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Promotion of a Protease–Antiprotease Imbalance in the Airways through Chronic Vaping

There is ongoing controversy in regard to the safety of electronic cigarettes (e-cigarettes) and vaping. Although originally promoted to help facilitate smoking cessation, a number of significant concerns have been highlighted, not least the uptake of these devices by previously nonsmoking youths and their high transfer to traditional smoking as a result of nicotine addiction (1).

Electronic nicotine delivery systems are aerosol-generating devices that heat, not burn, a solution containing a complex mixture of solvents and flavoring (a number of which have known toxicity) in addition to nicotine, the final composition in the aerosol of which is determined by temperature (2). Of note, the fine particles delivered by e-cigarettes are similar in size and concentration to tobacco smoke, and although the composition differs, the pattern of particle deposition in the lungs is similar (3). A number of studies now report e-cigarette exposure to be associated with airway irritation and inflammation, as well as mucus hypersecretion, and have been linked to an exacerbation of symptoms in those with chronic airways diseases such as cystic fibrosis, asthma, and chronic obstructive pulmonary disease (4).

Proteases and their inhibitors play pivotal regulatory roles in most physiological processes required for life. They act as nature's molecular scissors, processing other biological molecules leading to the synthesis, activation, or degradation of functionally important peptides and proteins, and play a vital role in tissue remodeling and wound healing. However, when the normally exquisite control of their action is lost, proteases can be key triggers or amplifiers of many important human diseases such as cancer, cardiovascular disease, rheumatoid arthritis, sepsis, and neurological disorders including Alzheimer disease and multiple sclerosis, with many proteases well-recognized as potential biomarkers of disease and/or therapeutic targets (5–7). In chronic airways diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis, a protease–antiprotease imbalance has long been associated with tissue injury and disease progression. Aberrant proteolytic activity resulting from high levels of neutrophil elastase (NE), in particular, is widely associated with episodes of acute exacerbation and pulmonary decline (8–10).

E-cigarette vapor extract has been shown to stimulate the release of MMP-9 (matrix metalloprotease-9) and interleukin 8 (CXCL8) from isolated neutrophils, as well as increase in NE and MMP-9 activity (11). MMP-9 and CXCL8 release caused by e-cigarette vapor extract prepared from different e-cigarette brands were found to be similar to, or in excess of, a cigarette smoke extract response. In addition, MMP-9 and CXCL8 was increased after exposure to e-cigarette vapor extract with and without nicotine, suggesting the involvement of other proinflammatory constituents.

In this issue of the *Journal*, Ghosh and colleagues (pp. 1392–1401) investigate the effect of chronic e-cigarette use on the protease–antiprotease balance in the airways of vapers (12). The study recruited never-smokers, current tobacco smokers, and e-cigarette users (vapers), with the latter group including both never-smokers and former tobacco smokers. Protease levels were quantified in BAL samples, as well as from immune cells stimulated with e-liquid components.

Protease levels were measured using Western blotting and activity by hydrolysis of peptide-based substrates ($\pm$protease class inhibitors), with gelatin zymography also used to assess the activity of MMP-2 and MMP-9. Importantly, serum nicotine, cotinine, and

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hydroxycotinine were measured to confirm tobacco/vape use; previous work by these authors reported a significant increase in the levels of nicotine and other metabolites in vapers serum, similar to levels observed in cigarette smokers (13).

Western blot analysis showed NE and MMP-2/9 to be significantly elevated in smokers and vapers (including those who reported no prior cigarette use) compared with nonsmokers. In addition, relevant protease inhibitors were measured: AAT (alpha-1 antitrypsin), SLPI (secretory leukocyte proteinase inhibitor), TIMP-1, and TIMP-2 (tissue inhibitor of metalloproteinases-1 and -2). Similar increases were not observed in protease inhibitor levels, indicating a net potential increase in proteolysis.

The number of proteases investigated was then expanded for spectrofluorimetric analysis, which included other enzymes associated with airway pathophysiology: serine (plasmin, trypsin-like, and chymotrypsin-like), cysteine (cathepsin B, S/L and K), and MMP-3 and MMP-12. Of these, only NE and MMP-2/MMP-9 were upregulated in both smokers and vapers, with cathepsin B increased only in the smokers samples tested. Work on immune cells was conducted using treatments of nicotine and/or a solution comprised of e-cigarette components (3.3 mM nicotine $\pm$3% propylene glycol/vegetable glycerine [PG/VG]), with results showing an increase in NE independent of PG/VG. Of note, mannitol was included as a control for potential increases in osmotic stress caused by PG/VG, but no effect on NE levels was observed. The increase in NE was subsequently shown to be associated with a rise in cytosolic calcium levels in response to nicotine; earlier studies by the same group found increases in intracellular Ca^{2+} in HEK293T cells exposed to cigarette smoke, and the tobacco smoke metabolites 1-NH_2-naphthalene, formaldehyde, nicotine, and nicotine-derived nitrosamine ketone (14).

A strength of the study was the robustness of their protease analyses and the measurement of nicotine and its metabolites cotinine and hydroxycotinine in serum, BAL, and sputum samples, which confirmed the tobacco/vape use of each participant. This also ensured that a physiologically relevant concentration of nicotine was used to treat neutrophils and alveolar macrophages. A limitation of the study, however, is the relatively small numbers recruited to each group and the fact that the vaper group also comprised former tobacco smokers. Furthermore, an inherent problem for researchers investigating the effect of e-cigarettes is the vast array of products and devices on offer, which makes it difficult to standardize exposure. The extensive number of formulations and flavorings further increases the level of complexity. A recent study reports e-cigarette products to have an average of 6.2 (SD = 3.6) flavoring chemicals, with the sweetest flavors having the greatest number: 21% of products tested contained flavoring chemicals with potential risk for inhalation toxicity (benzyl alcohol, benzaldehyde, and vanillin); other toxicants such as acrolein and diacetyl were also detected, and measurable levels of tobacco-specific nitrosamines, an important group of carcinogens in tobacco products, were present in 70% of tested products (15). The full significance of the inhalation of these complex mixtures of components on the protease–antiprotease balance, the proteome, lung tissue injury, and chronic airways disease progression in general will be difficult to determine.

The overall conclusions of the paper that NE and MMP2/9 levels are elevated in vapers, consistent with that seen in smokers, and that this protease imbalance has the potential to increase overall proteolysis in the lung makes a further contribution to the field and lends support to the argument that vaping is not any safer than tobacco smoking. Given that the link between dysregulated proteolysis and lung disease is well-established, coupled with the worrying trend of young, previous nonsmokers being attracted to vaping, the possible risk for another generational wave of chronic lung disease in the foreseeable future, must be considered. ■

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Toward Early Detection of Idiopathic Pulmonary Fibrosis

Since their emergence as a frequent and potentially clinically meaningful finding in computed tomography (CT) screenings of smokers a decade ago (1), interstitial lung abnormalities (ILAs) have drawn significant interest and controversy. A specific set of radiologic abnormalities on chest CT scans, ILAs are relatively common and can be found in up to 10% of lung cancer screenings and older smokers (2). ILAs have traditionally been taken lightly by physicians and affected individuals alike, as symptoms in subjects with ILA are often lacking or very mild, and the prognostic significance of ILA was unknown. This has changed in recent years with the increased recognition that individuals with ILAs are at higher risk of death and exhibit higher rates of lung restriction (3–5) and that on tissue histology they often exhibit fibrosis (6). The possibility that individuals with ILAs may represent a population at risk for subsequent development of idiopathic pulmonary fibrosis (IPF) or other interstitial lung disease (ILD) is of particular importance, because of the potential for more effective interventions when the disease is diagnosed early. The connection between ILAs and pulmonary fibrosis has been supported by radiologic progression of ILAs, the presence of ILAs in asymptomatic family members of individuals with familial pulmonary fibrosis, and the significant association of ILAs with rs35705920 in the promotor region of MUC5B (Mucin 5B, oligomeric mucus/gel-forming) (4), the same gene variant that accounts for approximately 30% of cases of IPF (7). However, so far, the genetic overlap between patients with ILAs and IPF has not been studied in detail.

In this issue of the *Journal*, Hobbs and colleagues (pp. 1402–1413) performed a meta-analysis using available genome-wide data of 1,699 subjects with ILA and 10,274 control subjects from six cohorts and compared the results with genetic associations in patients with IPF (8). Because subpleural ILAs are believed to be more clinically relevant, they performed the analysis of ILAs in general and subpleural ILAs separately. In the ILA analysis, they identified three genome-wide significant associations that included the known MUC5B promoter polymorphism rs35705950 and two novel loci: rs6886640 at 5q12 near IPO11 (importin 11) and rs73199442 at 3q13 near the long noncoding RNA FCF1P3 (FCF1 pseudogene 3). In the subpleural ILA analysis—in addition to MUC5B—they identified a genetic association at the 6q15 locus with rs7744971 near HTR1E (5-hydroxytryptamine receptor 1E). None of the novel ILA loci replicated in IPF genome-wide association studies. Of the 12 reported genome-wide association study loci for IPF, only the MUC5B variant reached genome-wide significance, whereas the genetic variants near DPP9 (dipeptidyl peptidase 9), DSP (desmoplakin), FAM13A (family with sequence similarity 13 member A), and IVD (isovaleryl-CoA dehydrogenase) were nominally associated with ILA.

The findings of this study have several major implications. The most important is that although individuals with ILAs represent a population at risk for IPF, they are not synonymous with the IPF population. Only a subset of individuals with ILA exhibit a genetic risk profile that is similar to individuals with IPF, whereas others exhibit genetic associations that do not occur in IPF: the reported odds ratio is 1.97 for rs35705950 for all ILAs, and 2.22 when subsetting to subpleural ILAs, but 4.84 for IPF. None of the other IPF risk loci were significant on a genome-wide level, and all of them had a lower odds ratio in ILA. This could suggest an ILA subpopulation that is at risk of developing IPF but is being diluted by a larger fraction of subjects with ILA who do not share the same genetic risk. The finding of three novel ILA genetic associations not observed in IPF also indicates a potentially distinct entity, possibly a predisposition to other non-IPF ILDs or even the presence of gene variants that reduce the probability of progression of ILAs to fibrosis and may be protective. Regardless of their potential functional relevance, the finding of variants associated with ILA but not IPF, if replicated, could be useful developing a polygenic genetic risk profile. This is important because, currently, chest CT screenings to detect early IPF are not clinically feasible or justified. The results of this study should encourage investigators to design further studies assessing whether genetic risk profiling, potentially combined with other noninvasive biomarkers, could be used to prioritize individuals for CT screening.

Although exciting and intriguing, this study has some limitations that should be highlighted. Of course, the most obvious limitation of the discovered novel ILA associations is the lack of an independent replication cohort, but the limitations regarding the negative results should not go unnoticed. Indeed, only MUC5B reached genome-wide significance in this study, but the main study population consisted of data obtained from several cohorts that were not designed to capture early ILD. These populations differed in the definitions of ILA, the depth of phenotyping, and the original aims of the studies. Thus, it is highly possible that although the strongest association (MUC5B) was able to emerge, other valid associations simply were drowned by the sea of differences and may emerge again if comparably sized future studies are designed to detect ILAs using standard definitions, adjudicated radiological reading, and patient phenotyping.

In summary, the study by Hobbs and colleagues (8) represents a major step toward better understanding ILAs as tools for defining

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