Effects of Short- and Long-Term Exposure to E-Cigarette Vapour

DZL – Deutsches Zentrum für Lungenforschung
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2019 Balzan Prize for Pathophysiology of Respiration: from Basic Sciences to the Bedside

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Administrating Institution: Deutsches Zentrum für Lungenforschung
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Erika von Mutius is a pediatrician and allergologist at the Helmholtz Center in Munich, and at the Ludwig-Maximilians-University of Munich. Klaus F. Rabe is Medical Director and Managing Director at Grosshansdorf Hospital and W3 Chair in Internal Medicine/Pulmonology at the University of Kiel. Werner Seeger is Director and Scientific Member at the Max Planck Institute for Heart and Lung Research, Bad Neuheim. Tobias Welte was Professor of Pulmonary Medicine and Head of the Department of Pulmonary and Infectious Disease at Hannover University School of Medicine.

General Report

The winners of the Balzan Prize 2019 – Erika von Mutius, Klaus Rabe, Werner Seeger and Tobias Welte – proposed to dedicate their Balzan Prize funds to the cross-disciplinary project *Effects of Short- and Long-Term Exposure to E-Cigarette Vapour*. The project is divided into three subprojects, listed below. A group of DZL early career scientists started Subprojects 1 and 2 in 2020; Subproject 3 started in 2021.

1. Early responses to e-cigarettes and their components: airway structural changes, signaling pathways and survival in *Drosophila melanogaster* in the context of risk alleles (DZL site ARCN)
2. Effect of short- and long-term exposure to e-cigarette vapour on inflammation, endothelial barrier function and structural alterations in murine lungs (DZL sites BREATH and UGMLC)
3. Association of e-cigarette use with respiratory status in lung-healthy subjects and pulmonary patients (DZL sites CPC-M and TLRC)

After temporary closures and delayed progress in 2020 and 2021 due to the COVID-19 pandemic, the project gained momentum and will continue until the end of 2024.

Scientific Report

Subproject 1: Early responses to e-cigarettes and their components: airway structural changes, signaling pathways and survival in *Drosophila melanogaster* in the context of risk alleles (Dr. Hanna Angstmann, Prof. Dr. Susanne Krauss-Etschmann,

As reported previously, marked morphological and airway transcriptional changes in the offspring of

mothers exposed to nicotine vapour (e-nicotine) in the team's experiments with *Drosophila* had been observed (El-Merhie *et al.*, 2021). had been observed. A more general transcriptional analysis also showed a sex-specific altered expression in the respiratory tract of *Drosophila* larvae. This supports the aforementioned published results and highlights the importance of considering the sexes separately. Because the above data showed that maternal e-nicotine negatively affects offspring, they began using

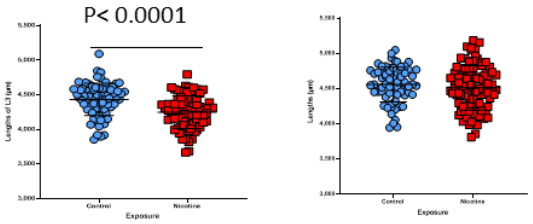

Figure 1: Length of L3 larvae (4 recplicates, mean +/- SD, unpaired t-test). Blue: water, Red: nicotine. Left: males, Right: females

commercially available ecigarettes. Care has been taken to use the same brand and composition as the project partners in UGMLC. Initial data indicate that the offspring of mothers treated with vapour are smaller. This was already visible when only the carrier liquid of e-cigarettes (propylene glycol and glycerine) has been used. Furthermore, the airways showed morphological changes. The team aims to confirm these results in replicate experiments. Using *Drosophila* larvae as a model for early life, they have further demonstrated that cigarette smoke enters the entire larval airway system, where it activates cyp18a1, which is homologous to human CYP1A1 to metabolize Csderived polycyclic aromatic hydrocarbons and further induces heat shock protein 70. These results have been published by Sirocko *et al.* (2022).

In a second aspect, they investigated whether paternal exposure to enicotine also has an influence on the offspring. The size of male offspring was highly and significantly reduced in the larvae (L3) stage (Figure 1), while female offspring were bigger than adult flies (Figure 2).

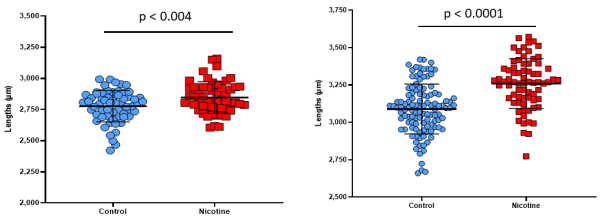

Figure 2: Length of adult offspring flies (2 replicates, mean +/- SD, unpaired t-test). Blue: water, Red: nicotine. Left: males, Right: females

Of note, the length of terminal airway cells, which are finger-shaped cells located at the tip of secondary branches and are responsible for gas exchange, was significantly reduced in males only (Figure 3). In addition, the number of "fingers" was reduced in these cells. To obtain insight into potential mechanisms underlying the structural changes, airways were isolated from these flies and are currently subjected to RNA sequencing.

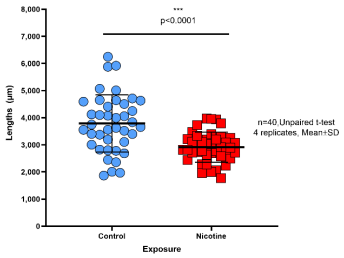

Figure 3: Length of terminal cells in male F1 generation

Next, an investigation was conducted to determine if airway structural changes in offspring translate into reduced physiological fitness. Male F1 larvae from enicotine-exposed fathers crawled significantly slower than male offspring form 'control' fathers, which has not been seen in female larvae. In adult flies of the F1 generation, however, female flies from e-nicotine-exposed fathers had a significantly reduced locomotor activity. In a next step, we plan to use lactate dehydrogenase reporter lines to indirectly assess if airway structural changes lead to tissue damage via reduced oxygen supply.

Subproject 2: **Effect of short- and long-term exposure to e-cigarette vapour on inflammation, endothelial barrier function and structural alterations in murine lungs – preliminary results** (Prof. Dr. Sascha David & Dr. Benjamin Seeliger, Hannover Medical School, DZL site BREATH; PD Dr. Natascha Sommer, Justus-Liebig University, Giessen, DZL site UGMLC)

1. BREATH subproject: Effect of exposure to nicotine containing e-cigarette vapour on endothelial barrier integrity in human-umbilical cord endothelial cells (HUVECs)

The subproject at BREATH investigates the impact of e-cigarette vapour extract (ECVE) on endothelial barrier integrity in human umbilical cord endothelial cells (HUVECs). Previous findings demonstrated a dose-dependent increase in supernatant angiopoietin-2 (ANGPT-2), a marker of endothelial damage, when HUVECs were exposed to ECVE with or without nicotine.

The project began with experiments on the interplay between ECVE-induced endothelial damage and inflammation (here: stimulation by lipopolysaccharide (LPS)). We analyzed the effect of preincubation with ECVE followed by lipopolysaccharide (LPS) stimulation on the Angiopoietin/TIE2 system as well as continuous trans-endothelial resistance (TER) measurements using an electrical cell impedance sensing (ECIS) device. Pre-incubation with ECVE medium increased the susceptibility of HUVECs to subsequent LPS stimulation by 20% with regard to ANGPT-2 production. The ANGPT2/TIE2 mRNA ratio increased significantly with nicotine exposure comparable to effects of LPS alone and was even higher with combined nicotine and LPS stimulation. On the functional level, pre-treatment with nicotine-containing ECVE resulted in an increased susceptibility to LPS-induced reduction in endothelial barrier integrity. To evaluate potential treatment options within the Angpt2/Tie2 pathway and ECVE-induced damage, the team induced phosphorylation of the protein kinase Akt by a monoclonal TIE2 agonist (PMC-403).

In a trans-well model with human PBMC and HUVECs, an alteration of the absolute transmigration of PBMC was not detected. However, ECVE selectively down-regulated CD4 T Cell transmigration. In the most recent experiments, it was found that trans-endothelial migration induced by LPS could be normalized by using a TIE-2 agonist (PMC-403). Interestingly, PMC-403 did not, however, inhibit ANGPT-2 production upon ECVE or LPS-stimulation, implying additional effects outside of ANGPT-2 induced capillary leakage.

In summary, these results suggest that ECVE induces endothelial activation and that nicotine-containing ECVE increases vulnerability to LPS-induced barrier breakdown, potentially increasing susceptibility to sepsis-induced capillary leakage. Moreover, nicotine-containing ECVE selectively down-regulates CD4 T Cell trans-endothelial migration. Using a TIE-2 agonist, the team was able to reverse this effect.

2. UGMLC subproject: Effect of short- and long-term exposure to e-cigarette vapour on inflammation, endothelial barrier function and structural alterations in murine lungs

The UGMLC subproject previously showed in isolated ventilated and perfused mouse lungs that direct inhalation of e-cigarette vapour (ECV) with nicotine induced endothelial dysfunction which was independent of an additional inflammatory bacterial (lipopolysaccharide, LPS) or viral co-stimulus. Now, the protective effect of a selective TIE2 receptor agonist (PCM-403) was investigated, which has been

provided via cooperation with Sascha David & PD Dr. Benjamin Seeliger (see above). Findings indicate that application of the agonist completely inhibited the increase of capillary permeability induced either by LPS application alone or by co-stimulation of LPS and e-cigarette inhalation (Figure 4). Currently, the effect of the agonist on ECV-induced endothelial dysfunction without LPS costimulation is currently being investigated.

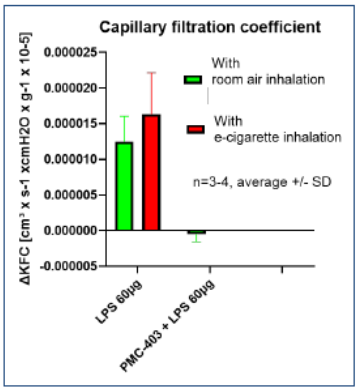

Figure 4: Capillary filtration coefficient (KFC) determined in isolated ventilated mouse lungs during direct inhalation of ecigarette vapour and co-stimulation with lipopolysaccharide (LPS). KFC was determined in presence or absence of the TIE2 agonist PMC-403.

Subproject 3: Association of e-cigarette use with respiratory status in lung-healthy subjects and pulmonary patients (Dr. Stefan Karrasch, LMU Munich, DZL site CPC-M, and Dr. Claudius Melzig, Heidelberg University Hospital, TLRC)

This subproject further elucidates the modalities of e-cigarette use in subjects from the German National Cohort (GNC, NAKO Gesundheitsstudie) based on self-reported information on smoking behaviour and individual smoking history from more than 64.000 adults aged 20-75 years. While the overall frequency of e-cigarette ever-use in these participants was almost 10%, there was a substantial heterogeneity in this percentage across study centers ranging from 6% up to 12% of e-cigarette everusers. Based on the observation that the majority of e-cigarette users were dual users (i.e., concomitantly also using conventional cigarettes), the team examined the correlation between frequencies of conventional smoking and e-cigarette use on study center level which showed a slight trend towards a positive association driven by current or former regular e-cigarette users and reaching statistical significance in this subgroup. Furthermore, not only was a higher volume of e-liquid consumed per day in dual-users as compared to e-cigarette only users observed, but a positive association between the number of conventional cigarettes smoked per day and e-liquid volume per day in current dual users was also found. On the other hand, no consistent association was found between the volume of e-liquid consumed per day and participants' age or the past duration of e-cigarette use. These findings indicate that in current users, e-cigarette consumption shows similar patterns to conventional smoking in terms of general use as well as regarding smoking intensity and that these relationships can be observed regardless of subjects' age.

Regarding effects on respiratory status, e-cigarette users more frequently report cough and wheezing or whistling beyond the known effects of conventional cigarette smoking, and these effects are driven by symptoms in patients with respiratory disease such as asthma or chronic obstructive pulmonary disease. Considering an overall increase in the frequency of reported respiratory symptoms such as cough and sputum production with increasing body mass index (BMI) even in participants without a diagnosed respiratory disease, more pronounced in women than in men, stronger effects of e-cigarette use on symptom levels in subjects with BMI < 25 kg/m2 as compared to overweight or obese subjects were observed. This relationship was primarily found for cough and sputum production; for wheezing or whistling the difference between BMI groups was smaller. However, in subjects without a diagnosed respiratory disease but with self-reported allergy to animal hair, stronger effects of e-cigarette use on

wheezing or whistling frequency as compared to those without even after adjustment for confounding factors such as age, sex, smoking and BMI were observed. These findings suggest that the effects of e-cigarettes on respiratory status beyond those of conventional smoking may depend on specific characteristics indicating higher individual susceptibility to adverse respiratory outcomes of e-cigarette use.

To enable identification of potential imaging biomarkers based on magnetic resonance imaging (MRI), a cross-validation approach including imaging subgroups of both the population-based GNC (ca. 30,000 participants) and the COSYCONET cohort of COPD patients (ca. 500 patients) was chosen. COSYCONET, the German COPD and SYstemic consequences-COmorbidities NETwork, is an associated partner of DZL. Besides clinical examinations and pulmonary function tests, MRI examinations of the thorax were performed in these subgroups of participants in both studies.

To derive lung-specific imaging parameters (lung volume and shape, vessel morphology, radiomics features) from underlying image data in GNC and COSYCONET, a voxel separation of the lungs from surrounding tissues is required (image segmentation). Deep learning based algorithms promise fast and fully automatic image segmentation. However, manual delineation of lung borders in a small number of participants is required as training data for the algorithm. A common approach for manual segmentation in both study cohorts was established and training datasets were created. Automatic lung segmentation was successfully performed and iteratively refined in the datasets of ca. 11,000 participants in the GNC and of ca. 500 patients in the COSYCONET study. However, a visual evaluation approach for automatic lung segmentations was chosen for final validation of lung segmentations. Therefore, various visualisation methods for time-efficient identification of segmentation errors were evaluated and a segmentation mask projection method was finally applied in our project. Analysis of lung shape variations based on the validated segmentations and association of MR-based lung volume and statistical lung shape parameters with smoking status and lung function tests are currently being carried out.

Outlook

During 2023, all subprojects proceeded as planned. Initially, the project was to be completed by the end of that year. Due to delays related to the pandemic situation, the date was extended until the end of 2024. In 2024, the results of the subprojects will be integrated. Moreover, work on the research manuscript giving an in-depth overview of research on e-cigarettes will be continued.

The research group is still convinced that the project addresses an important issue that has become even more topical recently. While tobacco producers try to sell e-cigarettes as healthy substitutes of tobacco smoking, their preliminary results show that ingredients of e-cigarette vapour contain substances which have profound physiological influence. Depending on the final results, the group, operating under the conviction that there is an urgent need for further research in this area, envisages pursuing their research on e-cigarettes beyond the scope of the Balzan Prize project.

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