nose-only exposure minimizes aerosol deposition on the animal fur, which could be subsequently taken up by grooming and contribute as an oral uptake or transdermal component to the total nicotine uptake. Aerosols were generated with commercially available 6-jet Collison nebulizers that function by applying pressure to force liquid solutions through small apertures resulting in the production of a fine aerosol. This mode of aerosol generation and exposure has been previously validated as being appropriate for studying nicotine exposure via inhalation (Shao et al., 2013), and was applied to nicotine aerosol generation in our prior 28-day inhalation study where stable aerosols were generated through the study conduct (Phillips et al., 2015). This setup allowed for exact control of the exposure conditions, including the reproducible exposure to three concentrations of the PG/VG (+/− nicotine) mixtures, which was confirmed both by analyzing the constituents in the aerosol and after the uptake biomarkers in the plasma/urine (Fig. 1). In future studies, the ability to control inhalation-route aerosol delivery of mixtures with precision could, for example, support comparative assessments of aerosols containing different flavor mixtures. With its experimental design, our study extends and complements previous toxicity studies for PG and VG, which assessed other modes of exposure (e.g., RTECS, 1985), inhalation of the individual compounds (e.g., Renne et al., 1992; Suber et al., 1989; Werley et al., 2011), or PG/VG in ECIG liquids (e.g., Werley et al., 2016).

significantly enriched for the 9 shared down-regulated proteins/genes. **(E)** Gene expression (GEX) and protein expression (PEX) profiles for functional clusters shared between GEX and PEX data. See methods for details on the identification of the six marked functional clusters. Fold-changes of the average nicotine effect and the group comparisons with the vehicle group are color-coded, and statistical significance is marked (adjusted p-value, x = < 0.05, * = < 0.01). **(F)** Gene expression profiles for the "chemokine receptors bind chemokines [REACTOME]" and "cytokine-cytokine receptor interaction [KEGG]" gene sets. Note that the respective proteins were not quantified, otherwise representation as in **E**. **(G)** Lipidomics response profiles in liver. Volcano plots (as in Fig. 3A) represent the group differences from vehicle exposure and the average nicotine effect on the liver lipidome. **(H)** Effects on concentrations of the different (conjugated) fatty acids with different length and degree of desaturation (carbon chain length: number of double bonds). Heatmap representation as in Fig. 6E. (**I**) Effects on lipid class concentrations as in Fig. 4D.

The PG/VG mixture was assessed at three test atmosphere target concentrations in this study: PG (mg/L)/VG (mg/L): 0.174/0.210 (low), 0.520/0.630 (medium), and 1.520/1.890 (high). These concentrations correspond approximately to a daily delivered dose of 50 (PG)/60 (VG) mg/kg (low), 150/181 (medium), and 438/544 mg/kg (high) (formula adapted from (Alexander et al., 2008): $D = (C \times RMV \times d)/BW$), where D is the dose (mg/kg), C is the concentration of constituent in aerosol (mg/L), RMV is the respiratory minute volume (for rats, 0.2 L/min was used), d is the duration of exposure (min), and BW is body weight (kg) (for rats, 0.25 kg was used). Calculating the human equivalent dose (HED) based on the body surface area by dividing the rat dose by a factor of 6.2 (CDER, 2005) yields a maximum HED of approximately 71 mg/kg for PG and 88 mg/kg for VG, or a total uptake of 4.3 g/day of PG and 5.3 g/day of VG for a 60-kg adult human. The delivered quantities exceed normal daily use of e-liquids: The majority of human users consume less than 4 mL e-liquid per day (Action on Smoking and Health, 2016) with variable ratios of PG and VG (Peace et al., 2016); 4 mL of a 100% (v/v) PG solution correspond to 4.2 g PG, and 4 mL of a 100% (v/v) VG solution correspond to 5.0 g VG.

The added nicotine concentration in the test atmosphere was targeted to be 23 μg/L (corresponding to approximately 6.6 mg/kg/day according to (Alexander et al., 2008)). The daily inhaled dose was close to the maximum tolerated dose (MTD) of 7.5 mg/kg for acute administration and approximately half of the 80% mortality dose (15 mg/kg) determined by single intratracheal instillation (Phillips et al., 2015). Calculating the human equivalent dose (HED) based on the body surface area by dividing the rat dose by a factor of 6.2 (CDER, 2005) yields a HED of 1.1 mg/kg, corresponding to a daily nicotine dose of 66 mg for a 60-kg adult human. As discussed above, the majority of human users consume less than 4 mL e-liquid per day (Action on Smoking and Health, 2016), and the European Tobacco Products Directive (TPD Article 20, 3. B) caps the maximum nicotine concentration in an e-liquid at 20 mg/mL (European Parliament and Council, 2014). Based on this, a maximum of 80 mg inhaled nicotine per day is to be expected, close to (121% of) the calculated HED for a 60-kg adult human. At these nicotine concentrations, a mild effect of nicotine was expected based on our previous study (Phillips et al., 2015).

Respiratory effects. At the three tested concentrations, PG/VG without nicotine did not show any clear effects on the measured endpoints in the respiratory system, including histopathology, lung inflammation, and the molecular responses measured by transcriptomics and proteomics. In a published 90-day rat inhalation study (for 6 h/day, 6 days/week), 1.1 mg/L and 2.2 mg/L PG in the aerosol (317 mg/kg and 634 mg/kg daily delivered dose, according to (Alexander et al., 2008), see above) caused an increase in the numbers of goblet cells or increased mucin production in the nose, and 2.2 mg/L PG (634 mg/kg daily delivered dose) caused nasal hemorrhage, possibly as a dehydration effect (Suber et al., 1989). In another rat inhalation study for PG, bleeding around the nose was observed after an acute (7-day) exposure, and squamous metaplasia in the larynx was observed after 28 days of PG exposure (Werley et al., 2011), which was only observed in the presence of nicotine in our study (see below). However, Werley et al. used a different exposure regimen, and the NOEL for PG in the 28-day study was estimated at 30 mg/L PG for 12 min per day (288 mg/kg daily delivered dose, according to (Alexander et al., 2008), see above). In a 13-week rat inhalation study with glycerol, Renne et al. mainly observed mild squamous metaplasia of the epithelium lining of the epiglottis for 0.662 mg/L glycerol (6 h/day, 5 days/week) (191 mg/kg daily delivered dose, according to (Alexander et al., 2008), see above), which was considered an adaptive response to the mild irritant effects of the aerosol. With this, the lack of effects of the PG/VG aerosol (or mild effects that did not reach significance), up to a daily delivered dose of 438 mg/kg PG and 544 mg/kg VG, is overall in line with the observation of only mild irritation effects in previous studies of the individual compounds.

The addition of nicotine to the PG/VG aerosol resulted in only a low incidence and mild severity of histopathological findings in the upper respiratory tract (Fig. 2). These findings were mainly localized in the larynx, but also included slightly higher counts of free lung cells for female rats exposed to nicotine-containing aerosols (Fig. 2F). Squamous metaplasia of the larynx is among the most sensitive endpoints for inhalation studies, reacts to a wide range of conditions, including dehydration by low-humidity air, and has been identified as a sensitive adaptive rather than toxicologically relevant response (Burger et al., 1989; Osimitz et al., 2007). These mild effects following exposure to the nicotine-containing aerosols should be placed in context by contrasting the findings of this study with those for cigarette smoke (CS) from the 3R4F reference cigarette in two previously published 90-day rat inhalation studies with a comparable exposure regimen (6 h per day, 5 days per week) (Kogel et al., 2016; Oviedo et al., 2016; Wong et al., 2016). In these studies, exposure to mainstream 3R4F smoke at a concentration of 23 μg/L nicotine caused severe squamous cell hyperplasia-metaplasia in the larynx and severe lung inflammation, including an increase in free lung cells (up to >5-fold higher free cell numbers for 3R4F vs. sham exposure) and a dramatic increase in BALF neutrophils, as well as several significantly affected cytokines in the lavage fluid.

Similarly, while CS from 3R4F clearly affected the lung transcriptome in these previous studies (e.g., >1200 significantly differentially regulated transcripts in female rats exposed to smoke from 3R4F (23 μg/L nicotine) vs. Sham (Kogel et al., 2016)), only very few significantly differentially expressed transcripts could be identified in the current study (Fig. 3). However, the aggregated analysis of the nicotine exposure effects across all exposure groups allowed for identification of the low-level effects of nicotine: These included up-regulation of the transcripts for xenobiotic enzymes (Cyp1a1 and Fmo3) and down-regulation of T-cell-associated genes. This is consistent with a previous rat inhalation study, where the up-regulation of Cyp1a1 following nicotine aerosol exposure had been identified as the clearest concentration-dependent effect of nicotine in the lung tissue (Phillips et al., 2015). To our knowledge, Fmo3 up-regulation has not been reported as a marker of nicotine exposure in rat lungs. Fmo3 is known as the enzyme that catalyzes nicotine-N′-oxide formation in the liver (Yildiz, 2004), and has been hypothesized to contribute to the metabolism of nicotine to nicotine-N′-oxide in rat lung microvascular endothelial cells (Ochiai et al., 2006). Thus, Fmo3 induction is likely to be functionally associated with the xenobiotic response of the lung tissue to nicotine exposure. T-cell-associated genes were down-regulated following nicotine exposure in lung tissue (Fig. 3). This is in line with observations in our previous nicotine aerosol exposure study (Phillips et al., 2015) and reproduces a generally observed exposure effect for nicotine-containing aerosols in the lung (e.g., Kogel et al., 2016). This observation could either reflect the down-regulation of T-cell receptor components or a more general decrease in T-cell numbers in the lung following nicotine exposure. Possibly, this finding is linked to the previously noted anti-inflammatory effects of nicotine in the lung immune system, including its suppression of T-cell responses (e.g., Singh et al., 2000; Sopori et al., 1998).

Hematology and clinical chemistry. While hematology and clinical chemistry did not show any significant and consistent effects for PG/VG exposure, the exposure to nicotine-containing aerosols compared with vehicle exposure (and comparing PG/VG aerosols at corresponding concentrations with and without nicotine), especially in female rats, resulted in a significant reduction in

blood glucose and lipid levels, including cholesterol and triacylglycerols (Fig. 4). Similar effects of nicotine-containing aerosols have been observed in previous rat inhalation studies (Oviedo et al., 2016; Phillips et al., 2015; Wong et al., 2016). Unexpectedly, exposure to nicotine-containing PG/VG aerosols also resulted in increased body weight in female rats and in increased food consumption in both male and female rats (Supplementary Fig. 2), but these effects were not observed in Phillips et al. (2015) and, generally, nicotine is more commonly reported to exert a suppressive effect on food consumption and weight gain in the literature (e.g Grunberg et al., 1986).

Liver effects. In line with the other endpoints, the PG/VG aerosols did not show clear and significant effects on liver-related endpoints. However, rats exposed to PG/VG aerosols with added nicotine exhibited several hepatic effects, including increased liver weight, increased liver enzyme activities in plasma, and increased vacuolation of liver cells compared with vehicle exposure (Fig. 5). Similar effects of nicotine on the liver have been reported in a previous nicotine inhalation study (Phillips et al., 2015) and were, more generally, also observed in previous rat inhalation studies for other nicotine-containing aerosols, including CS (Oviedo et al., 2016; Wong et al., 2016). Hepatocyte vacuolization could be considered an adverse effect as it is consistently seen after mild and subacute liver injury. However, several lines of evidence point to the possibility that hepatocyte swelling with nonlipidic vacuolization may reflect a cellular adaptation, rather than a degenerative change. Observations in animal and human livers suggest vacuolated hepatocytes observed during liver injury are cells adaptively altered to resist further insult. In particular, Nayak et al. suggest hepatocyte vacuolization may reflect an adaptive cellular response (Nayak et al., 1996). For example, morphological and biochemical investigations have shown that cytoplasmic vacuolation of hepatocytes following low doses of CCl4 was due to excess accumulation of glycogen. Low dose CCl4 exposed cells lacked features of degeneration or regeneration, and were much less susceptible to injury by larger subsequent CCl4 doses, as assessed by structural and serum enzyme analyses (Nayak et al., 1996). In our previous studies, we have also demonstrated that both severity score and incidence of nicotine-induced cytoplasmic vacuolization in liver cells reverted back to baseline levels after the 42 days recovery period (Wong et al., 2016). Reversibility is an important factor in the holistic interpretation of toxicity studies and given that hepatocyte vacuolization induced by high dose of nicotine has been shown to be readily and completely reversible on cessation of treatment this would indicate a lower level of concern (Lewis et al., 2002).

Transcriptomics analysis of the liver showed that these changes were associated with alterations in the metabolic state of the liver (see below). In addition, PG/VG with nicotine exposure resulted in down-regulation of chemokine and cytokine gene sets in the liver (Fig. 6), including the chemokines Cxcl9 and Cxcl14, and the prolactin (Prlr), Ifngr2, and oncostatin M (Osmr) receptors. Down-regulation of cytokine and interferon signaling gene sets by nicotine exposure has also been observed in (Phillips et al., 2015) and may, together with the aforementioned down-regulation of T-cell markers in the lung, be related to the effect of nicotine on the immune system (Han and Lau, 2014).

General metabolic shift. Exposure to the nicotine-containing aerosols resulted in a general shift of the metabolic state, most prominently in female rats – which might be due to the higher plasma nicotine concentrations, and thus higher systemic exposure, achieved under these inhalation conditions in the female rats (see 3.1). Nicotine-exposed female rats displayed increased body weights that were associated with increased food consumption. At the same time, levels of glucose and a broad range of lipid classes such as triacylglycerols and cholesterol were reduced in blood. Systems toxicology investigations indicated that nicotine exposure also affected metabolic pathways the in liver, including up-regulation of fatty acid beta-oxidation, cholesterol synthesis, gluconeogenesis, and ketone body formation pathways. Similar effects of nicotine exposure were also observed in a previous nicotine/pyruvate inhalation study (Phillips et al., 2015). In that study, the metabolic effects of the nicotine-containing aerosols, which were also most pronounced in female rats, included a reduction in blood glucose, cholesterol, and other plasma lipid levels. Overall consistent with our results, effects on liver metabolism pathways in that study included up-regulation of cholesterol biosynthesis, oxidative phosphorylation, and gluconeogenesis, as well as effects on lipid and steroid metabolism. Similar metabolic effects were also observed in previous CS exposure studies, and have been attributed to a nicotine effect, potentially conjoined with a systemic stress response (Everds et al., 2013; Wong et al., 2016) (see below). Consistent with our results, Golli et al. observed decreased cholesterol plasma levels and increased liver gluconeogenesis enzymes (Pck1) for rats exposed intraperitoneally to e-liquids with nicotine for 28 days (Golli et al., 2016). However, other responses to nicotine exposure were different in that study, including the observed effects on food consumption, body weight gain, and glucose plasma levels. In addition, several non-inhalation nicotine exposure studies found increases in lipid and cholesterol levels in plasma (Adluri et al., 2008; Ashakumary and Vijayammal, 1997; Chattopadhyay and Chattopadhyay, 2008; Valenca et al., 2008). Overall, we can conclude that nicotine exposure affects lipid and glucose metabolism in a complex manner. However, the resulting (and experimentally observed) physiological states are likely dependent on the route, concentration, and duration of exposure, among other factors. Possibly contributing to the differences in the final induced metabolic states are the complex effects of nicotine on the cholinergic nervous system, interlinked with the physiological response to general exposure stress, as discussed in the next section.

Systemic stress. Nicotine exerts complex effects on the stress response of animals (al'Absi et al., 2013; Picciotto et al., 2002). Nicotine has been reported to affect different stress regulation systems, most prominently the hypothalamic–pituitary–adrenal (HPA) axis (Faraday et al., 2005; Matta et al., 1998; Rhodes et al., 2001; Rohleder and Kirschbaum, 2006). Likewise, cold stress or heat stress has been shown to increase the blood glucagon level in rats, concomitant with increases in free fatty acids and glycerin (Kuroshima et al., 1981). While free fatty acids were not measured in the current study, the observed elevated glycerin levels in plasma are consistent with such a metabolic stress response. Moreover, in healthy non-smokers, nicotine infusion was observed to increase serum levels of noradrenaline, adrenaline, glycerol, and free fatty acids, while the plasma levels of glucagon, insulin, glucose, pyruvate, lactate, and cortisol did not significantly change (Andersson et al., 1993). In our study, consistent with an effect of the nicotine-containing aerosols on these stress systems, we observed several clinical responses that have been identified previously as stress response phenotypes (Everds et al., 2013; Selye, 1936), including a decrease in thymus weight and an increase in adrenal gland weight (Supplementary Fig. 10). Notably, complex interactions of nicotine and other sources of stress have been observed (e.g., Chen et al., 2008; Cheng et al., 2005; Faraday et al., 1999), which could have further modulated (induced or suppressed) the stress-related effects in our study, in which the rats were concurrently exposed to the stress of the nose-only exposure procedure and nicotine-containing aerosols. For example, the HPA axis is functionally interlinked with adipose tissue metabolism via leptin (Spinedi and Gaillard, 1998), and glucocorticoids produced

by the adrenal cortex affect metabolism (e.g., increased gluconeogenesis in the liver) and modulate the activity of the immune system (Nicolaides et al., 2015; Rose and Herzig, 2013). Therefore, we cannot exclude the possibility that some of the observed metabolic and immune-related effects of nicotine exposure may be explained by the complex modulation of stress response pathways by nicotine.

Limitations of the study. A general challenge in toxicological assessment studies is to judge the possible implications, especially of observed mild effects—here, for example, the histopathological effects observed for the nicotine-containing aerosols on the larynx and free lung cells. Above, we contrasted the effects of the tested aerosols with the much more pronounced effects observed following exposure to CS in a previous study. However, to further improve comparability of the effect sizes in future studies—e.g., between ECIG aerosols and CS—it would be beneficial to include exposure to CS as a reference. In particular, systems toxicology endpoints would benefit from such a "positive control" for the induced exposure effects. Using a Collison nebulizer for aerosol generation, the current study assessed the effects of the unaltered main ECIG liquid components. However, depending on the ECIG design used for aerosol generation, overheating of the liquid may occur, which can then, for example, result in the formation of aldehydes (Ahmad et al., 2016; Bekki et al., 2014; Farsalinos et al., 2015). Thus, for a given ECIG/e-liquid combination, beyond the assessment of the pure compounds, it will be pertinent to also specifically assess to what extent thermal decomposition products are generated and how they affect the toxicity of the generated aerosol. Another deviation of our nebulizer-generated test aerosol from genuine ECIG aerosols produced by the dedicated electronic nicotine delivery systems is the larger particle size, which may result at least in part from hygroscopic growth due to the dilution, and from differences in the measurement methods (Fuoco et al., 2014; Lerner et al., 2015a; Manigrasso et al., 2015), or the lack of trace metal (such as copper) contamination from heater elements (Lerner et al., 2015a). Thus, the present paper reports the basic inhalation toxicity of the e-liquids as the first part of a multi-stage assessment approach (see introduction). To fully assess the risk of ECIG aerosols, additional studies will be required, which would also include the contributions from the delivery devices.

5. Conclusion

A 90-day rat inhalation study according to OECD TG 413 (OECD, 2009b) for the toxicological assessment of nebulized PG/VG aerosols with and without nicotine was conducted. Standard toxicological endpoints were complemented with systems toxicological analyses using transcriptomics, proteomics, and lipidomics of lung tissue, liver tissue, and serum. Both standard and systems toxicology endpoints demonstrated very limited biological effects of PG/VG aerosol with no signs of toxicity. Systems toxicology analyses detected biological effects of nicotine exposure, which included up-regulation of the xenobiotic-metabolizing enzymes Cyp1a1 and Fmo3 in the lung and metabolic effects, likely interlinked with a generalized stress response to nicotine present in the exposure aerosols.

Altogether, under the conditions of this 90-day SD rat study, several nicotine related responses have been observed but taking in to account the overall weight of evidence no adverse effects were observed for PG/VG/nicotine up to 438/544/6.7 mg/kg/day, since the vast majority of the effects observed are considered to be adaptive changes caused by the nicotine levels and they have been shown to be reversible on cessation of treatment.

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Authors' contributions

BP, BT, UK, DS, SL, DS, ME, SK, EV, MCP, JH, and PV contributed to the conception or design of the work. BP, UK, DS, JH, CN, EG, AE, and NVI contributed to the data collection. BP, BT, UK, DS, PL, YX, DS, CN, WS, FM, and PV contributed to the data analysis and interpretation. BP, BT, UK, and PV drafted the article. All authors critically revised and approved the article.

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Appendix A. Supplementary data

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Transparency document

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