Examining the effects of cigarette smoke on mouse lens through a multi OMIC approach

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Here, we report a multi OMIC (transcriptome, proteome, and metabolome) approach to investigate molecular changes in lens fiber cells (FC) of mice exposed to cigarette smoke (CS). Pregnant mice were placed in a whole-body smoke chamber and a few days later pups were born, which were exposed to CS for 5 hours/day, 5 days/week for a total of 3½ months. We examined the mice exposed to CS for CS-related cataractogenesis after completion of the CS exposure but no cataracts were observed. Lenses of CS-exposed and age-matched, untreated control mice were extracted and lens FC were subjected to multi OMIC profiling. We identified 348 genes, 130 proteins, and 14 metabolites exhibiting significant (p < 0.05) differential levels in lens FC of mice exposed to CS, corresponding to 3.6%, 4.3%, and 5.0% of the total genes, protein, and metabolites, respectively identified in this study. Our multi OMIC approach confirmed that only a small fraction of the transcriptome, the proteome, and the metabolome was perturbed in the lens FC of mice exposed to CS, which suggests that exposure of CS had a minimal effect on the mouse lens. It is worth noting that while our results confirm that CS exposure does not have a substantial impact on the molecular landscape of the mouse lens FC, we cannot rule out that CS exposure for longer durations and/or in combination with other morbidities or environmental factors would have a more robust effect and/or result in cataractogenesis.

Cataract is a clouding or opacity of an otherwise transparent lens of the eye. Cataracts can be classified into the following two types: congenital cataracts caused by mutations in genes essential for development of the lens and maintenance of its transparency1–3, and age-related cataracts, a multifactorial disorder involving genetic susceptibility loci and environmental factors i.e., cigarette smoke (CS), and ultraviolet (UV) exposure, etc. 4–6. Age-related cataract represents a significant burden of blindness worldwide that will grow as the age expectancy and population increases globally7.

CS has been identified as an important risk factor for development of cataracts8,9; however, the molecular mechanism of this association remains elusive. CS is a complex mixture of metal ions and different compounds responsible for the generation of reactive oxygen species (ROS)10. Trace and heavy metals have been reported in rat lens exposed to tobacco smoke11. In a recent study p-benzoquinone induced changes are reported as a causative factor of CS-related cataractogenesis in guinea pig lens12.

The ocular lens consists of two, morphologically distinct, cell subpopulations: a monolayer of epithelial cells on the anterior side of the lens, and terminally differentiated fibers cells (FC) that account for the majority of lens volume13. Lens FC are generated throughout life by differentiation of lens epithelial cells; however, since lens FC are anuclear and organelle-free, they do not have the ability to repair, and therefore, damage to lens FC results in the opacification of an otherwise transparent lens13.

Here, we adopted a multi OMIC (transcriptome, proteome, and metabolome) approach to investigate molecular changes in lens FC of mice exposed to CS. We identified 348 genes, 130 proteins and 14 metabolites exhibiting significant (p < 0.05) differential levels including diminished levels of the branched-chain amino acid (BCAA)-related metabolites in lens FC of mice exposed to CS. To the best of our knowledge, this is the first report of a multi OMIC approach to investigate changes at a molecular level in lens FC of mice exposed to CS.

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Results and discussion

Here, we evaluate changes at a molecular level in lens FC of mice exposed to CS through a multi OMIC (transcriptome, proteome, and metabolome) approach. A total of 32 newborn pups were exposed to CS in a smoke chamber for 5 hours/day, 5 days/week for a total of 3½ months (105 days). The mice exposed to CS were examined for CS-related cataractogenesis. The CS-exposed and age-matched, untreated control (Ct) mice were euthanized, lenses were extracted, lens FC were separated from the epithelium and used for next-generation RNA sequencing-based transcriptome, mass spectrometry-based proteome, and mass spectrometry-based metabolome profiling. The replicates of FC samples from both CS-exposed and untreated Ct mice consisted of lens from equal number of male and female mice.

We performed next-generation RNA sequencing (RNA-Seq)-based transcriptome, mass spectrometry-based proteome, and mass spectrometry-based metabolome profiling of lens FC exposed to CS. The lenses were extracted from CS-exposed and untreated Ct mice and FC were separated from lens epithelium under a microscope. The extracted lens FC were maintained in distinct pools from either four right or four left eyes to serve as biological replicates. As illustrated in Fig. 1, the right eyes were used for transcriptome while the corresponding left eyes were used for proteome profiling. As 25 mg of lens FC mass is required for the metabolome analysis, each biological replicate from either CS-exposed or untreated Ct mice consisted of lens FC from both eyes of four mice.

The next-generation sequencing resulted in 424.07 × and 397.36 × sequence coverage for lens FC of CS-exposed and untreated Ct mice, respectively. The mapped reads were assembled into transcripts, and gene expression was measured and normalized using the fragment per kilobase per million mapped reads (FPKM) method, which identified the expression (≥ 1.0 FPKM) of 9531 and 9590 genes in lens FC of CS-exposed and untreated Ct mice, respectively (Supplementary Data 1). The analysis identified 348 differentially expressed (DE) genes (p < 0.05), including 186 down- and 162 up-regulated genes in lens FC of mice exposed to CS (Supplementary Data 1). Moreover, an additional analysis was performed to determine the differential gene expression in lens FC of mice exposed to CS. The standard deviation (SD) was calculated for each transcript expression fold change (log2) as a deviation from its mean of 0 or no change. The analysis revealed 332 DE genes (± 2 SD), including

**Figure 1.** Illustration of the workflow adopted to investigate the effects of cigarette smoke (CS) on mouse lens fiber cells (FC) through a multi OMIC (transcriptome, proteome, and metabolome) approach. A total of 32 newborn mice were exposed to CS in a smoke chamber for 5 hours/day, 5 days/week for a total of 3½ months (105 days). The mice exposed to CS exposure were examined for CS-related cataractogenesis. The CS-exposed and age-matched, untreated control (Ct) mice were euthanized, lenses were extracted, lens FC were separated from the epithelium and used for next-generation RNA sequencing-based transcriptome, mass spectrometry-based proteome, and mass spectrometry-based metabolome profiling. The replicates of FC samples from both CS-exposed and untreated Ct mice consisted of lens from equal number of male and female mice.
201 down- and 131 up-regulated genes in lens FC of mice exposed to CS (Fig. 3A). Col4a4, Col4a5, and Col6a3 were among the down-regulated genes in lens FC of mice exposed to CS (Supplementary Data 1). Mutations in Col4a4 and Col4a5 result in Alport syndrome along with developmental cataracts. Additionally, we identified the differential expression of Prmt3, Rarb, Caprin2, Gdi2, Oat, Herc1, and Agl in lens FC of mice exposed to CS (Supplementary Data 1). It is worth noting that loss of Prmt3, Rarb, Caprin2, Gdi2, Oat, Herc1, and Agl have been associated with abnormal lens FC morphology and cataracts in mice (http://www.informatics.jax.org/).

In parallel, mass spectrometry generated a total of 84,063 peptide spectrum matches (PSMs), yielding 23,882 total peptides, corresponding to 2323 proteins. We had previously reported 5404 proteins in the mouse lens proteome14, and since the number of proteins identified here was significantly less (i.e., 2323 vs. 5404), we performed a second 8-plex TMT experiment to rule out the possibility of a technical error. We detected 2352 proteins in the second TMT experiment, a nearly similar protein count identified in the first TMT experiment.

Figure 2. Evaluation of lens phenotype in cigarette smoke (CS)-exposed and age-matched, untreated control (Ct) mice. The 32 mice exposed to CS were examined for CS-related cataractogenesis after completion of CS exposure and age-matched untreated Ct mice but no cataracts were observed. The images of the lens of mice exposed to CS (CS1–32) were similar to images of the lens of age-matched, untreated Ct mice (Ct1–4). The four adult female mice exposed to CS were examined, 9-months post-exposure for development of cataracts but no cataracts were observed in these mice (CS33–36). The koR and koL illustrate the cataractous lens (right and left eyes, respectively) from a cataract-causing gene knockout mouse.
Perhaps, the age of the mice i.e., embryonic, and early postnatal lens compared to 3 months old mice lens and/or the use of the whole lens (lens epithelium and FC used in previously reported study) compared to lens FC used in the current study may have contributed to the difference in protein counts.

The combined analysis of both proteome datasets identified a total of 130 proteins with differential levels (p < 0.05), including 42 exhibiting elevated and 88 diminished levels in lens FC of mice exposed to CS (Supplementary Data 2). To further validate the DE protein, we reanalyzed the proteome datasets by calculating the SD for each protein fold change (log2) as a deviation from its mean of 0 or no change. The analysis revealed 101 DE proteins (> ± 2SD), including 59 exhibiting elevated and 42 diminished levels in lens FC of mice exposed to CS (Fig. 3B). We identified the elevated levels of CTNN2, a delta catenin protein in lens FC of mice exposed to CS (Supplementary Data 2) that has recently been associated with age-related cortical cataracts15. Additionally, we identified a decreased levels of CHORDC1, CRYGB, GCLC, HSD17B4, PANK4, PCBD1, OARD1, and HIP1R in lens FC of mice exposed to CS (Supplementary Data 2). Loss of CHORDC1, CRYGB, GCLC, HSD17B4, PANK4, PCBD1, OARD1, and HIP1R has been associated with abnormal lens morphology and cataracts in mice (http:// www.informatics.jax.org/). The proteome also revealed diminished levels of GCLC, PCBD1, CRYGB, and PANK4 in lens FC of mice exposed to CS (Supplementary Data 2). Fan and colleagues recently identified nuclear cataracts in lens-specific Gclc knockout mice16. Bayle and colleagues reported hyperphenylalaninemia and cataracts in Pcbd1 knockout mice17. Liu et al. reported a point mutation in Crygb responsible for dominant nuclear cataracts in mice18. Sun and colleagues reported a mutation in PANK4 liable for congenital posterior cataracts and importantly the Pank4 null mice develop cataracts19.

Next, we completed the mass spectrometry-based metabolome profiling of lens FC of mice exposed CS. We identified a total of 280 metabolites in lens FC of mice exposed to CS (Supplementary Data 3). Among the 280
metabolites identified in lens FC of mice exposed to CS, we detected the differential levels of 14 metabolites (p < 0.05), including eight metabolites exhibiting higher levels and six metabolites displaying lower levels (Supplementary Data 3). We identified a decrease in branched-chain amino acid (BCAA)-related metabolites in lens FC of mice exposed to CS (Fig. 4A) including isoleucine, leucine, and valine, which are essential amino acids with key roles in protein synthesis, energy metabolism, and cell signaling.

The BCAA metabolites have reported being involved in oxidative metabolism and energy production through a catabolic pathway involving branched-chain α-keto acid dehydrogenase (BCKD), a multi-enzyme complex, which catalyzes BCAAs to the corresponding acyl-CoA derivatives (Fig. 4B). We identified diminished levels of alpha-hydroxyisocaproate, 1-carboxyethylleucine, isoleucine, leucine, and valine in lens FC of mice exposed to CS (Fig. 4C–G)
The machine was adjusted to produce sidestream (89%) and mainstream (11%) aerosol. The premise of the experiment was based on the results of two independent studies in mice exposed to CS, we sought evidence of CS exposure by examining Cadmium (Cd), and Nickel (Ni) in the lungs of mice exposed to CS. The metal ion concentrations are normalized for sample mass and dilution. The metal ion concentrations are a mean value of five scans. RSD (relative standard deviation) is standard deviation divided by the mean value of the five scans (concentration in Table 1) and reported as a percentage. The detection limits were 0.2, and 2.0 ppb for Cd, and Ni respectively. Ct-ml control mouse lungs, CS-ml cigarette smoke-exposed mouse lungs, ppb parts per billion (also equilivalent to ng/g).

| Sample   | Cadmium Concentration (ppb) | RSD | Nickel Concentration (ppb) | RSD |
|----------|-----------------------------|-----|-----------------------------|-----|
| Ct-ml-1  | 0.253                       | 26.0| 12.150                      | 10.2|
| Ct-ml-2  | 0.519                       | 19.0| 16.455                      | 4.8 |
| Ct-ml-3  | 0.196                       | 19.5| 9.977                       | 7.7 |
| Ct-ml-4  | 0.538                       | 12.1| 19.550                      | 8.3 |
| CS-ml-1  | 1.487                       | 3.6 | 20.152                      | 5.1 |
| CS-ml-2  | 1.366                       | 8.6 | 20.863                      | 2.0 |
| CS-ml-3  | 1.541                       | 10.1| 23.134                      | 8.7 |
| CS-ml-4  | 1.178                       | 8.2 | 25.652                      | 5.0 |

Table 1. Mass spectrometry-based metal ions analysis of lungs from mice exposed to cigarette smoke (CS) and age-matched, untreated control mice. The quantification shows differential concentrations of cadmium (Cd), and nickel (Ni) in the lungs of mice exposed to CS. The metal ion concentrations are normalized for sample mass and dilution. The metal ion concentrations are a mean value of five scans. RSD (relative standard deviation) is standard deviation divided by the mean value of the five scans (concentration in Table 1) and reported as a percentage. The detection limits were 0.2, and 2.0 ppb for Cd, and Ni respectively.

Since we observed a minimal effect of CS exposure on the molecular landscape of lens FC with no cataracts in mice exposed to CS, we sought evidence of CS exposure by examining Cadmium (Cd), and Nickel (Ni) in the lungs of mice exposed to CS. The premise of the experiment was based on the results of two independent studies reporting a 3.1- and 1.7-fold increase in Cd concentration in the serum of tobacco cigarette smokers. The premise was also based on the identification of Cd and Ni in mainstream particulates of cigarettes containing tobacco.

We performed total metal quantification in lungs of mice exposed to CS using an Agilent 7500cx inductively coupled plasma mass spectrometer. The analysis identified a 3.70-fold \((p = 0.0001)\) and a 1.54-fold \((p = 0.01)\) higher concentration of Cd and Ni, respectively in the lungs of mice exposed to CS compared with untreated Ct mice (Table 1 and Supplementary Table 3). The statistically significantly higher concentrations of Cd and Ni in the lungs of mice exposed to CS are in line with chronic exposure to CS.

In summary, we report results of a multi OMIC approach to examine the molecular landscape of mice lens FC exposed to CS for 3½ months, which confirmed a minimal effect on the molecular landscape of lens FC. However, we cannot rule out that CS exposure for longer durations \((i.e., > 3½ months)\) and/or in combination with other morbidities or environmental factors would have a more robust effect and/or result in cataractogenesis.

Materials and methods

Animals included in the study. The use of mice in this study was approved by the Johns Hopkins Animal Care and Use Committee (ACUC; Baltimore, MD, USA), and all experiments were performed in accordance with the approved protocol by the Institutional Review Board (IRB) of the Johns Hopkins University School of Medicine (Baltimore, MD) and consistent with the Association of Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. The C57BL/6J mouse strain (Stock # 000664; Jackson laboratory) was used for all experiments.

The study design included placing pregnant mice (gestation days 18–20) in a whole-body smoke chamber and a few days later pups were born. Of these, four adult females along with 32 pups (16 males and 16 females) were exposed to CS for 5 hours/day, 5 days/week, and remained in the chamber for a total of 3½ months (105 days). In parallel, age-matched, four adult female mice and 32 (16 males and 16 females) pups were housed in the animal facility for 3½ months that served as untreated controls.

Exposure to CS in a whole-body smoke chamber. The mice were exposed to CS as described. The smoke chamber contained a smoking machine (TE-10, Teague Enterprises, Davis, CA) that burns five cigarettes (2R4F reference cigarettes (2.45 mg nicotine/cigarette; Tobacco Research Institute, University of Kentucky) at a time, taking two-second duration puffs at a flow rate of 1.05 l/min, to provide a standard puff of 35 cm³, providing a total of eight puffs per minute. The machine was adjusted to produce sidestream (89%) and mainstream...
smoke (11%). The chamber atmosphere was monitored to maintain total suspended particulate at 90 mg/m³ and carbon monoxide at 350 ppm.

Evaluation of lens phenotype in CS-exposed and untreated Ct mice. The mice were examined for CS-related cataractogenesis after completion of CS exposure. The eyes of CS-exposed and untreated Ct mice were dilated using tropicamide (1%) and phenylephrine (2.5%) followed by anesthesia by ketamine/xylazine (100 mg/kg body weight for ketamine and 16 mg/kg body weight for xylazine). The lenses were examined with a slit-lamp microscope and images were taken by Phoenix Micron III Retinal Imaging Microscope (Phoenix Research Labs, Pleasanton, CA, USA).

Extraction of the lenses and separation of lens FC. The mice were anesthetized with isoflurane and euthanized through cervical dislocation. The lenses were extracted from CS-exposed and untreated Ct mice and FC were separated from the lens epithelium using forceps under a microscope. The lens FC from CS-exposed and untreated Ct mice was maintained at −80 °C in distinct pools (i.e., biological replicates), each consisting of FC of the right eye or the left eye.

We used four biological replicates (two male and two female) of the CS-exposed and untreated Ct mice, each consisting of lens FC of the right eyes for transcriptome profiling and their respective left eyes for proteome profiling. As a minimum of 25 mg of lens FC mass was required for metabolome analysis, each of the four biological replicates of the CS-exposed and untreated Ct mice consisted of eight lens FC (both right and left eyes) from four male or four female mice.

Transcriptome profiling. Next generation RNA-Seq of lens FC of CS-exposed and untreated Ct mice was performed commercially by Novogene Corporation Inc. (Sacramento, CA). A total of four biological replicates, each consisting of pooled lens FC of the right eyes of four CS-exposed and untreated Ct mice was used for transcriptome profiling and the RNA-Seq data were analyzed as described.

The FPKM expression values were imported in the Spotfire DecisionSite with Functional Genomics (TIBCO Spotfire, Boston, MA) software for further evaluation and graphical representation. All transcripts log2-fold changes between lens FC of CS-exposed and untreated Ct mice were analyzed to determine the SD from their mean of 0, which represents no change.

Proteome profiling. Four biological replicates, each consisting of pooled lens FC of the left eyes of four CS-exposed and four untreated Ct mice were used for proteome profiling. A total of 200 μg of protein for each sample was used for each replicate. The reduction and alkylation of the proteins were conducted with 10 mM of tris (2-Carboxyethyl) phosphine hydrochloride and 40 mM of chloroacetamide for 1 h at room temperature. The sample was used for each replicate. The reduction and alkylation of the proteins were conducted with 10 mM of tris (2-Carboxyethyl) phosphine hydrochloride and 40 mM of chloroacetamide for 1 h at room temperature. Mass spectrometry-based proteome profiling was completed as described.

The abundance values of reporter ion intensities from the 8-plex TMT experiment were imported into Partek Genomics Suite v6.6 (Partek, Inc., St. Louis, MO, USA) for protein annotation and differential expression analysis. The normalized reporter ion intensities were examined for SD to investigate the differential expression in lens FC of mice exposed to CS compared with lens FC of untreated Ct mice. The p values were estimated by a two-tailed t-test, assuming a hypothesized mean of 0 change. The normalized ratios were converted to a log2 scale (becoming the conventional “log-ratios” or “log2 fold changes”) for statistical and graphic representation.

Metabolome profiling. Four biological replicates, each consisting of pooled lens FC, wet mass (25 mg) of four CS-exposed and untreated Ct mice (right and left) were used for metabolome profiling. The FC were frozen immediately at −80 °C until further processing for metabolome profiling. Metabolome profiling and data analysis were performed commercially by Metabolon, Inc. (Morrisville, NC, USA) as described.

Sample preparation involved metabolite extraction with methanol and the resulting extracts were analyzed on an accurate mass global metabolomics platform consisting of multiple arms differing by chromatography method and mass spectrometry ionization mode. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard using software developed at Metabolon.

Metal ion analysis. Metal ion quantification and data analysis were performed by the Biomarker Mass Spectrometry Facility (UNC-Chapel Hill, NC). A total of four biological replicates, each consisting of pooled mouse lungs wet mass (250–460 mg) of four CS-exposed and untreated Ct mice were used for metal ion analysis. Mouse lungs were frozen immediately at −80 °C upon extraction, until further processing. The samples were digested with a combination of concentrated nitric acid, 30% hydrogen peroxide, and heat. The digested samples were diluted to 4 ml with deionized water. Quantification of total Cd, and Ni, was performed using Agilent 7500cx inductively coupled plasma mass spectrometer (ICP-MS; Santa Clara, CA) as described.

ARRIVE guidelines. This study was completed in compliance with the ARRIVE guidelines.

Data availability RNA-Seq raw reads and processed data of lens FC of CS-exposed and untreated Ct mice have been deposited in the NCBI Gene Expression Omnibus and are accessible through the GEO accession number GSE144818. The mass spectrometry data of lens FC of CS-exposed and untreated Ct mice have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017414 and PXD020421.
The metabolome raw data of lens FC of CS-exposed and untreated Ct mice have been deposited in the Metabo-Lights repository and are accessible through study identifier MTBLS207.

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Author contributions
S.Y.K., M.A., and S.A.R.: conceived and designed the experiments; C.H.N., J.T.H., and S.A.R.: contributed reagents, materials, and analytical tools; S.Y.K., M.A., Y.J., T.R., A.J.S., B.O.I., P.H.C., and C.H.N.: performed experiments; S.Y.K., M.A., Y.J., T.R., A.J.S., B.O.I., P.H.C., C.H.N., and S.A.R.: analyzed the data; S.Y.K., M.A., Y.J., T.R., A.J.S., B.O.I., P.H.C., C.H.N., J.T.H., and S.A.R.: contributed to writing the manuscript.

Competing interests
The authors declare no competing interests.

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