RESEARCH ARTICLE

Toxicity of aerosols of nicotine and pyruvic acid (separate and combined) in Sprague–Dawley rats in a 28-day OECD 412 inhalation study and assessment of systems toxicology

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Abstract
Toxicity of nebulized nicotine (Nic) and nicotine/pyruvic acid mixtures (Nic/Pyr) was characterized in a 28-day Organization for Economic Co-operation and Development 412 inhalation study with additional transcriptomic and lipidomic analyses. Sprague–Dawley rats were nose-only exposed, 6 h/day, 5 days/week to filtered air, saline, nicotine (50 μg/l), sodium pyruvate (NaPyr, 33.9 μg/l) or equimolar Nic/Pyr mixtures (18, 25 and 50 μg nicotine/l). Saline and NaPyr caused no health effects, but rats exposed to nicotine-containing aerosols had decreased body weight gains and concentration-dependent increases in liver weight. Blood neutrophil counts were increased and lymphocyte counts decreased in rats exposed to nicotine; activities of alkaline phosphatase and alanine aminotransferase were increased, and levels of cholesterol and glucose decreased. The only histopathologic finding in non-respiratory tract organs was increased liver vacuolation and glycogen content. Respiratory tract findings upon nicotine exposure (but also some phosphate-buffered saline aerosol effects) were observed only in the larynx and were limited to adaptive changes. Gene expression changes in the lung and liver were very weak. Nic and Nic/Pyr caused few significant changes (including Cyp1a1 gene upregulation). Changes were predominantly related to energy metabolism and fatty acid metabolism but did not indicate an obvious toxicity-related response. Nicotine exposure lowered plasma lipids, including cholesteryl ester (CE) and free cholesterol and, in the liver, phospholipids and sphingolipids. Nic, NaPyr and Nic/Pyr decreased hepatic triacylglycerol and CE. In the lung, Nic and Nic/Pyr increased CE levels. These data suggest that only minor biologic effects related to inhalation of Nic or Nic/Pyr aerosols were observed in this 28-day study.

Keywords
Nicotine, pyruvic acid, sub-chronic inhalation, systems toxicology

Introduction
Cigarette smoking can cause severe, life-threatening diseases. Health consequences are caused primarily by smoke constituents other than nicotine. Therefore, provision of nicotine during nicotine replacement therapies (NRTs) or by using electronic nicotine-delivery systems (e-cigarettes) may be considered a less harmful way to facilitate smoking cessation or to reduce harm by smoking fewer tobacco cigarettes (Benowitz & Goniewicz, 2013; Franck et al., 2014). Nicotine administration via inhalation is an attractive approach for an NRT because it can deliver nicotine rapidly to the brain, thus better mimicking the effects of its discontinuous/episodic exposure achieved if smoking cigarettes.

Aerosols of nicotine pyruvate have been proposed as improved forms of NRT for those who are not willing to stop smoking. Such aerosols have favorable pharmacodynamic properties and subjective responses (Rose et al., 2010). Along with pharmacodynamic parameters and sensory evaluations/subjective responses, limited tolerability testing and safety assessment for acute exposure has been reported by Rose et al. (2010) but the toxicity of repeated or extended use of aerosols of nicotine pyruvate has not been investigated.

Remarkably, the inhalation toxicity of nicotine aerosols and vapors has been investigated only sparsely (Salturk et al., 2015; Waldum et al., 1996; Werley et al., 2014). In addition, Callahan-Lyon (2014) concluded that “Data on short-term health effects are limited and there are no adequate data on long-term effects”. For toxicity testing of cigarette smoke (including the composition, ingredients and varying nicotine delivery levels), numerous repeated-dose (usually 21–35 days) or subchronic (90 days) inhalation studies on rats have been published. In contrast, only two inhalation studies
with nicotine aerosol in rats were identified in a recent PubMed literature search. In one study, Chowdhury and colleagues exposed male Sprague–Dawley (SD) rats in groups of five to aerosols of nebulized saline or nicotine dissolved in saline twice daily for 15, 30, 45 and 60 min for 21 days. Then, they 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). Recently, the acute toxicity (LC\textsubscript{50} test) and pharmacokinetics of inhaled nicotine aerosol after a single bolus-like exposure of 2-min duration were determined (Shao et al., 2013).

Most of the studies on nicotine toxicity have applied non-inhalation routes of exposure. Such routes have usually been subcutaneous, intravenous or intraperitoneal injection, but subcutaneous osmotic minipumps and oral administration have also been used (Shao et al., 2013). Inhaled pyruvic acid, in the form of sodium pyruvate, has been tested for safety and therapeutic efficacy as an anti-inflammatory and anti-oxidant therapy for chronic obstructive pulmonary disease, and was shown to be well tolerated in a clinical study with daily administration for \( \leq 6 \) weeks (Votto et al., 2008). This study addresses the need for more comprehensive data on the toxicity of inhaled aerosols of nicotine and pyruvic acid, alone or in combination.

This is the first report on a comprehensive repeated-dose inhalation study on aerosols of nicotine and nicotine pyruvate that includes respiratory, hepatic and other systemic toxicity endpoints. This 28-day inhalation study following Organization for Economic Co-operation and Development (OECD) Testing Guideline 412 (OECD TG 412) (OECD, 2009b) for repeated-dose toxicity testing was designed to fully characterize the toxicity of a test article by an inhalation route (OECD, 2009b).

Taking into account the concepts of systems toxicology in the risk assessment of products (Hoeng et al., 2013; Krewski et al., 2009; Stephens et al., 2012; Sturla et al., 2014), the objectives of this study were to determine and characterize the toxicity of inhaled aerosols containing nicotine and pyruvic acid, alone or in combination, in an OECD TG 412 study that included additional molecular endpoints (transcriptomics and lipidomics in satellite treatment groups of male rats) to obtain additional insights in the molecular mechanisms underlying toxicity. A similar ‘OECD plus’ (OECD+) approach comparing conventional mainstream cigarette smoke with an aerosol from a tobacco-heating prototypic modified risk tobacco product (pMRTP) has been conducted recently in a 28-day rat inhalation study. Classical toxicology endpoints as required by OECD TG 412 were combined with gene-expression (array-based transcriptomics) and computational modeling (Kogel et al., 2014). This approach has been demonstrated to be suitable to characterize the molecular mechanisms underlying the observed adaptive and inflammatory exposure effects of cigarette smoke on the respiratory tract, and deemed sufficiently sensitive to detect subtle effects of pMRTP exposure that were not measurable by the classical toxicity endpoints according to the OECD guideline.

Here, we extend high-density molecular analysis by lipidomics to capture the anticipated effects of inhaled nicotine on lipid metabolism in the liver. Such effects have been observed for non-inhalation nicotine administrations: in 1990, Winders and Grunberg (1990) speculated that nicotine has a selective effect on body fat. Infusion of nicotine has been shown to increase basal lipolysis in rats by 78% (Sztalryd et al., 1996). Conversely, Wistar rats that were given nicotine in drinking water had increased plasma levels of triacylglycerol (TAG) and total cholesterol, as well as accumulated fat globules in the liver (steatosis) (Valenc et al., 2008). A similar observation with regard to increased lipid levels has also been found in SD rats supplied with nicotine in drinking water (Ashakumary & Vijayammal, 1997; Chattopadhyay & Chattopadhyay, 2008) and in Wistar rats injected subcutaneously with nicotine (Balakrishnan & Menon, 2007b). Conversely, Friedman et al. (2012) found that nicotine injection to male C57BL/6 mice did not affect levels of TAGs or lipid accumulation in the liver. In this study, inclusion of lipidomics allowed, for the first time, investigation of the effects of inhaled nicotine (alone or in combination with pyruvic acid) on lipid profiles in the plasma, lung and liver of male SD rats.

Our combined assessment of classical toxicological endpoints with additional transcriptomic and lipidomic investigations in one study allows for simultaneous investigation of a multitude of mechanistic parameters and biomarkers. They can be correlated directly with toxicological outcomes, thereby avoiding the need for separate mechanistic studies with a higher number of animals, including those for bridging between studies, positive and negative controls and confirmation of biologic outcomes.

**Methods**

**Study design**

A 28-day repeated-dose OECD TG412 (OECD, 2009b) inhalation toxicity study was conducted using 70 male and 70 female rats to determine potential local and systemic effects after exposure to nicotine and pyruvic acid (alone or in combination).

The study comprised two negative control groups [one sham group exposed to filtered conditioned fresh air, and one vehicle group exposed to aerosolized phosphate-buffered saline (PBS)]; three nicotine/pyruvic acid groups (Nic/Pyr) exposed to aerosols containing 18, 25 and 50 \( \mu \)g nicotine/l and 9.8, 13.6 and 27.1 \( \mu \)g pyruvic acid/l; and two reference groups [nicotine aerosol (50 \( \mu \)g nicotine/l) (Nic) and sodium pyruvate aerosol (33.9 \( \mu \)g sodium pyruvate/l) (Pyr)] (Table 1). For OECD-required endpoints, 10 male and 10 female mice were allocated to each group. To characterize exposure-related effects at the molecular level (transcriptomics and lipidomics), 56 male rats (8 per group, separate from OECD) were allocated to the seven experimental groups (Table 1) (OECD+). To save animals, we used only male rats in these explorative add-on molecular study groups; in future studies, a sex comparison might also be included.

At appropriate time points/defined intervals, data were collected for clinical observations, toxicokinetics, clinical pathology, gross pathology, organ weights and histopathology. An overview of all investigations and endpoints of the combined OECD and OECD+ study is provided in Table 2.
The study was conducted in compliance with the OECD Principles on Good Laboratory Practice (GLP; as revised in 1997), with the exception of bronchoalveolar lavage fluid (BALF) analytics using RodentMAP® and the exploratory transcriptomics and lipidomics investigations.

### Aerosol generation

Aerosols were generated using Collison Nebulizers (BGI, Waltham, MA) and diluted with filtered, conditioned fresh air (Figure 1) to obtain the target concentrations of nicotine, pyruvic acid or pyruvate (Table 1). A solution of sodium pyruvate was used instead of pyruvic acid as a reference due to the low pH of pyruvic acid. The solution of sodium pyruvate had a physiologic pH, as did the nicotine solution, in PBS. A physiologic pH is required to avoid pH effects unrelated to the toxicity of a specific compound. Aerosols, generated from the respective stock solutions in PBS with a commercially available Collison three- or six-jet nebulizer (BGI), were delivered to animals in flow-past nose-only exposure chambers. A similar approach to generate nicotine aerosols for acute inhalation exposure has been described (Shao et al., 2013). Details of aerosol generation and qualification of the inhalation set-up are provided in Supplementary material.

### Analytical characterization of test atmospheres

To characterize the test atmosphere and verify reproducibility of the generation and dilution of aerosols, several analytical parameters were determined at the breathing area of the rats in the exposure chambers (Supplemental Table S1). Nicotine concentrations were determined by capillary gas chromatography after trapping on sulfuric-acid-impregnated silica gel (Majeed et al., 2014). Levels of pyruvic acid and pyruvate were measured by enzymatic means by a commercial Pyruvate Detection kit (Cayman Chemicals, Ann Arbor, MI) in total particulate matter (TPM) samples of the aerosols collected on Cambridge filter pads. For details of the frequency of determinations and additional parameters, see Supplementary Table S1.

### Animals and exposure

Six week-old outbred male (n = 126) and nulliparous, non-pregnant female (n = 70) SD rats [Crl:CD(SD)], bred under specific pathogen-free conditions, with a mean body weight of 174.4 g (males) and 156.3 g (females), were obtained from Charles River Laboratories (Raleigh, NC). The study was undertaken 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 were approved by the Animal Care and Use Committee of PMIRL.

All rats were identified individually by transponders implanted subcutaneously. They were housed and exposed in the laboratory animal facility with restricted access and under

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**Table 1. Study design.**

| Exposure group                        | Nicotine (µg/L) | Pyruvic acid (µg/L) | Sodium pyruvate (µg/L) | Molarity (all) (µmol/L) | OECD endpoints | OECD+ endpoints |
|---------------------------------------|-----------------|---------------------|------------------------|-------------------------|----------------|-----------------|
| Sham (fresh air)                      | 0               | 0                   | 0                      | 0                       | 10/10          | 8               |
| PBS                                   | 0               | 0                   | 0                      | 0                       | 10/10          | 8               |
| Nicotine                              | 50              | 0                   | 0                      | 0.308                   | 10/10          | 8               |
| Sodium pyruvate                       | 0               | 0                   | 33.9                   | 0.308                   | 10/10          | 8               |
| Nicotine and pyruvic acid low         | 18              | 9.8                 | 0                      | 0.111                   | 10/10          | 8               |
| Nicotine and pyruvic acid med         | 25              | 13.6                | 0                      | 0.154                   | 10/10          | 8               |
| Nicotine and pyruvic acid high        | 50              | 27.1                | 0                      | 0.308                   | 10/10          | 8               |

OECD, Organization for Economic Co-operation and Development; PBS, phosphate-buffered saline.

**Table 2. Measurements and endpoints.**

| Category                          | Parameter                                      | OECD | OECD+ |
|-----------------------------------|------------------------------------------------|------|-------|
| Test atmosphere                   | Total particulate matter, nicotine, temperature, flow rate, relative humidity, particle size distribution | X    | X     |
| Exposure                          | Nicotine metabolites (urine)                    | X    |       |
| In-life observations              | Body weight, feed consumption                   | X    | X     |
| Systemic effects                  | Hematology, organ weights, histopathology (non-respiratory tract) | X    |       |
|                                  | Clinical chemistry                              | X    | X     |
| Respiratory tract-related effects | Respiratory physiology (minute volume, tidal volume, respiratory frequency, peak inspiratory flow) | X    |       |
|                                  | Lung inflammation (free lung cells in bronchoalveolar lavage, analytes) | X    |       |
|                                  | Histopathology                                  | X    |       |
|                                  | Transcriptomics, lipidomics                     | X    |       |
| Liver-related effects             | Liver weight                                    | X    |       |
|                                  | Histopathology                                  | X    |       |
|                                  | Transcriptomics, lipidomics                     | X    |       |

OECD, Organization for Economic Co-operation and Development; OECD+, Organization for Economic Co-operation and Development Extended.
specific hygiene conditions. The laboratory air was high-efficency particulate absorption-filtered, and temperature (21–22°C) and humidity (52–55%) were regulated. A light–dark cycle of 12 h was in operation. Exposure was carried out in the light phase of the cycle. Rats were housed in pairs in cages with softwood (spruce and fir) granulate bedding (Lignocel® BK 8-15; Rettenmaier & Soehne, Rosenberg, Germany) and environmental enrichment was provided. A gamma-irradiated pellet diet (T2914C irradiated rodent diet; Harlan Laboratories, Bicester, UK) and sterilized drinking water were provided ad libitum except during exposure.

A total of 126 male and 70 female rats were randomized (stratified by sex and body weight) into one of the groups (Table 1). They were allowed to acclimatize to their environment for ≥17 days before exposure to test aerosols. Mean body weights of rats in groups on the day of randomization were 248.5–250.2 g (males) and 199.5–200.2 g (females) with a maximum relative standard deviation of 6.0% in male groups and 4.1% in female groups.

Rats were exposed nose-only to aerosol or fresh air according to Table 1. A nose-only exposure mode was used to ensure maximum reproducibility of uptake of aerosols by inhalation. A 7-day adaptation period using ascending concentrations was implemented to reach the target concentration of the aerosol.

Dose range was based on two dose range-finding studies (data not shown). The first study was based on OECD guideline 403 (OECD, 2009a) and determined the acute toxicity of nicotine and nicotine/pyruvic acid mixtures after intra-tracheal instillation. Administration of nicotine alone at the lowest dose (1.5 mg/kg) resulted in no deaths, whereas medium (7.5 mg/kg) and high doses (15 mg/kg) produced 40 and 80% mortality, respectively. Nicotine exposure-related mortality was reduced if nicotine was co-administered with pyruvic acid or sodium pyruvate. All deaths occurred on study day-1 during the first 2 h after administration.

In a 4-day dose range-finding inhalation study (data not shown), two groups of 8- to 9-week-old rats (three rats per sex and group) were exposed to aerosols of nicotine/pyruvic acid, or of sodium pyruvate, with daily increasing concentrations up to a maximum target concentration of 0.465 μmol/l (equivalent to 75 μg nicotine/l and 40.7 μg pyruvic acid/l). Two rats (1 male and 1 female) served as untreated controls. Body weight, signs of toxicity/neuronal toxicity (including labored breathing, tremor, convulsions, Straub tail) and urinary nicotine metabolites were monitored during the study, and necropsy undertaken after the final exposure. In addition, histopathology from the larynx (the most sensitive site for the detection of the irritation-based effects of inhaled aerosol particles in the respiratory tract) revealed only mild-to-moderate adaptive changes (epithelial hyperplasia and metaplasia at various sites) but no degeneration or ulceration. Based on signs of distress (reduced body temperature, decreased activity and weakness) at the maximum concentration of nicotine/pyruvic acid aerosol of this range-finding study, 0.310 μmol/l (equivalent to 50 μg nicotine/l and 27.1 μg pyruvate/l) was chosen as the highest exposure level for the 28-day study (sodium pyruvate aerosol was well tolerated at all concentrations).

In-life observations

Body weights, feed consumption, and health status of rats were monitored throughout the study (Table 3). With respect to the parameters employed to measure the uptake and exposure of aerosol (once per study), urinary pyruvate and nicotine metabolites were determined in 24-h urine samples collected from eight male and eight female rats per group using the Enzymatic Pyruvate Detection kit, and high-performance liquid chromatography after derivatization of 1,3 diethyl-2-thiobarbituric acid for nicotine metabolites (Rustemeier et al., 1993), respectively.

Respiratory physiology

Respiratory physiology was measured once in the study using head-out plethysmography (EMKA Technologies, Paris,
France) from each rat for evaluation of breathing rate, minute volume, tidal volume and peak inspiratory flow. Modified plethysmograph exposure restraint tubes are used, which permit the continuous inhalation of the test aerosol during the monitoring of the tube pressure (EMKA technologies pressure transducer) using a fitted latex cuff at the neck of the animal (head-out plethysmography). After a period of acclimatization to the plethysmograph of approximately 30 min, the breathing patterns of the animals were recorded in a series of $10 \times 30$ s measurement periods. Data acquisition was performed using the IOX 2.9.4.32 software package (Cisco Systems, Inc., San Jose, CA). A poor seal at the plethysmograph cuff (as determined during a leak test before and after measurement) or irregular or inconsistent breathing patterns during the 10 measurement cycles were flagged by the evaluation software and suppressed.

### OECD parameters of general and systemic toxicity

All rats in the OECD study (70 males and 70 females) were euthanized after 28 days of exposure ($\approx 16–24$ h after the final exposure) according to OECD TG 412 (OECD, 2009b). Tissues were collected between 16 and 24 h. There was no diet deprivation before dissection to avoid any impact that this regimen may have on clinical chemical parameters.

Several parameters were checked to investigate systemic and local effects in all rats. Hematology included differential cell count (Supplemental Table S2). Blood samples were taken from rats under pentobarbital anesthesia from the retro-orbital venous plexus, and analyzed using the UniCel® DxC 600i system (Beckman Coulter, Fullerton, CA). For some blood samples, hematology data was unavailable or suppressed due to (i) insufficient sample volume for analysis, (ii) clot formation in the blood and (iii) rejection by the instrument analytical software (abnormal scattergram). For assessment of blood coagulation, hemostasis and clinical chemistry in serum (Supplemental Table S3), blood samples were taken from rats under pentobarbital anesthesia by exsanguination via the abdominal aorta, and analyzed using the UniCel® DxC 600i system (Beckman Coulter Inc., Brea, CA). In some instances, data was unavailable for analysis due to insufficient volume, or to significant hemolysis, resulting in group sizes ranging from $N = 8$ to 10.

For urinalyses, urine was collected once during the study (to coincide with urine collection for metabolic studies) and tested using LabStrip U11 Plus Semi-quantitative Urine Test Strips (Analyticon Biotechnologies, Muehlenberg, Germany) (Supplemental Table S4). Urinalyses data were not reported because fecal contamination of samples during collection rendered the data difficult to interpret.

After exsanguination, organ weights were collected for lungs (with trachea and larynx), liver, kidneys, adrenal glands, heart, spleen, thymus, brain and testes (Supplemental Tables S5 and S6). Gross pathology included examination of the external surface of the body, all orifices, as well as the cranial, thoracic and abdominal cavities and their contents.

### Histopathology

Non-respiratory tract organs were collected according to OECD 412 guidelines (OECD, 2009b), (Supplemental Table S6) and fixed in 4% formaldehyde solution except for the sternum and testes (which were fixed in Schaffer solution and Bouin solution, respectively).

Histopathology of organs in the respiratory tract included nasal passages with nasal-associated lymphoid tissue, larynx, trachea and lungs (Supplemental Tables S5 and S7). Respiratory tract organs (nose, larynx, trachea and left lung) were fixed in ethanol glycerol acetic acid formaldehyde solution (EGAFS). Lungs with larynx and trachea were removed together. After determination of weight, the right lung was separated for bronchoalveolar lavage (BAL). The left lung was fixed by instillation via the left bronchus with EGAFS and subsequent immersion. The tracheal bifurcation and larynx were fixed separately in EGAFS. After fixation, tissues were transferred to 70% ethanol and processed for paraffin embedding.

Histologic sections of respiratory tract organs were prepared at defined levels (Supplemental Table S7) and stained with hematoxylin and eosin (H&E). In addition, sections from the nose at level 1, trachea at level 4 (bifurcation) and left lung were stained with Alcian blue/periodic acid–Schiff’s (PAS) reagent and evaluated by histopathologic means. Thickness of the epithelium of the larynx was determined at two sites (ventral portion of the vocal cords and ventrolateral wall of the arytenoid portion) from the level of the arytenoid projections.

Histopathologic assessment of respiratory tract organs and assessment of liver vacuolation (evaluation 1) was undertaken by a board-certified (American College of Veterinary Pathologists, ACVP) veterinary pathologist (Hall Consulting, Mount Airy, MD) in a blinded fashion. Severity was graded on a scale from 0 (normal), 1 (minimal), 2 (mild), 3 (moderate) to 4 (severe). Histopathologic assessment of the

| Parameter | Method | Frequency | Number of rats | Remarks |
|-----------|--------|-----------|----------------|---------|
| Body weight | Gravimetry | ≥Twice/week | All rats | Individually |
| Feed consumption | Gravimetry | Once/week | All groups | Determined per group |
| In-life observations | Non-systematic observation | Daily | All rats | – |
| | Group observations according to checklist | Daily | All rats | Immediately after removal from exposure tube* |
| | Individual observations according to checklist | Daily | Two rats selected systematically per group per day | Immediately after removal from exposure tube* |
| Mortality | Observation | Daily | All rats | – |

*Except rats selected for urine collection on the day of determination.
liver was done independently in-house by a board-certified (ACVP) veterinary pathologist (evaluation 2; for details of the evaluation criteria, see Supplementary material). To investigate further vacuole content, PAS and PAS–diastase (PASD) staining (for glycogen) as well as Oil-Red-O staining (for lipids) were carried out. Histopathologic assessment of non respiratory tract organs was undertaken by the Laboratory of Pharmacology and Toxicology (Hamburg, Germany).

**Lung lavage and analyses of lung inflammation**

The right lung was cannulated and instilled with lavage medium [PBS with 0.325% bovine serum albumin (BSA)] for collection of free lung cells from the first-to-fifth cycle of BAL from the right lung of all animals (cycle-1, PBS; cycles 2–5, PBS + 0.325% BSA). Cells were collected and analyzed by flow cytometry for viability and differential leukocyte count, as described previously (Friedrichs et al., 2006; Kogel et al., 2014). Cell-free bronchoalveolar lavage fluid (BALF) from cycle-1 (remaining after centrifugation of cells for BALF) was frozen, and submitted to Myriad Rules-Based Medicine (Austin, TX) for exploratory (non-GLP) analyses of a panel of 60 selected proteins (RodentMAP v3.0; Life Technologies, Carlsbad, CA; Supplemental Table S8).

**Transcriptomics**

**RNA preparation**

Total RNA was extracted from homogenized tissues of lung and liver using TRIzol™ and quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Integrity of isolated RNA was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Quality of RNA samples was considered acceptable if the RNA Integrity Number (RIN) score was >6.0.

**Microarray preparation**

Transcriptome analyses were done using a GeneChip® HT 3’ IVT Express kit (Affymetrix, Santa Clara, CA) following manufacturer instructions. Biotinylated fragmented cRNA (10 µg) was hybridized to GeneChip Rat Genome 230 arrays (Affymetrix) for 16 h. Arrays were imaged using a 3000 7G Scanner (Affymetrix). All scanned images were inspected for artifacts visually before analyses.

**Analyses of gene-expression data**

Raw data files were read by the ReadAffy function of the Affy package (Gautier et al., 2004) belonging to the Bioconductor Suite of microarray analysis tools ( Gentleman et al., 2004) available for the R statistical environment (R Development Core Team, 2007). Quality was controlled by generating and examining RNA degradation plots (AffyRNAdeg function of the Affy package), NUSE and RLE plots [AffyPLM package (Brettschneider et al., 2008)] by calculating MA(RLE) values.

Robust microarray analysis (RMA) background correction and quantile normalization was used to generate values for microarray expression (Irizarry et al., 2003) from all arrays that passed the quality control checks. An overall linear model was fitted to the gene-expression profiles, and specific contrasts of interest were evaluated to generate raw p values for each probe set on the expression array (Smyth, 2004). Then, the Benjamini–Hochberg false discovery rate (FDR) method was used to correct for multiple testing effects.

**Lipidomics**

**Sample treatment and lipid extraction**

To further evaluate the small changes observed in the groups exposed to high nicotine concentrations, an extensive lipidomics evaluation was performed on all study groups with the exception of the nicotine and pyruvic acid low exposure group (which had minimal effects during in life and transcriptomics evaluation). Lung and liver tissues as well as blood plasma were snap-frozen immediately after dissection. Then, they were pulverized using a CP02 CryoPrep Dry system (Covaris, Woburn, MA) and resuspended in ice-cold methanol containing 0.1% butyldihydroxytoluene. Ceramides, cerebrosides, glycolipids, glycerophospholipids and cholesterol esters (CEs) were extracted using a modification of the Folch method for extracting lipids, as described previously (Jung et al., 2011), and carried out on a Microlab® Star Robot (Hamilton Robotics, Reno, NV). Gangliosides were extracted according to the method described by Fong et al. (2009) with minor modifications. Eicosanoids were extracted as described previously (Deems et al., 2007).

**Mass spectrometric analyses (shotgun lipidomics) and processing**

Lipid extracts were analyzed using a Hybrid Triple Quadrupole/Linear Ion Trap Mass Spectrometer (QTRAP® 5500; AB SCIEX, Framingham, MA) (Zora Biosciences, Espoo, Finland) equipped with a robotic nanoflow ion source (TriVersa NanoMate HD; Advion, Ithaca, NY), as described previously (Stahlman et al., 2009). Targeted molecular lipids were analyzed on a 5500 QTRAP Spectrometer equipped with an ultra-high-pressure liquid chromatography system (CTC HTC PAL Autosampler and Rheos Allegro Pump) using a multiple reaction monitoring-based method in negative ion mode.

Mass spectrometry data files were processed using LipidView™ version 1.1 (Shanghai AB Sciex Analytical Instrument Trading Co., Shanghai, China) and MultiQuant™ version 2.0 (AB SCIEX) to generate a list of lipid names and peak areas. Lipids were normalized to their respective internal standards and tissue weight. Concentrations of molecular lipids are presented as nmol/mg lung or liver. For statistical analyses, a Wilcoxon rank-sum test was conducted for each lipid for comparing study groups. Monte-Carlo estimation of exact p values was undertaken. Multiple testing was controlled with FDR q values.

**Statistical analyses**

For minute volume and nicotine metabolites, males in OECD and OECD + groups were combined to compute statistics. For clinical chemistry and hematology endpoints, OECD and OECD + male groups are shown separately if the sample size was n >3, except for the OECD + male sodium pyruvate
group, which had a sample size of 3. For histopathology endpoints, no OECD + rats were investigated, so statistics refer to OECD animals only.

For group comparisons, a specific approach was used for continuous variables. That is, each of the six non-sham groups was compared individually against sham. Then, to compare group X against sham, the normality of data was investigated by a Shapiro–Wilk test on the residuals of the two groups combined (allowing a gain in power for this normality test compared with applying it individually to the two groups being compared). If the Shapiro–Wilk test led to \( p \geq 5\% \), then a \( t \)-test accounting for variance heterogeneity was computed (results are displayed in graphs by *\( p < 0.05 \), **\( p < 0.01 \) and ***\( p < 0.001 \)). If the Shapiro–Wilk test led to \( p < 5\% \), then a non-parametric test was performed, i.e. the Mann–Whitney–Wilcoxon rank sum test (its exact version was implemented and, given the long computation that this could generate, Monte-Carlo simulations were used to estimate the result of this test; this approach was also used for lipidomic data and usually results in quite similar values compared with the asymptotic Wilcoxon test). If a non-parametric test was used, the signs +, ++ or +++ are displayed in the graph or table instead of *, ** or ***, respectively. All \( p \) values are unadjusted. For histopathologic scores (categorical data), all comparisons were made against sham by a Cochran–Mantel–Haenszel test.

**Results and discussion**

**Concentrations, particle size of aerosols and daily doses in test atmospheres**

Target concentrations of nicotine, pyruvic acid and sodium pyruvate in test and reference aerosols are shown in Figure 2. Background values for pyruvate in sham, PBS and nicotine groups were close to or below the lower limit of quantitation (LLOQ) of the assay.

The particle (droplet) size of PBS aerosol did not change when pyruvic acid and nicotine, or nicotine and sodium pyruvate was added (Figure 3). Mass median aerodynamic diameters (MMADs) were 1.4–2.0 \( \mu m \) with values of geometric standard deviation (GSD) varying from 1.7 to 2.2 (Figure 3), thereby confirming that the aerosols were equally respirable. The OECD recommends a MMAD of 1–3 \( \mu m \) with a GSD of 1.5–3.0 (OECD, 2009b).

Based on the formula described by Alexander et al. (2008), the concentration of nicotine (50 \( \mu g/l \)) generated a
delivered dose (DD) of nicotine aerosol equivalent to 13.6 mg nicotine/kg body weight, calculated as follows:

\[ DD = \left( \frac{C \times RMV \times D}{BW} \right) \]

where \( DD \) = delivered dose (mg/kg); \( C \) = concentration of substance in air (mg/l); \( RMV \) = respiratory minute volume (l/min); \( D \) = duration of exposure (min); and \( BW \) = body weight (kg). \[ DD = \left( \frac{0.05 \text{ mg/l} \times 0.194 \text{ l/min} \times 360 \text{ min}}{0.25 \text{ kg}} \right) = 13.6 \text{ mg/(kg BW)}, \text{ or } 3.4 \text{ mg/rat (250 g BW)}. \]

The daily inhaled dose (13.6 mg/kg BW) of nicotine in nicotine and nicotine/pyruvic acid (high) groups (each with 50 \( \mu \)g/l nicotine) was well above the maximum tolerated dose (MTD) of 7.5 mg/kg for acute administration and close to the 80% mortality dose (15 mg/kg) as determined by single intratracheal instillation in our MTD study. In one acute (20-min) inhalation toxicity study with high concentrations of nicotine aerosols, the LC\textsubscript{50} was 2300 \( \mu \)g/l (Shao et al., 2013). This finding demonstrates that inhalation studies with extended exposure periods and lower concentrations are better suited for investigation of the long-term effects of nicotine-containing aerosols than bolus-like inhalation and non-inhalation administration studies, for example, via subcutaneous or intraperitoneal injections or intratracheal instillation, which cause an acute toxic effect owing to transient, very high concentrations.

The human equivalent dose (HED) can be calculated based on the body surface area by dividing the rat dose by a factor of 6.2 (CDER, 2005): \( \text{HED} = 13.6 \text{ mg/kg/6.2} = 2.2 \text{ mg/kg.} \) This value translates to a daily nicotine dose of 132 mg for a 60-kg adult human, equivalent to \( \approx 130 \) conventional cigarettes smoked per day.

**Biomonitoring and in-life observations**

**General behavior**

Typical signs of exposure-related stress were observed in both sexes: secretion from the Harderian glands, wet fur and sporadic reduction in reflexes (pinna and corneal). Additional possible nicotine effects were observed in females: high prevalence of cold/shivering (tremors) during adaptation but very infrequent in subsequent weeks (two observations). Mortality (two technical deaths) was observed during the first week of the study; the two rats were replaced, and all 140 rats of the OECD part completed the study.

**Changes in body weight**

All rats had an increase in body weight throughout the exposure period (Figure 4). In male rats, no effect compared with sham groups was evident for PBS and sodium pyruvate, nicotine caused a slight, significant reduction in body-weight gain from day-16 onwards and nicotine/pyruvic acid tended to reduce body weight (not concentration-dependent) at the three concentration levels tested; significance was observed for the medium concentration of the nicotine/pyruvic acid aerosol from day 12 onwards. In female rats, there were no obvious trends for PBS, nicotine and nicotine/pyruvic acid groups, but a transient (significant from day 16 to 23) reduction in body-weight gain was noted. Note, while all rats were allocated at the same time, the OECD + animals exposure start date began 1 week later than that for the OECD animals, resulting in a higher starting body weight for the ‘‘plus’’ groups. No significant differences in feed consumption relative to body weight were observed in any group compared with sham (data not shown). A significant decrease in body weight gain has also been observed in male Wistar rats exposed to daily nicotine injections (2.5 mg/kg, s.c.) for 22 weeks (Muthukumaran et al., 2008).

**Respiratory physiology**

There were no consistent test substance-related changes in respiratory physiology. Slight increases in tidal volume and respiratory frequency were only significant for a few conditions but their product, respiratory minute volume, was increased significantly for all male exposure groups except PBS and nicotine/pyruvic acid (high), and for all female groups except sodium pyruvate and nicotine/pyruvic acid (medium) (Figure 5). These results suggest a weak general aerosol effect rather than a test substance-related effect, and are opposite to the decreases in respiratory minute volume that are, in general, observed for cigarette smoke (Kogel et al., 2014; Terpstra et al., 2003; Vanscheeuwijk et al., 2002) or other irritant inhalants (Alarie, 1973).

**Aerosol uptake**

Recovery of total nicotine metabolites in urine was proportional to the exposure concentrations of nicotine (Figure 6). As shown above, the daily DD of nicotine for an exposure concentration of 50 \( \mu \)g nicotine/L can be calculated to be at most 3.4 mg/rat, assuming a respiratory minute volume of 194 ml, body weight of 250 g and 100% retention, however, the actual nicotine retention efficiency may be lower in rats (due to lower deposition fractions of inhaled aerosol particles (Chen et al., 1989) than in humans, where nearly 100% of the inhaled nicotine dose from cigarette smoke were retained in the respiratory tract (Baker & Dixon, 2006; Feng et al., 2007). Given that 4500 nmol nicotine metabolites were excreted after one exposure (inhaled dose 3.4 mg or 21 000 nmol nicotine), and that the selected metabolites analyzed...
within 24 h represent less than 50% of the total amount of i.v. administered nicotine (Schepers et al., 1993), the minimum dose of retained nicotine can be calculated to be greater than 9000 nmol or 1.5 mg per rat and day.

Rats exposed to nicotine alone (50 μg/l) and the combined nicotine/pyruvic acid (high) group (which also contained 50 μg nicotine/l) showed similar recovery of total metabolites, therefore suggesting that pyruvic acid did not have any effect on nicotine uptake in male or female rats. Relative amounts of metabolites (Figure 7) showed the prevalence of cotinine (COT), nicotine-N'-oxide (NN'O) and with lower quantities of nornicotine (NNc), norcotinine, (NCOT) and 3'-hydroxyco-
tinine (3'HOCOT). There were slight differences in the distribution of metabolites for the three nicotine/pyruvic acid test-atmosphere concentrations studied, principally for NN'O and 3'HOCOT \( p \leq 0.05 \) for nicotine/pyruvic acid (low) versus nicotine/pyruvic acid (high) or nicotine alone. However, these differences are consistent with the relative variability of metabolites observed in historic studies with cigarette smoke (Vanscheeuwijk et al., 2002).
For pyruvate analyses, we used an exploratory method that was – unlike the other methods applied in this study – not validated formally. Detected levels of pyruvate in urine (data not shown) did not differ from sham in male rats ($p > 0.05$). Several female groups had higher levels of pyruvate than that in the sham group, but this was not in an exposure concentration-dependent manner. Furthermore, there was no way to distinguish the exogenous source of pyruvate (pyruvic acid or sodium pyruvate from the exposure) from physiologic pyruvate. Therefore, measurement of urinary pyruvate using the applied method is not reliable for monitoring uptake of pyruvic acid or sodium pyruvate.

**General and systemic effects of aerosol exposure**

**Macroscopic observations of organs and organ weights**

There were several macroscopic observations of organs at necropsy, but they were specifically related to neither treatment nor exposure concentration (data not shown). The predominant observation was reddish discoloration of the
liver, but this did not translate to any obvious findings at the histopathologic level. Other findings included bladder stones, tissue discoloration and patches and nodules in various tissues.

During dissection, absolute weights of the following organs were measured, and organ weights relative to body weight were calculated after exsanguination: lung (with larynx and trachea), liver, heart, left and right adrenal glands, left and right kidney, thymus, brain, spleen and testes. For the following organs, there were no control or test-atmosphere effects on organ weight relative to body weight in male or female rats: larynx, trachea and lung, left adrenal gland and testes. Some isolated changes compared with sham groups were observed for the heart [higher in male rats in the nicotine/pyruvic acid (medium) group], left kidney [higher in female rats in the nicotine/pyruvic acid (high) group] and brain (higher in male rats in the nicotine group). For a compilation of all relative organ weights, see Supplemental Table S9A and B.

Liver weight was increased in both sexes in nicotine and most nicotine/pyruvic acid groups in a nicotine concentration-dependent manner; a pyruvate effect was not observed. A nicotine concentration-dependent increase was observed in the nicotine group and nicotine/pyruvic acid (medium and high) groups only for the right adrenal gland for both sexes. A small nicotine concentration-dependent increase was observed in nicotine/pyruvic acid (low, medium and high) groups for the right kidney in female rats (Supplemental Table S9B). A nicotine concentration-dependent decrease in relative thymus weight was observed in both sexes for nicotine and nicotine/pyruvic acid groups [not significant for male rats in the nicotine/pyruvic acid (low) group]. Spleen weight in male rats was decreased in groups exposed to high concentrations of nicotine irrespective of the presence of pyruvic acid (Supplemental Table S9A). Spleen weights in female rats elicited a different response, with higher weights after exposure to PBS or sodium pyruvate (Supplemental Table S9B). This result in females, however, appeared to be due to the low spleen weight in the sham group compared with PBS groups for both sexes. Decreased weight of the spleen (males) and thymus (male and females) in combination with increased weight of the adrenal gland (male and females) was highly suggestive of a secondary chronic stress-related response and not a primary nicotine-dependent effect. Thymus and adrenal gland weights in toxicity studies are considered to be sensitive parameters that indicate stress (Everds et al., 2013).

**Hematology**

Red blood cell (RBC) parameters [erythrocyte count, blood hemoglobin concentration, mean corpuscular volume (MCV), hemoglobin volume, mean corpuscular hemoglobin (MCH)] did not demonstrate any consistent exposure-related effects in male rats of all OECD exposure groups, with similar results in the OECD+ groups with the exception of a decreased MCV value in the nicotine/pyruvic acid (medium) exposure group, and increased erythrocyte count in the nicotine/pyruvic acid high group. In female rats, no effect was seen in blood hemoglobin concentration or erythrocyte count, but the MCV, and MCH (Figure 8) were increased significantly in the nicotine group; erythrocyte volume was also increased for the three nicotine/pyruvic acid groups and the mean erythrocyte hemoglobin volume was increased for the nicotine/pyruvic acid (high) group (Figure 8). Such increases in these RBC parameters as observed in female rats can only occur secondary to acute stress effects (Everds et al., 2013).

Parameters for white blood cells showed minimal exposure-related effects for total leukocyte counts in the OECD males and females groups (Figure 9C). Neutrophil counts exhibited a nicotine-dependent response: significant increases occurred in nicotine and nicotine/pyruvic acid (high) groups in both sexes, and in the nicotine/pyruvic acid (low) group in male rats only (Figure 9A). Lymphocyte count was significantly decreased for female rats in the nicotine group only, but there was a similar trend (not significant) for the nicotine/pyruvic acid (high) group in both sexes, and for the nicotine/pyruvic acid (medium) group in male rats only (Figure 9B). Alterations in peripheral neutrophil (increase) and lymphocyte (decrease) counts likely reflected a secondary stress-related response and not a primary nicotine-related effect. Leukogram profiles induced by stress in rats typically manifest as lymphopenia and neutrophilia (as seen here) and are driven typically by glucocorticoids (Everds et al., 2013).

**Clinical chemistry**

For detailed representation of all results, see Supplemental Tables S10 and S11. Markers of hepatic damage in plasma, alkaline phosphatase (ALP) and alanine aminotransferase (ALT) showed increased activities by nicotine and nicotine/pyruvic acid (medium and high) but not by sodium pyruvate in both sexes, and ALT was also increased by nicotine/pyruvic acid in female rats (Figure 10A and B). Aspartate aminotransferase (AST) activity was increased only in females exposed to nicotine/pyruvic acid (high). Serum albumin (but not total protein) concentration was decreased significantly only in females exposed to nicotine (Supplemental Tables 10 and 11). ALT is used frequently as an indicator of liver injury (Akkaya et al., 2007; Ennulat et al., 2010). Upon injury, ALT is released into the circulation via cytoplasmic blebbing (Emnulat et al., 2010). However, ALT is also an important enzyme that catalyzes a crucial reaction in gluconeogenesis and the urea cycle (Ennulat et al., 2010). Canbakan et al. (2010) suggested that ALT may not be the best indicator of liver damage based on the observation that serum levels of ALT do not correlate with parameters of apoptosis and oxidative stress in the liver as measured in 50 patients with non-alcoholic fatty liver disease. However, in mice, ALT has been shown to increase accompanied by ethanol-induced steatosis, but not to increase further upon co-treatment with nicotine, although lipid accumulation and deposition of collagen I are increased by nicotine (Lu et al., 2013).

Approximately 2-fold increases in levels of ALP, AST and ALT accompanied by oxidative stress and lipid peroxidation in plasma, liver, kidneys and lung have been observed in rats after subcutaneous injection of 2.5 mg nicotine/day over 154 days (Balakrishman & Menon, 2007a; Kalpana et al., 2007; Muthukumaran et al., 2008). In our study using inhaled nicotine, increases in levels of ALP and ALT had a similar magnitude but the increase in AST levels was lower.
Whether the increase of circulating levels of ALT was due to liver injury or a more robust gluconeogenesis/urea cycle is – in the absence of morphologic correlates – not possible to distinguish (Ennulat et al., 2010).

Total cholesterol (but not triglyceride concentration) was decreased in response to nicotine (no effect was observed with pyruvic acid) in both sexes but the decrease was not concentration-dependent (Figure 10C). With regard to plasma levels of cholesterol, the observed effect of inhaled nicotine was the opposite of the effects of administered nicotine noted in the literature. That is, an approximately 2-fold increase in plasma levels of cholesterol and a 1.5-fold increase in triglycerides has been reported after chronic (154 days) nicotine injection (Balakrishnan & Menon, 2007a; Kalpana et al., 2007), whereas we found decreased levels of free cholesterol (FC) and steryl ester levels in plasma after 28-day nicotine inhalation.

Similar effects to those observed for cholesterol were seen for glucose in male rats only. In female rats, glucose levels were decreased significantly only in the nicotine/pyruvic acid (high) group (Figure 10D). Urea levels were increased only in male rats exposed to PBS or nicotine without or with the addition of pyruvic acid (data not shown). Bilirubin levels were decreased in male rats in the nicotine/pyruvic acid (high) group only (Supplemental Table S10).

A few changes in the concentrations of salts and ions were observed. In male rats, sodium concentration was decreased marginally in the sodium pyruvate group and slightly increased in nicotine/pyruvic acid (medium and high) groups. Phosphorus concentration was decreased significantly in female rats in the nicotine/pyruvate (high) group only. Remaining parameters of clinical chemistry showed no effect in response to test atmospheres. Clinical chemistry was also investigated in an additional group of male rats designated for molecular analyses (transcriptomics and lipidomics). Those results were very similar to the ones in the OECD cohort (e.g. for ALP, ALT, cholesterol and glucose; Figure 10). No significant changes were observed in all mineral levels in blood (Supplemental Tables S10 and S11).

Urinalyses

Data are not reported due to fecal contamination of samples during collection. However, this contamination did not interfere with determination of nicotine metabolites, as the values were in accordance with historical data.
Free lung cells and inflammatory mediators in BALF

The total number of free lung cells in BALF in response to aerosol from nicotine/pyruvic acid, or either of the control aerosols (nicotine, pyruvate and PBS), did not show any difference relative to sham-exposed rats (Supplemental Figure S1A). Differential cell counts revealed non-significant trends for higher lymphocyte numbers in female rats in nicotine/pyruvic acid (low and medium) groups, as well as for higher neutrophil numbers in nicotine and nicotine/pyruvic acid groups (females), and in nicotine and medium nicotine/pyruvic acid groups (males) (Supplemental Figure S1B). However, these trends did not follow a consistent concentration-related response. Eosinophil numbers in female rats were increased in all exposure groups (including PBS control). Significance was reached for free lung cell counts in response to the nicotine/pyruvic acid low aerosol concentration. Neither of the control aerosols showed any difference relative to sham-exposed rats in terms of absolute levels of eosinophils. There was, however, a mild increase in relative levels of neutrophils and macrophages recovered from the BALF of nicotine/pyruvic acid-exposed females in the nicotine/pyruvic acid (low) group (which exhibited the strongest increase). For males, no significant changes compared with sham groups were observed. Even the highest number of eosinophils in female rats in the nicotine/pyruvic acid (low) group represented only ≈0.025% of total free lung cells and this can, therefore, be considered a non-toxicologically relevant change.

In terms of profiling of inflammatory mediators, 60 analytes were measured in BALF from all exposure groups and the sham group. Most analytes were below the LLOQ or did not show exposure-related effects. Among the 11 analytes showing differences between exposure groups and sham, only monocyte chemotactic protein-3 [MCP-3, aka. chemokine (c–c motif) ligand 7] (increase), myeloperoxidase (increase) and vascular endothelial growth factor (decrease) showed trends or concentration-dependent effects in response to nicotine exposure (alone or in combination with pyruvic acid) (Supplemental Figure S1C and D). These changes are not indicative of exposure-related inflammatory processes.

Histopathology of the respiratory tract

Only slight differences were observed in the respiratory tract in nicotine/pyruvic acid-, nicotine- and sodium pyruvate-exposed rats compared with sham-exposed rats. These exposure-related differences were limited to the larynx.

Figure 9. Hematology: white blood cells. Differential leukocyte counts (absolute) for male and female rats. (A) Neutrophils, (B) lymphocytes, (C) leukocytes. Mean ± SEM, n = 6–10; p-values obtained by t-test accounting for variance heterogeneity or by Wilcoxon exact MC. ***/+++p < 0.001; **/++p < 0.01; */+p < 0.05.
All histopathologic findings in the respiratory tract are provided in Supplemental Tables S12 and S13. Highlights of the findings (significantly different from the sham-exposed rats) are summarized below.

**Base of epiglottis, mid level**

Minimal-to-mild hyperplasia of the ventral squamous epithelium in all exposure groups (except sodium pyruvate) in females (Figure 11A), and minimal squamous metaplasia of the ventrolateral epithelium in all nicotine-exposed rats, independent of pyruvic acid) (Figure 11B) was noted.

**Base of the epiglottis, distal level**

Minimal squamous hyperplasia of the ventral epithelium in all nicotine-containing groups was observed (Figure 11C).

**Arytenoid projections, vocal cords**

Minimal-to-mild squamous epithelial hyperplasia of the ventral portion was observed in males [nicotine, nicotine/pyruvic acid (high)] and females [nicotine, nicotine/pyruvic acid (low, medium and high)] (Figure 11D). In accordance with morphologic observations, histomorphometry revealed significant increases in the epithelial thickness of the ventral portion of the vocal cords for males from nicotine and nicotine/pyruvic acid (high) and females from nicotine/pyruvic acid (medium) groups (Supplemental Tables S14 and S15).

Mild hyperplasia (mean score ≤ 1.5) of the squamous epithelium at the base of the epiglottis reflects the high sensitivity of this site for any particulate inhalant. Hyperplasia can be considered mainly particulate-induced irritation that occurred in all aerosol inhalation groups (including the PBS control). Effects observed for the other three endpoints can be considered nicotine-related because they increased in severity in all nicotine-containing aerosol-exposed groups but not in PBS and sodium pyruvate groups. These effects were relatively mild (mean scores ≤ 1.5) and independent of the concentration of nicotine and pyruvic acid. Similar findings have been reported from rats following a 14-days inhalation of an aerosol containing 58 μg nicotine/l (Werley et al., 2014).

In accordance with the absence of increases in inflammatory cells in the BALF, histologic investigation of the lung did neither reveal signs of exposure-related inflammation, nor were there observable adaptive changes. Changes observed at the other respiratory tract tissues (nose, trachea, tracheal...
bifurcation and lung; Supplemental Tables S12 and S13) assessed were considered incidental. With regard to the absence of exposure-related histopathologic changes in the lung after inhalation of nicotine-containing aerosols at relatively high daily doses, continuous exposure appeared to facilitate rapid absorption and detoxification of nicotine to prevent inflammatory effects, destructive pulmonary effects or irritation. Absence of pulmonary changes in this study is in contrast to the reported effects of subchronic administration of nicotine via non-inhalation routes in which the entire daily dose (usually ≤ 20% of the inhaled daily dose, 13.6 mg/kg) was delivered as a single bolus with very high transient concentrations, which could explain the observed toxic effects. For example, the authors of a 22-week nicotine (2.5 mg/kg/day, s.c.) study reported severe histologic changes in the lung (fibrosis, emphysema, lymphocyte infiltration and macrophage accumulation) and even adenocarcinoma has been reported as a nicotine-related effect (Sudheer et al., 2008). Other authors have observed no proliferative changes but instead neutrophil infiltration, edema and fibrosis in rat lungs after repeated intra-tracheal instillation of nicotine for ≤30 weeks (Yokohira et al., 2012).

Histopathology of the liver and other non-respiratory tract organs

After assessment of all non-respiratory tract organs and tissues (Supplemental Table S16A and B), the only significant finding was in the liver. Two independent evaluations were undertaken and both led to very similar conclusions. Overall, cytoplasmic vacuolation of hepatocytes was observed in the livers of most rats of the study, and increased vacuolation could be observed in rats exposed to nicotine and sodium pyruvate. Sex-related differences were minimal. Hepatocyte vacuolation had a slightly predominant centrilobular pattern, and a diffuse, lace-like appearance within each cell (Figure 12, upper row). In vacuolated cells, granular PAS-positive material appeared in aggregates and individual stippled material within the hepatocyte cytoplasm was seen. Larger PAS-positive aggregates were observed in cytoplasmic vacuoles (Figure 12, middle row). Diastase digestion permitted identification of the material as glycogen (Figure 12, lower row).

In evaluation 1, the severity of vacuolation increased from a mean score of ≤1 in sham and PBS control groups until up

![Figure 11. Histopathology of the respiratory tract organs and larynx. Mean values are given; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.](image_url)
to 3 (in female rats) with increasing nicotine concentration (Figure 13A–C). Vacuolation was also increased in the livers of female rats that inhaled sodium pyruvate compared with sham and PBS control females, but this difference was small. A similar effect was not seen in males, and sodium pyruvate was not considered to have contributed to this change.

In evaluation 2, increased vacuolation was observed in nicotine alone, nicotine/pyruvic acid (high) and nicotine/pyruvic acid (medium) groups. In the livers of female rats, a severity score of 2 was assigned to the sodium pyruvate group. Vacuoles with this score typically had a centrilobular pattern. Severity of vacuolation increased with increasing nicotine concentration in the livers of female and male rats, with a more pronounced response in female nicotine/pyruvic acid groups (Figure 13D). In addition, cytoplasmic glycogen granules were observed within non-vacuolated and vacuolated hepatocytes. Glycogen granules positively identified in non-vacuolated hepatocytes (score 1) were small, had a finely stippled appearance, and were distributed evenly throughout the cytoplasm of an individual hepatocyte. Within vacuolated hepatocytes, glycogen granules appeared as large, dense aggregates with non-uniform distribution in cytoplasm (Figure 13D). Severity of cytoplasmic glycogen increased with nicotine concentration in male and female rats, with a more pronounced response in female nicotine/pyruvic acid groups (Figure 13D). In addition, cytoplasmic glycogen granules were observed within non-vacuolated and vacuolated hepatocytes. Glycogen granules positively identified in non-vacuolated hepatocytes (score 1) were small, had a finely stippled appearance, and were distributed evenly throughout the cytoplasm of an individual hepatocyte. Within vacuolated hepatocytes, glycogen granules appeared as large, dense aggregates with non-uniform distribution in cytoplasm (Figure 13D). Severity of cytoplasmic glycogen increased with nicotine concentration in male and female rats, with a more pronounced response in females (Figure 13D). Positive correlation between the severity of cytoplasmic vacuoles and severity of cytoplasmic glycogen was identified: higher severity scores for vacuoles correlated with higher severity scores for glycogen.

Lipid staining with Oil-Red-O was very weak in all treatment groups and no differences compared with sham groups were observed (Supplemental Table S17). The only observed additional findings in the liver were lympho-histiocytic infiltration and congestion, being within the normal range of variation and therefore interpreted as coincidental and not exposure related (Supplemental Table S16) while no signs of fibrosis or changes in stellate cells were observed in any group. Induction of fibrogenic processes by nicotine exposure has been observed in human primary hepatic stellate cells in vitro (Soeda et al., 2012). As noted in the lung, liver histopathology revealed only weak effects of inhalation exposure to nicotine-containing aerosols, a finding that is in contrast to published results seen after repeated subcutaneous administration of single daily doses of nicotine. Such observations included (in addition to vacuolation) portal inflammation, steatosis and focal degeneration (Sudheer et al., 2008). All remaining findings in non-respiratory tract organs and tissues were considered coincidental, and not related to test substances.

**Systemic versus lung toxicity of test aerosols**

Exposure to nicotine-containing aerosols (with or without pyruvic acid) indicated increased liver weight and slight hematologic changes in rats (e.g., increases in the numbers of neutrophils and decreases in the numbers of lymphocytes). Effects of nicotine or pyruvate (or a combination thereof) did not significantly affect the numbers of free lung cells and most of the inflammatory mediators in the BALF of rats. Most of the analytes fell below the LLOQ, thereby making interpretation of results difficult. Histologic examination revealed adaptive changes only in the larynx (hyperplasia and metaplasia) and liver (vacuolation/glycogen content); signs of tissue destruction or inflammation were not observed.

Analyses of clinical chemistry results showed several parameters responded, in a concentration-dependent manner,
to test aerosols. Exposure to nicotine (with or without pyruvic acid) caused increases in the activities of liver enzymes in plasma: ALP (maximum, 1.5- to 2-fold) and ALT (maximum, 1.5-fold). With the exception of ALP, increases in liver enzymes (AST, ALP) in plasma in our 28-day inhalation study at high daily nicotine doses (13.5 mg/kg/day) were less pronounced than in a 154-day nicotine exposure study with daily subcutaneous nicotine injections of a much lower daily dose (2.5 mg/kg/day) (Balakrishnan & Menon, 2007a,b). Moreover, plasma levels of cholesterol were increased 2-fold in the nicotine injection study, whereas we observed a 25–35% decrease, together with a 20–30% decrease in glucose, in nicotine-exposed groups, with reductions being more pronounced in female than in male rats. This finding is

Figure 13. Liver histopathology. Vacuolation of hepatocytes. A–C refer to evaluation 1. (A) Severity of vacuolation. (B) Glycogen content of vacuoles was confirmed by staining (PAS, PAS-C and PAS-diastase). D and E refer to evaluation 2. (D) Cytoplasmic vacuolation. (E) Cytoplasmic glycogen content. Mean ± SE. All comparisons against sham; Cochran–Mantel–Haenszel test: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
also consistent with the reduced levels of cholesterol/CE seen in the liver and plasma lipidomic analyses. Taken together, in the nicotine groups, plasma levels of ALT and ALP were increased whereas those of cholesterol and glucose were decreased, concomitant with increased hepatocellular vacuolation along with altered intracytoplasmic deposition of glycogen. ALP is an indicator of cholestasis in rats and humans which, in this study, could be secondary to diffuse swelling of hepatocytes, causing compression of bile canaliculi and ducts (as seen morphologically).

In the respiratory tract, histopathology demonstrated that the mild irritant/particle effects of nicotine-containing aerosols were limited to the sensitive sites of the larynx, and that there were no signs of irritation or inflammation in the lung. These findings are in contrast to results from non-inhalation nicotine-exposure studies in which the authors reported severe histologic changes in the lung (Sudheer et al., 2008; Yokohira et al., 2012). The lack of such severe effects in our nicotine-inhalation study may be explained (at least in part) by continuous administration of the dose over 6h at a well-tolerated concentration that avoids the high bolus-like concentrations obtained if the total daily dose is administered via a single injection/instillation. Reduced toxicity of nicotine upon inhalation administration as compared with a single bolus was also underlined by our acute toxicity study for intra-tracheal instillation of nicotine in which a single dose of 15 mg/kg elicited 80% mortality, whereas an inhaled dose of 13.6 mg/kg/day was well tolerated and did not cause any deaths.

Transcriptomics

Lung

Figure 14 shows the system response profiles as volcano plots for gene expression in the lungs of rats exposed to nicotine or pyruvate or a combination thereof. Changes in gene expression were subtle. Few significant changes in gene expression were observed in nicotine and nicotine/pyruvic acid (medium and high) groups as compared with the PBS group (Figure 14).

One of the two significantly upregulated genes in the nicotine group was RGD1566401. Its human homolog, MEG3, has been shown to be methylated in immortalized lung epithelial cells exposed to cigarette smoke (Hu et al., 2009). Two downregulated genes are related to growth arrest and differentiation (Gas7) and to differentiation of ciliated cells (LamC3). Interestingly, the largest fold change (even though nicotine had FDR <0.05) was observed for the cytochrome P450 family, member A1 (Cyp1a1) gene in all nicotine-containing groups. Moreover, gene expression

Figure 14. System response profiles for lung transcriptomics. Volcano plots of the gene expression in the lungs of male rats after 28-day exposure to nicotine or pyruvate, or a combination thereof, as compared with vehicle control (PBS group). For each gene, change in gene expression, calculated as the log2 (fold change), is plotted on the x-axis. Blue dots indicate non-significant changes in gene levels between comparison groups; red dots indicate significantly upregulated genes in one group as compared with the other group; green dots indicate significantly downregulated genes in one group as compared with the other group.
to be downregulated (as most of the differentially regulated transcripts are unique to the PBS versus sham comparison, and which appear to be downregulated (as most of the differentially regulated gene sets) in most of the nicotine and nicotine/pyruvic acid versus PBS comparisons. Among the few upregulated gene sets in nicotine-exposed groups, those related to energy and lipid metabolism are predominant, particularly in the nicotine/pyruvic acid (low) group. Nicotine alone was associated with downregulation of numerous Community 12 gene sets (signal transduction, immunity and defense), most of which disappeared in combination with pyruvic acid; only one subset was also seen in the nicotine/pyruvic acid (high) group, suggesting a possible nicotine effect. Many gene sets were enriched in single treatment groups only – without an obvious relationship to the concentration of nicotine or pyruvic acid. This can (at least in part) be attributed to the overall low impact of exposures on differential gene expression in the lung.

A more context-specific (but hypothesis-driven) evaluation was also undertaken. These network-based transcriptomic analyses leverage a set of causal biological network models constructed to capture perturbations in signaling processes known to occur in lung cells upon exposure to a range of biologically active substances such as environmental toxins, drugs and cigarette smoke (Gebel et al., 2013; Park et al., 2013; Schlage et al., 2011; Westra et al., 2011, 2013). The biological impact factor (BIF) indicates the overall strength (relative to the control) of perturbations of the biological mechanisms represented by the set of network models. The BIF comprises NPAs that were calculated for the biological mechanisms represented in the set of network and subnetwork models (Martin et al., 2012, 2014). We have recently published a detailed description of its application in a 28-day rat inhalation study (Kogel et al., 2014).

The highest BIF was observed for nicotine versus PBS and, in Figure 16. Figure 16B shows the breakdown of the BIF into the NPAs of the specific biological processes/subnetworks belonging to the set of biological network models (only processes that were significantly perturbed in at least one comparison are shown). For PBS versus sham, perturbations were seen for the immune regulation of tissue repair, macrophage activation and barrier defense of epithelial cells, as well as for protein synthesis in autophagy. These perturbations together make up for a weak BIF that may reflect a general compensatory response to the inhaled aerosol.

NPAs for sodium pyruvate versus PBS did not reach significance in any of the impacted biological processes. However, nicotine and nicotine/pyruvic acid (high) groups had several common significant NPAs for biological processes related to stress, proliferation and senescence of cells, inflammatory processes, tissue repair and angiogenesis (Figure 16B). For nicotine/pyruvic acid (low and medium) groups versus PBS, there was no overall concentration-related NPA magnitude, with a few exceptions: senescence regulation by tumor suppressors, inflammatory processes–mucus hypersecretion or cell stress–oxidative stress. When interpreting these signaling perturbations, such analyses are quite sensitive [compared with the few significant differentially expressed genes (DEGs) seen in system response profiles] and NPA values are shown as relative values per subnetwork, which may exaggerate their absolute contributions that sum up to a BIF for the nicotine/pyruvic acid (high) versus PBS, which is increased with increasing nicotine concentrations (Figure 15). CYP1A1 is a major inducible phase-I xenobiotic-metabolizing enzyme in extrahepatic tissues (including the respiratory tract) and is important for the metabolism of environmental compounds (including cigarette smoke) (Ding & Kaminsky, 2003; Thum et al., 2006). The liver is the principal site of nicotine metabolism, and the major nicotine-metabolizing hepatic phase-I enzyme in Humans is CYP2A6 (Benowitz et al., 2009; Devore & Scott, 2012). However, it has been shown that, under certain conditions, CYP1A1 can be induced in lung tissue not only by classical inducers such as the polycyclic aromatic hydrocarbons and dioxins, but also by nicotine (Iba et al., 1998, 1999; Price et al., 2004; Weems & Yost, 2010; Wei et al., 2002), even though it has been reported that the major nicotine-metabolizing enzyme in the respiratory tract is CYP2A13 (Bao et al., 2005; Devore & Scott, 2012). In the nicotine/pyruvic acid (high) group, only Esm1 (endocan) was upregulated significantly, whereas five genes were downregulated, two of which (Mki67 and Cdkn3) are related to regulation of the cell cycle.

In our previous OECD study, an aerosol with nicotine concentration of 23 μg/L [similar to the nicotine concentration in the nicotine/pyruvic acid (medium) group] from a pMRTP-based tobacco heating technology also did not elicit significant differential gene expression in rat lungs under commonly applied statistical thresholds. Only threshold-free approaches such as gene set enrichment analysis (GSEA) or causal biological network model analysis were suitable to reveal weak exposure effects on gene expression (Kogel et al., 2014). A similar evaluation [GSEA as a hypothesis-free approach and targeted, network-based network perturbation amplitude (NPA) analysis] was conducted here. Results from GSEA (Supplemental Figure S2) suggest several upregulated transcription-related and G-protein signaling-related gene sets unique to the PBS versus sham comparison, and which appear to be downregulated (as most of the differentially regulated gene sets) in most of the nicotine and nicotine/pyruvic acid versus PBS comparisons.
approximately half of the reference BIF for nicotine versus PBS. Moreover, these biological impacts characterize molecular mechanisms in the lung that can obviously compensate for the toxic effects of inhalants because there were no observable morphologic, inflammatory or functional changes in the lung under these exposure conditions.

Liver

Figure 17 shows the system response profiles (volcano plots) for gene expression in the liver. The number of gene-expression changes versus PBS control was small and increased with the concentration of nicotine exposure, but was not influenced by a combination of nicotine with pyruvic acid (low and medium) or by sodium pyruvate alone. Significant DEGs were observed only for the nicotine versus PBS (1 up, 1 down), nicotine with pyruvic acid (medium) versus PBS (1 down) and nicotine with pyruvic acid (high) versus PBS (10 up, 8 down) comparisons. These results suggest that nicotine inhalation alone had a marginal effect on the liver transcriptome, and that nicotine with pyruvic acid (high) also had only a weak effect when usual thresholds were applied.

Threshold-free GSEA was conducted to investigate, with higher sensitivity, the biological mechanism associated with these exposures (Figure 18). Approximately half of the significantly enriched gene sets from the nicotine with pyruvic acid versus PBS comparison (9 of 20) were also seen for the nicotine versus PBS comparison, and three upregulated gene sets were shared across all nicotine-containing exposures: oxidative phosphorylation, 39S-ribosomal subunit–mitochondrial, as well as valine–leucine and
valine–isoleucine degradation. Among other gene sets, significant upregulation was seen in nicotine versus PBS and/or nicotine with pyruvic acid (high) versus PBS. Most of these instances of upregulation were related not only to metabolic reactions (energy, xenobiotics and lipids), but also to gluconeogenesis, biosynthesis of lipids and proteins and the cell cycle. Two commonly downregulated gene sets were related to the immune system (cytokine signaling and interferon signaling). Sodium pyruvate had a unique downregulating impact on all protein biosynthesis gene sets in C9. Moreover, it downregulated C10 cell cycle-related gene sets which, in contrast, were all upregulated when exposed to nicotine with pyruvic acid (high).

With regard to increased liver enzymes, morphologic findings and increased liver weights, the observed decrease in levels of cholesterol and glucose would support a functional alteration in the liver as a result of nicotine exposure that leads to a reduction in the synthesis of cholesterol and glucose as well as a change in fatty-acid metabolism (as indicated, e.g. by increased gene expression of Cyp4a1 and Decr1), and to changes in energy metabolism (as suggested by GSEA). Conversely, increases in the expression of genes related to gluconeogenesis and lipid biosynthesis could be interpreted as compensatory activation of these pathways.

We also examined gene expression of the major xenobiotic metabolism enzymes on microarrays, including those that have been reported to metabolize nicotine in rats (Figure 19). Cyp2b1 and Cyp2b2 have been reported to be the major enzymes that convert nicotine to cotinine in rats (Mwenifumbo & Tyndale, 2009). Involvement of Cyp2a6 in nicotine metabolism has not been observed in rat livers (Howard et al., 2001; Joshi & Tyndale, 2006; Micu et al., 2003), a finding that is in contrast to the situation in Humans [where CYP2A6 is the major hepatic nicotine-metabolizing enzyme (Benowitz et al., 2009; Devore & Scott, 2012; Nakajima et al., 1996)]. CYP enzymes that were observed to be responsible for nicotine metabolism in rat livers or in a reconstituted system (e.g. Cyp1a2, Cyp2c6, Cyp2c11) (Hammond et al., 1991; Iba et al., 1999; Nakajima et al., 1998; Nakayama et al., 1993; Schoedel & Tyndale, 2003) are included in this figure, as well as Cyp1a1 and Cyp4a1 (related to metabolism of fatty acids). Levels of flavin-containing monooxygenases (FMOs), aldehyde oxidases (AOXs) and UDP-glucuronosyltransferases (UGTs) were also examined. Ochiai et al. (2006) reported that the FMO enzyme is responsible for the conversion of nicotine to nicotine N\(^{-}\)-oxide in microvascular endothelial cells in rat lungs. In mice, FMO1 is responsible for this reaction (Itoh et al., 1997). Moreover, although AOX genes are often examined in humans, the gene
is conserved from monkeys, cows, chickens to rodents (mice and rats) and frogs (NCBI gene database). Brandange & Lindblom (1979) reported that the AOX1 gene is responsible for the conversion of nicotine-5'-iminium ion to cotinine.

With the two exceptions mentioned below, all observed increases did not reach significance under the usual threshold (FDR < 0.05). The strongest increase was observed for fatty acid metabolism-related Cyp4A1 only in nicotine and nicotine/pyruvic acid (high) groups, with significance noted only for the latter. For the typical nicotine-metabolizing enzymes Cyp2b2, Cyp1a2 and Cyp2c11, similarly high increases in gene expression (though not reaching significance) were seen for both exposure groups receiving a high nicotine concentration and, just as in the lung, Cyp1a1 was concentration-dependently upregulated also in the liver (significantly so in the nicotine/pyruvic acid (high) group). The
Cyp2e1 gene was upregulated only marginally (Figure 19). Activity of the Cyp2E1 enzyme has been reported to be induced in rat livers by nicotine, but this upregulation appeared to occur at the post-transcriptional level (Howard et al., 2001; Joshi & Tyndale, 2006; Micu et al., 2003). For Acox1 and Ugt1a1, only very weak increases could be seen. All these findings from gene chips should be considered with caution and confirmation; for example, real-time-quantitative polymerase chain reaction, is required to corroborate these non-significant results.

Blood
System response profiles revealed that all of the changes in gene expression in blood samples were very small and did not reach significance (Supplemental Figure S3). The nicotine/pyruvic acid (high) group exhibited a very slight change in the gene profile. However, this differential gene expression would be unlikely to have biological implications.

Systemic versus lung gene expression changes
The effect of pyruvate and nicotine aerosol (alone or as a mixture) upon gene expression was not substantial as compared with the PBS group in all tissues (blood, liver and lung). The greatest alteration in gene expression was detected in the liver in the nicotine/pyruvic acid (high) group, related mainly to perturbation of xenobiotics, lipids and energy metabolism. Although the weak metabolic changes were primarily associated with nicotine concentration, an interaction between nicotine and pyruvate was observed, particularly in the nicotine/pyruvic acid (high) group, thereby suggesting some alteration in energy metabolism in the liver (including induction of beta-oxidation of hepatic lipids). Interestingly, expression of the Cyp1a1 gene was also dose-dependently increased in response to nicotine exposure (with and without pyruvic acid). This observation is consistent with authors reporting the role of nicotine in inducing CYP1A1 expression in lung tissue (Price et al., 2004; Weems & Yost, 2010; Wei et al., 2002). In the lung, exposure to nicotine or pyruvate affected gene expression only marginally, and the most prominent upregulation was for Cyp1a1.

Lipidomics
Lung, liver and plasma samples from all groups except the nicotine/pyruvic acid (low) group were analyzed using the following technologies/platforms: shotgun lipidomics, ceramide and cerebroside lipidomics, sphingosine lipidomics, eicosanoid lipidomics, ganglioside lipidomics and free cholesterol quantification. Combined results for the relative differences of the general lipid classes are shown in Figures 20–22. A further breakdown into subclasses is provided in Supplementary material.

Lung
Exposure to nicotine or nicotine/pyruvic acid compared with PBS resulted in significantly increased levels of CE in rat lungs (Figure 20). A similar trend was observed in response to exposure to sodium pyruvate but this difference did not reach significance.

Other significant exposure-related differences at the general lipid class level [increases in diacylglycerol (DAG), alkanyl-linked phosphatidylethanolamine and phosphatidyethanolamine] were confined to the nicotine/pyruvic acid (medium) group, and to the sodium pyruvate group (decrease in ganglioside D1). In all other lipid classes, no significant exposure-related changes were observed at the lipid class level (Figure 20). However, a breakdown into the subclass level revealed significant changes for specific lipid species (e.g., significant increase in the arachidonic acid metabolite 12-hydroxyeicosatetraenoic acid, and in specific sphingolipids in the nicotine and pyruvic acid (high) group). For details, see the Supplementary material and Supplemental Figures S4–S6.

Liver
At the general lipid class level, nicotine alone or sodium pyruvate alone was associated mainly with decreased levels of steryl esters (CE) and TAG (Figure 21). Nicotine/pyruvic acid (medium and high) or nicotine alone reduced the level of CE more efficiently than sodium pyruvate alone. Moreover, all exposure groups containing nicotine and/or pyruvate caused a significant reduction in TAG. Only nicotine alone also caused a decrease in the amounts of ceramides, “globo-series” (Gb3) and phosphoserines (PS) in the liver. No significant differences were observed for the PBS versus sham comparison. These findings are in contrast to some results obtained for non-inhalation nicotine administration: significant increases in hepatic levels of cholesterol, free fatty acids, phospholipids and triglycerides, accompanied by histopathologic changes have been seen in Wistar rats injected daily (s.c.) with nicotine for 154 days (Adluri et al., 2008; Balakrishnan & Menon, 2007b). Conversely, Friedman et al. (2012) found that nicotine injection into male C57BL/6 mice did not affect hepatic levels of TAGs or hepatic lipid accumulation. Additional results from subclass-level analyses are provided in Supplementary material and Supplemental Figures S7–S9.

Plasma
Exposure to nicotine alone or as a mixture with pyruvic acid significantly reduced levels of several main lipid classes in plasma: FC, CE and phosphatidylcholine (PC) (Figure 22). Moreover, nicotine and nicotine/pyruvic acid (high) groups had significant decreases in levels of DAG and sphingomyelin, suggesting that nicotine may be the reason for decreased lipid levels in plasma (Figure 22). A decrease in levels of phosphatidyl inositol was more pronounced in the nicotine/pyruvic acid groups but did not reach significance in the nicotine or sodium pyruvate groups, suggesting some combinatorial activity (Figure 22).

Effects of nicotine and pyruvic acid or sodium pyruvate were also examined at the molecular subclass level (i.e., lipid species) (Supplementary material and Supplemental Figures S10–S12).

Our results suggesting a broad decrease in levels of various plasma lipids upon nicotine inhalation are in contrast with published effects of non-inhalation administration of nicotine. That is, Wistar rats given nicotine in drinking water had
increased plasma levels of TAG and total cholesterol, as well as accumulated fat globules in the liver (steatosis) (Valenca et al., 2008). A similar observation of increased plasma levels of lipids was found in SD rats with nicotine administered via the oral route for 90 days or by subcutaneous injection for 15 days (Ashakumary & Vijayammal, 1997; Chattopadhyay & Chattopadhyay, 2008).

### Systemic versus lung lipid changes

Effects of pyruvate- and nicotine-containing aerosols (alone or as a mixture) upon lipid levels in the plasma, liver, and lung after 28 days of exposure were more pronounced than the changes in gene expression. In plasma, nicotine-containing aerosols decreased (often concentration-dependently) the

![Figure 20](image-url)  
**Figure 20.** Relative difference (%) of the general lipid classes in rat lungs upon exposure to nicotine or nicotine pyruvate. Horizontal bar graphs show relative increase (red) or decrease (blue) of lipid levels in the sodium pyruvate, nicotine, and nicotine/pyruvic acid groups as compared with the PBS group (vehicle control). *p < 0.05. For abbreviations, see in the Figure.

![Figure 21](image-url)  
**Figure 21.** Relative difference (%) of general classes of lipids in rat livers. Horizontal bar graphs show relative increase (red) or decrease (blue) of lipid levels in sodium pyruvate, nicotine, and nicotine/pyruvic acid groups as compared with the PBS group (vehicle control). *p < 0.05. For abbreviations, see in the Figure.
levels of lipids from major plasma lipid classes (FC, CE, PC), whereas pyruvate alone did not affect plasma lipid levels. Thus, the effects observed by exposure to nicotine/pyruvic acid seemed to be driven by nicotine. A lowering effect of nicotine was observed in major lipid classes of plasma. In the liver, exposure to pyruvate and nicotine reduced hepatic levels of CE and TAG, suggesting that both can alter lipid metabolism. Nicotine had an overall lipid-lowering effect, and was shown to specifically alter sphingolipid contents and to lower lipids containing fatty acyl 20:4 in the liver and plasma. This observed lipid-lowering effect of inhaled nicotine contrasts with reported effects of non-inhalation nicotine administration which, in general, increase lipids and cholesterol in the liver and plasma (Adluri et al., 2008; Ashakumary & Vijayamall, 1997; Balakrishnan & Menon, 2007b; Chattopadhyay & Chattopadhyay, 2008; Valenca et al., 2008).

In the lung, more subtle effects on the lipidome were observed. Unlike in plasma and the liver, increases in CE and of several minor phospholipid and sphingolipid species were observed upon nicotine exposure. In the literature, an increase in the concentration of free fatty acids in the lung has been reported for rats exposed to nicotine subcutaneously for 154 days (Balakrishnan & Menon, 2007b).

Summary and conclusions

The objective of this study was to determine classical toxicity parameters (according to OECD TG 412) in combination with systems toxicological endpoints in SD rats after exposure to nebulized nicotine and pyruvic acid dissolved in PBS, compared with nebulized nicotine- and pyruvic acid- sham- and vehicle-exposed groups. Rats were exposed (nose-only) to test substances (nicotine and pyruvic acid at three concentrations) or to reference aerosols (nicotine alone or sodium pyruvate alone) in a 28-day inhalation study. The additional systems biology approach was undertaken to identify gene-expression network perturbations and lipidomics changes that may reveal the mechanistic details of exposure-related changes.

In conclusion, the nebulizer approach was successful in generating nicotine-containing aerosols with sufficiently high nicotine concentrations. All aerosols were inhaled efficiently and well tolerated by rats during the exposure period. Analyses of the hematology, serum and BALF analytes did not suggest extensive toxic effects by nicotine and pyruvate aerosols at comparably high nicotine doses that exceeded the equivalent nicotine doses achievable by heavy smoking. Histologic analyses revealed mild irritation in the larynx, but no signs of irritation, inflammation or injury were seen in the lung. In the liver, a nicotine-related increase in glycogen vacuoles was detected, but there were no signs of fibrosis or lipid accumulation. These results further suggest that aerosols of nicotine or pyruvate (or a combination thereof) did not result in substantial toxicity or damage-related alteration of gene-expression profiles in the blood, liver and lung. There was some indication that inhalation of nicotine plus pyruvic acid at a high concentration resulted in alteration of energy and lipid metabolism in the liver. Lipidomics analyses suggested a general, slight increase of lipid species in the lung. However, a decrease in lipid species including cholesterol, DAG, PC and sphingomyelin, as well as decreased levels of arachidonic acid-containing lipids, cholesterol and TAG were observed in the blood and liver, respectively.

Nicotine inhalation in general resulted in a greater impact than that of pyruvate due to the following observations: (i) effects were, in general, observed in nicotine-only and nicotine/pyruvic acid groups, (ii) changes were, in general, concentration-dependent (nicotine and nicotine/pyruvic acid low, medium, high), (iii) effects were not seen in the sodium pyruvate-exposed reference group and (iv) a few interaction effects were seen on the molecular level for the nicotine/ pyruvic acid (high) group, but they were not reflected by the endpoints of toxicity.

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Declarations of interest

The authors declare that there are no conflicts of interest.

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Supplementary material available online