CIGARETTE SMOKE, E-CIGARETTE/E-VAPING AND COVID-19: RISKS AND IMPLICATIONS IN THIS NEW ERA

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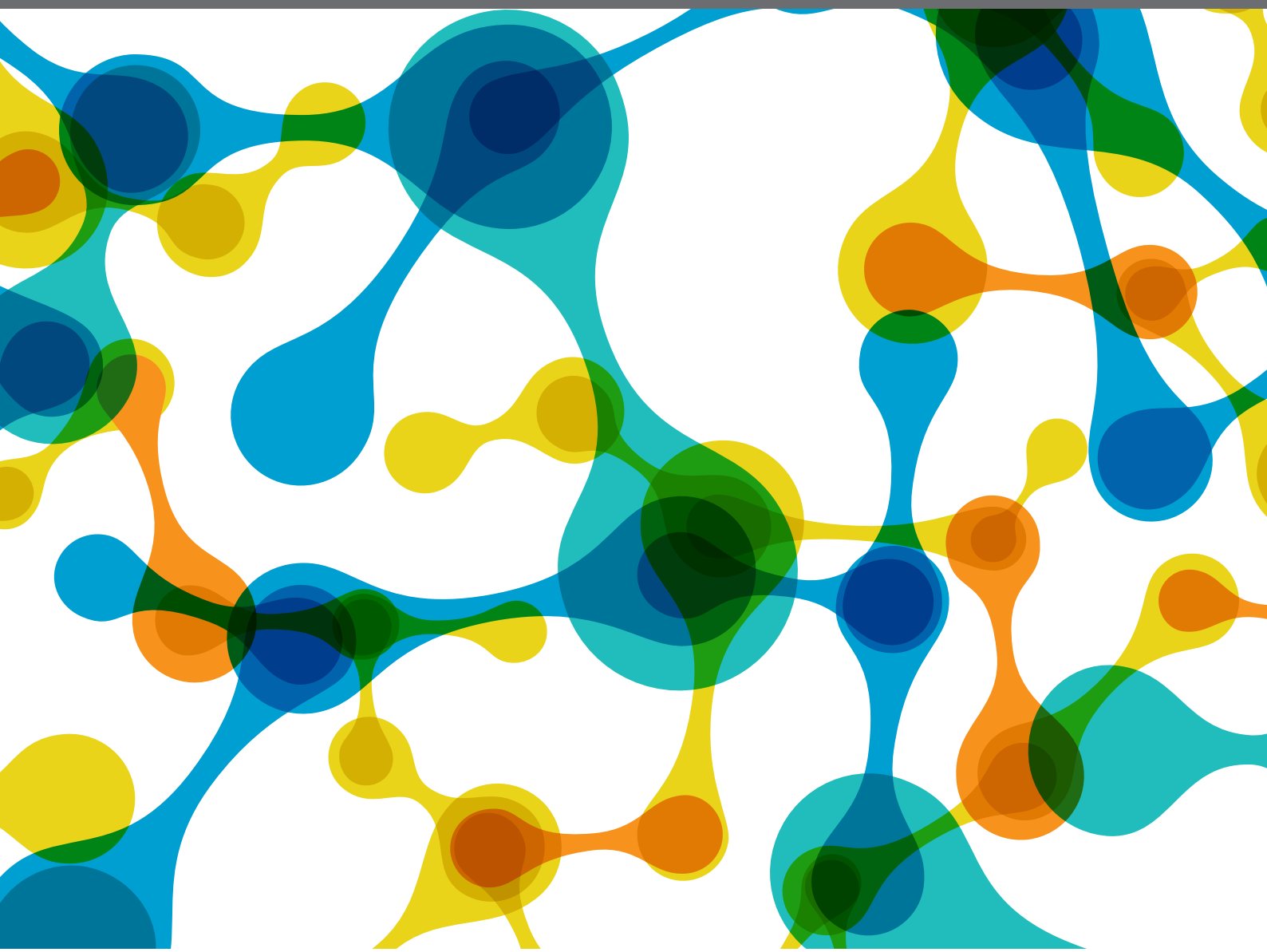

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CIGARETTE SMOKE, E-CIGARETTE/E-VAPING AND COVID-19: RISKS AND IMPLICATIONS IN THIS NEW ERA

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EDITORIAL
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Editorial: Cigarette Smoke, E-Cigarette/E-Vaping and COVID-19: Risks and Implications in This New Era

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Editorial on the Research Topic

Cigarette Smoke, E-Cigarette/E-Vaping and COVID-19: Risks and Implications in This New Era

In December 2019, a cluster of pneumonia was reported in Wuhan, China. The new disease, COVID-19, is caused by the novel coronavirus SARS-CoV-2 and rapidly spread to become pandemic in March 2020. Worldwide, on August 4, 2021, confirmed cases and deaths exceed 199 and 4 million, respectively, and there are 548,167 new cases and over 3 billion vaccine doses administrated (https://covid19.who.int). Cigarette smoking (CS) is a universal public health concern too. Because exposure to CS increases the incidence and mortality from infections (Jiang et al., 2020), CS could rise the risk for COVOD-19. Yet, the relationship between smoking and COVID-19 has been controversial. Lower prevalence in hospitalized COVID-19 patients (Goyal et al., 2020) and lower risk of SARS-CoV-2 infection (Lee et al., 2021) were reported among smokers. However, a meta-analysis (WHO, 2020) suggests an association between smoking and COVID-19 severity outcomes. The U.S. Centers for Disease Control and Prevention and the Food and Drug Administration list smoking as a factor likely to make COVID-19 worse. This Research Topic presents new findings and reviews evidence on the role of smoking/vaping on COVID-19 prognosis, diagnosis, and outcomes.

Neither smoking tobacco nor vaping have health benefits. In fact, both damage the respiratory, cardiovascular, and other organ systems. Furthermore, smoking often occurs with drinking. About 15% of ingested alcohol is exhaled or metabolized in the lung where it impairs neutrophil recruitment and compromises epithelial barrier function (Sisson, 2007; Muthumalage et al., 2019). In a study of 1,500+ COVID-19 patients with smoking or alcohol consumption histories, Dai et al. show more severe COVID-19 and higher probability of death in smokers than non-smokers. While alcohol consumption is not associated with severity or mortality in this study, Wetzel and Wyatt find that e-cigarette users are at high risk for binge and chronic alcohol consumption. They postulate that dual vs. single substance use might promote tissue damage, decrease immune

response and pathogen clearance, and increase IL-6 and IL-8 production and inflammation, which would elevate COVID-19 complications risk. Xie et al. highlight the importance of smokers' and alcohol consumers' behaviors that facilitate the spread of viruses. Peaks in COVID-19 cases and deaths in U.S. occurred after the (premature) opening in summer 2020 and the holidays in January 2021 (https://covidtracking.com/data/charts/2-metrics-7-day-average-curves). These events likely promoted physical closeness and crowding while drinking, hand-to-mouth contact, and smoke-induced lung insult that might increase susceptibility to COVID-19. This hypothesis could be tested by comparing with data during periods of lockdowns and social distancing. Xie et al. note that isolation and its negative effect on mental health make quitting smoking difficult and contribute to smoking and drinking.

Some COVID-19 patients suffer long-COVID-19, which manifests as symptoms that last weeks or months after being infected with SARS-CoV-2 or can appear weeks after infection. The cause and role of smoking in long COVID-19 is unknown. Compared to non-smokers, Kaur et al. find persistent high levels and activity of ACE2, the SARS-CoV-2 receptor, and of eotaxin and monocyte chemoattractant protein-1 in the sera of COVID-19 patients with smoking history. The relevance of these alterations on long-COVID-19 deserves attention.

Vaping, the inhalation of e-cigarette aerosols, associates with lung inflammation and injury. Teenagers and young adults who vape and/or smoke vs. non-users are 2.6–9 times more likely to obtain a COVID-19 test because of experiencing symptoms (Gaiha et al., 2020). Furthermore, youth who ever used e-cigarettes alone or plus conventional cigarettes are 5 or 6.8 times more likely, respectively, to receive a COVID-19 diagnosis than their peers who did not use. In this Research Topic, Masso-Silva et al. show changes in lung gene expression of mice exposed to e-cigarette aerosols that indicate neutrophil activation, a potential driver of acute lung injury in COVID-19. They also show vaping mint increases expression of ACE2. Moreover, Sivaraman et al. demonstrate that pre-exposure to vaped e-liquid vs. vehicle alone results in worse pulmonary inflammation and mortality in mice infected with the murine-tropic coronavirus MHV-A59. Together, these preclinical investigations indicate that vapers could have augmented susceptibility to SARS-CoV-2 infection and be at higher risk of developing severe COVID-19.

The presence of comorbidities and the relatively small studies' sample size had limited the generation of robust conclusions about COVID-19 and smoking association. Our Research Topic contains four reports with variable number of patients. In a study of 622 COVID-19 patients at an academic hospital in China, Peng et al. find that smokers have higher mortality risk than non-smokers after adjusting for age, sex, and underlying diseases. Smokers exhibit greater levels of leucocytes, hemoglobin and creatinine, and lower erythrocyte sedimentation rate than non-smokers. Zhong et al. study 91 patients between 3 months and 76 years of age in a tertiary hospital in China during the first 3 months of the outbreak. Severe disease occurs in people 65 or older, married, and with or below primary school education. While smoking, drinking, or betel quid chewing have no significant relationship with disease severity, diabetes and being retired/unemployed associate with worse COVID-19. In a longitudinal study of 10 controls and 25 COVID-19 adults with no comorbidities in Brazil, Alberca et al. show no difference in the number of leucocytes among current or former smokers and COPD patients during the hospital stay. However, smokers and COPD patients are at risk of kidney injury, and death occurs only among smokers. Although small, this study shows that, in the absence of comorbidities, smoking or COPD influences COVID-19 course. Replicating these results in larger populations and different demographics would be compelling. Together, these studies reveal that COVID-19 might present differently in distinct settings or countries or phases of the pandemic.

Children carry and transmit SARS-CoV-2. Compared to adults, COVID-19 in children is milder with lower hospitalization rates and death in U.S. and Europe. Potential protective factors are the transfer of maternal antibodies and antiviral proteins in the milk (Root-Bernstein, 2020a), administration of certain vaccines (Root-Bernstein, 2020b), lower ACE2 expression (Molloy and Bearer, 2020), and absence of underlying diseases in general. However, children are exposed to secondhand or direct CS, which may deteriorate pre-existing conditions or lead to respiratory problems and infections in at-risk patients. Schiliro et al. review that pediatric COVID-19 cases continue to increase, its symptoms are non-specific, and about 50% of cases happen with respiratory coinfections. While 80% of North America's pediatric intensive care unit admissions have developmental delay and/or genetic anomalies, only 4% suffer from chronic lung disease such as asthma. Nevertheless, multisystem inflammatory syndrome in children resembling Kawasaki disease can develop after COVID-19 diagnosis.

The course of the pandemic will shift toward improved outcomes as more people worldwide are vaccinated, effective therapies developed, and the mechanisms of disease understood. Unfortunately, new virus variants emerge, and old and new inhalation modalities are adopted with unknown health consequences. Thirupathi et al. show that physical exercise attenuates lung and muscle inflammation and histopathology in mice chronically exposed to hand-rolled cornhusk cigarette smoke. Elegant pre-clinical studies by Tian et al. elucidate the role of the CD73/adenosine A_{2B} receptor/PKCa/ERK pathway in cigarette smoke-mediated airway epithelial injury.

In conclusion, this Research Topic provides a view on how cigarette smoke, e-cigarette, or vaping can predispose and worsen COVID-19 and the negative effect of dual use of smoking with alcohol on this disease. Gaps in knowledge will close as preclinical and clinical investigations are completed and new studies implemented. ClinicalTrials.gov lists seven interventional or behavioral studies on COVID-19 and either smoking or tobacco use or nicotine. No study on COVID-19

and e-cigarette/vaping is registered. A study on immune senescence association with prior smoking as a susceptibility factor in COVID-19 is underway (NCT04403386), Longitudinal studies are necessary to understand long-COVID and develop appropriate treatments. Success of COVID-19 vaccination globally will reduce the risk of SARS-CoV-2 infection and spread of COVID-19 and improve long-COVID-19 symptoms. Because achievement of these goals will take months if not years, every effort to quit smoking and vaping should be a priority.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and read and approved it for publication.

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REVIEW
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Dual Substance Use of Electronic Cigarettes and Alcohol

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Electronic cigarettes (ECs) are a modern nicotine delivery system that rapidly grew in widespread use, particularly in younger populations. Given the long history of the comorbidity of alcohol and nicotine use, the rising prevalence of ECs raises the question as to their role in the consumption of alcohol. Of the numerous models of ECs available, JUUL is the most popular. This narrative review aims to determine current trends in literature regarding the relationship between EC and alcohol dual use, as well as hypothesize potential pathogenic tissue damage and summarize areas for future study, including second-hand vapor exposure and calling for standardization among studies. In summary, EC users are more likely to participate in hazardous drinking and are at higher risk for alcohol use disorder (AUD). We surmise the pathogenic damage of dual use may exhibit an additive effect, particularly in pathogen clearance from the lungs, increased inflammation and decreased immune response, physical damage to epithelial cells, and exacerbation of chronic obstructive pulmonary disease (COPD)-like illnesses. A better understanding of pathogenic damages is critical to understand the risks placed on dual users when exposed to respiratory pathogens, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Keywords: alcohol misuse, eCIG, vaping, ethanol, polysubstance use

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INTRODUCTION

The use of traditional cigarettes is one of the largest influencers of public health, contributing to more deaths per year than HIV, illicit drug use, alcohol use, motor vehicle injuries, and firearm-related injuries combined (Centers for Disease Control and Prevention, 2018). Cigarettes were subjected to a century of medical research regarding adverse health effects and nicotine addiction, along with establishing comorbidities of additional substance use. A significant body of literature demonstrates a high comorbidity between traditional cigarette smoking and harmful levels of alcohol consumption across a wide age demographic, including adolescents and young adults (Weinberger et al., 2015; Banks et al., 2017). The use of nicotine enhances the pleasurable effects felt during alcohol consumption and increases cravings for it (Thrul et al., 2019). Nicotine, one of the major addictive chemicals in cigarettes, activates the mesolimbic pathway in the brain, releasing dopamine and reinforcing addictive behavior (Sagheddu et al., 2019). Chronic alcohol consumption affects organs throughout the body, including the brain where long-term alcohol exposure induces cellular changes in neuronal pathways related to stress, motivation, and reward. Dopamine is released during alcohol consumption from the mesolimbic pathway, reinforcing alcohol ingesting behavior (Gilpin and Koob, 2008). Significant activation of pleasure and reward pathways in the brain

may suggest why greater than 80% of alcohol-dependent individuals report smoking cigarettes (Romberger and Grant, 2004), daily smokers have a 17% greater risk of relapse to alcohol abuse, and smokers have a 95% greater risk of alcohol dependence when compared with non-smokers (Weinberger et al., 2015). In addition, both daily and non-daily smoking are associated with higher levels of chronic alcohol use and binge drinking, respectively (Banks et al., 2017).

Modern advancements in nicotine delivery systems sparked the creation of electronic cigarettes (ECs). First developed in 2006, ECs rapidly grew in popularity accompanied by a commensurate decrease in traditional cigarette use (Bradford et al., 2019). Prevalence rates in adolescents have increased 46% since 2014, paralleling a 48.5 and 77.8% increase in U.S. middle and high school–aged students, respectively (Dai and Leventhal, 2019). Less risk, higher popularity, and social acceptance have been cited as factors contributing to their rapid increase in popularity (Kong et al., 2015; Gorukanti et al., 2017). The rising popularity of ECs can be seen in newly created devices, such as JUUL. In 2015, JUUL, a new retail brand of EC, emerged onto the U.S. market and quickly acquired 76% of the market by the end of 2018 (Huang et al., 2019). JUUL pods utilize a proprietary nicotine salt that closely resembles free-acid nicotine and allows for more rapid absorption and delivery of nicotine to the brain. The meteoric increase in popularity has led to the term "JUULing," which describes the action of using a JUUL (Teitell, 2017). It has become synonymous with the term "vaping" or the action of using an EC with reference to JUUL as "the iPhone of e-cigarettes" (Radding, 2015). Despite similar or higher nicotine levels than cigarettes, 39.3% of adolescents perceived JUUL as less harmful and 29.3% believed JUUL was less addictive compared with traditional cigarettes (Russell et al., 2020). Overall, the perceived safety of EC devices, rapid growth in popularity, attractive flavors, and sleek design present a significant, unknown public health concern requiring further investigation.

Given the relative newness of ECs, there is a limited body of literature detailing the role ECs play in alcohol consumption, how inaccurate perceptions of ECs contribute to risk-taking behaviors related to alcohol consumption, and the pathogenic damages that occur during dual use. In this narrative, a background will be presented using existing alcohol, smoking, and EC studies to identify potential similarities and trends, hypothesize mechanisms of damage for future study, and identify additional areas for study related to EC and alcohol dual use.

TREND OF EC AND ALCOHOL DUAL USE

EC users have an increased risk of alcohol misuse (**Figure 1**), such as binge drinking or chronic use, when compared with non-EC users (Lanza and Teeter, 2018; Mehra et al., 2019). While the literature is replete for this trend with the use of traditional cigarettes and alcohol consumption, a similar public health issue may exist for EC use and requires future study. EC users reported higher alcohol use disorders identification test (AUDIT) scores, suggesting EC users are at a higher risk for an alcohol use disorder (AUD) when compared with non-users (Hershberger et al., 2016). Age of initiation was identified as a factor contributing to alcohol misuse, with a younger age of onset more likely to demonstrate lifetime alcohol use (McCabe et al., 2018). An alarming trend was identified in the increase in EC use in Canadian high school students from 2014 to 2018, along with an increase in EC and alcohol dual use from 2017 to 2018 (Zuckermann et al., 2019).

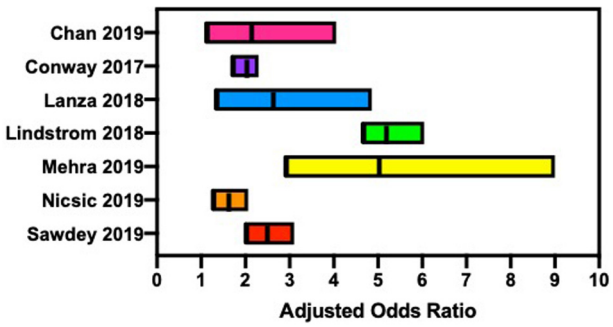

FIGURE 1 | Several reports suggest that electronic cigarette users are at an increased risk for hazardous alcohol consumption, such as binge drinking and chronic alcohol consumption.

Data from the Nicotine and Other Substance Interaction Expectancy Questionnaire (NOSIE) assessed expectancies of EC and alcohol use in adults living within a community dwelling. Compared with non-EC users, EC users had significantly higher problematic alcohol use ($p < 0.05$), and combined EC and alcohol use were significantly related to problematic alcohol consumption ($p < 0.05$) (Hershberger et al., 2016). Data collected from 2,299 U.S. high school seniors examined the association between early onset of EC use and the use of other substances. A higher percentage of students who began EC use in ninth grade or earlier were more likely to report current or lifetime substance use, including alcohol (McCabe et al., 2018). Using data from the Population Assessment of Tobacco and Health (PATH) study, wave 1 (2013–2014), mental health problems related to tobacco use, including ECs, were compared with non-users. EC users were more likely to report internalization problems [adjusted odds ratio (AOR) 1.9] and substance use problems (AOR 3.4) when compared with non-users (Conway et al., 2017). These current trends may suggest a key public health concern regarding the dual use of EC and alcohol, similar to that of smoking and alcohol consumption, that needs to be investigated further.

EFFECTS OF NICOTINE AND SMOKING ON THE LUNGS

Although fundamentally different, it has been suggested that the body's response to EC use may be similar to that of cigarettes with a few unique differences (Reidel et al., 2018). To better understand the potential harmful effects of EC and alcohol on the lungs despite little empirical data, it is important to better understand the harmful effects nicotine products have on the lungs, particularly cigarette smoking. Cigarette smoking

has deleterious effects on the lungs and is the leading cause of preventable death in the United States (Centers for Disease Control and Prevention, 2018). Smoking is significantly linked to lung cancer, with 80% of lung cancers in women and 90% of lung cancers in men caused by cigarette smoking (Lopez et al., 1994; Egleston et al., 2009). Besides nicotine in cigarettes, thousands of additional chemicals, such as reactive aldehydes, are shown to have carcinogenic properties (Phillips, 1996). When present in the body, these chemicals form reactive intermediates in tissues that can lead to DNA damage and cancer (Phillips, 1996). The smoke from cigarette combustion contains a high level of free radicals that can induce oxidative injury, cell membrane destruction, and inflammation within lung tissues (Munnia et al., 2006). Free radicals can induce lipid peroxidation, causing the oxidation of lipids in cell membranes, creating reactive oxygen species (ROS) and oxidative damage (Munnia et al., 2006). Additionally, ROS can interfere with the normal function of innate immune system cells, such as macrophages, neutrophils, monocytes, and eosinophils (Galvin and Franks, 2009). Macrophage -killing capacity diminishes and the recruitment of previously mentioned cells to the site of inflammation is compromised in smokers (Galvin and Franks, 2009). High levels of oxidative stress potentially play a role in the progression and exacerbation of chronic obstructive pulmonary disease (COPD) (Rahman, 2005). COPD is the third leading cause of death in the United States and, due to the long-term nature of care required, is projected to cost the U.S. health care system $49.0 billion in 2020 (Centers for Disease Control and Prevention, 2018). Given the relative newness of ECs into the market, little is known on the addictiveness of other chemicals found in ECs besides nicotine. While it is true that cigarettes contain a large number of chemicals with a variety of properties, further work is still needed to analyze the local and systemic effects of EC chemicals in the body. Secondly, the wide range of products available to the EC consumer with varying chemical compositions make standardization of testing for researchers quite difficult. Standardization of products will reduce variation between experiments and allow for a better understanding of other EC chemical effects on the body besides nicotine.

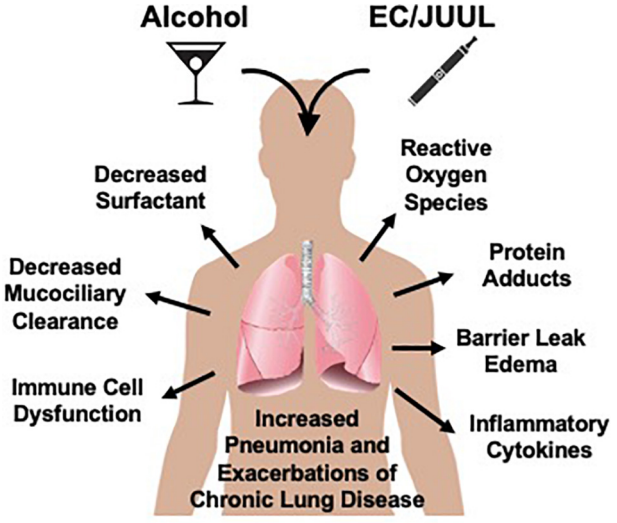

FIGURE 2 | Potential for electronic cigarettes (EC) to enhance lung tissue injury. Alcohol negatively impacts the lung via direct and indirect mechanisms. As with cigarette smoking, EC generate compounds known to enhance alcohol-mediated lung injury leading to both increased risk for infections and pneumonia as well as the exacerbation of existing chronic inflammatory lung diseases such as bronchitis, emphysema, and COPD.

EFFECTS OF ALCOHOL ON THE LUNGS

Moderate consumption of alcohol is typically socially acceptable and practiced by the majority of people. According to the Office of Disease Prevention and Health Promotion (ODPHP), moderate consumption is defined as two equivalent servings for men and one serving for women daily (U.S. Departments of Agriculture and Health and Human Services, 2015). However, heavy drinking, defined as more than 15 equivalent servings per week for men and 8 equivalent servings per week for women, for an extended period of time causes significant health problems throughout the body (U.S. Departments of Agriculture and Health and Human Services, 2015). The lungs are no exception (U.S. Departments of Agriculture and Health and Human Services, 2015; **Figure 2**). Alcohol interferes with normal innate lung immunity, particularly the physical barriers and cellular functions. Alcohol is principally metabolized by alcohol dehydrogenase in the liver after first pass via the hepatic portal vein, and during chronic alcohol consumption, by cytochrome P450 2E1 (CYP2E1) (Kaphalia and Calhoun, 2013). Up to 15% of ingested alcohol, however, is metabolized by CYP2E1 in the lungs or excreted directly via exhalation. The metabolism of alcohol produces acetaldehyde, superoxides, hydrogen radicals, and hydrogen peroxides (Aytacoglu et al., 2006), further promoting lipid peroxidation and generation of malondialdehyde (MDA) and 4-hydroxylnonenal (4-HNE) (Zheng et al., 2014). Chronic alcohol consumption is associated with bacterial pneumonia (Lujan et al., 2010), viral lung infections such as respiratory syncytial virus (RSV) (Simet and Sisson, 2015), and accumulation of fluid in the lungs due to epithelial barrier dysfunction as seen in acute respiratory distress syndrome (ARDS) (Guidot and Hart, 2005).

The first line of defense against microbial pathogens and debris in the respiratory system is the physical barriers and mucociliary transport. Chronic exposure to alcohol disrupts epithelial barrier function, allowing for paracellular leak from capillaries into the alveolar space (Guidot et al., 2000). Epithelial barriers, exposed to biologically relevant levels of alcohol for long periods, demonstrate an increased expression of sodium channels, which may serve as a counteractive measure against paracellular leak (Guidot et al., 2000). Once the tissue is inflamed, however, these mechanisms become overwhelmed and fluid accumulates within the alveolar spaces (Guidot et al., 2000). Cilia, present on the cell surface, maintain a clear airway by removing pathogens and inhaled debris out of the airway. Initial exposure to alcohol generates nitric oxide (NO) and activates a protein kinase A-dependent signaling pathway, increasing ciliary beat frequency (CBF) (Simet et al., 2013). While short-term exposure

increases CBF, exposure to long-term, excessive quantities of alcohol desensitize lung airway cilia and decrease CBF when exposed to pathogens (Wyatt and Sisson, 2001). Impaired epithelial barrier and cilia function due to chronic alcohol consumption leaves the lungs more susceptible to pathogens.

Chronic alcohol also interferes with the cellular response of the innate immune system, with alveolar macrophages considered the second line of defense to invading pathogens (Rubins, 2003). Macrophages and neutrophils, key immune cells, exposed to alcohol are unable to optimally phagocytize bacteria (Joshi et al., 2009). Additionally, such macrophages have diminished release of cytokines and chemokines, which recruit other immune cells (D'Souza et al., 1996), as well as neutrophil chemoattractants to attract neutrophils to the site of inflammation (Craig et al., 2009). Thus, alcohol interferes with the recruitment of neutrophils to the lungs and increases risk for infection. Chronic alcohol also causes macrophages to undergo oxidative stress due to depletion of glutathione (GSH) stores, leading to accumulation of ROS (Yeh et al., 2007). Alcohol also interferes with granulocyte colony-stimulating factor (G-CSF), a key factor in the production of granulocytes, suppressing neutrophil production and interfering with killing potential (Bagby et al., 1998).

DUAL EFFECTS OF SMOKING AND ALCOHOL

Both the metabolism of alcohol and the inhalation of cigarette smoke contribute to significant aldehyde exposure to the lungs. Alcohol metabolism by CYP2E1 leads to the generation of superoxides, hydrogen radicals, and hydrogen peroxides (Aytacoglu et al., 2006). This, in turn, promotes lipid peroxidation and generation of MDA and 4-HNE (Zheng et al., 2014). Cigarette smoke contains high concentrations of aldehydes, such as butyraldehyde, isobutyraldehyde, propionaldehyde, acetaldehyde, formaldehyde, acrolein, propanal, and MDA (Fujioka and Shibamoto, 2006). These aldehydes further promote lipid peroxidation and generate more MDA and 4-HNE. High levels of oxidative stress from carbonyl accumulation may exacerbate COPD symptoms in dual users, with alcohol shown to be an independent risk factor for COPD (Tabak et al., 2001).

The major pathological implication of aldehyde exposure on the lungs includes oxidative stress, immune dysfunction, membrane disruption, histone modification, and mitochondrial dysfunction. Acetaldehyde, a component of cigarette smoke and metabolic product of alcohol breakdown, has epigenetic and genetic toxic effects (Shukla and Lim, 2013) and can inhibit key mitochondrial reactions and functions (Manzo-Avalos and Saavedra-Molina, 2010). Exposure to acetaldehyde induces the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) from macrophages and IL-8 from human bronchial epithelial cells (Facchinetti et al., 2007; Moretto et al., 2009). Acetaldehyde inhibits neutrophil apoptosis, prevents neutrophil-mediated killing of pathogens, and contributes to neutrophil accumulation, resulting in a delay in resolution of inflammation (Finkelstein et al., 2005).

High levels of these reactive aldehydes form adducts on macromolecules present in the lungs, leading to an inflammatory response. Acetaldehyde and MDA form adducts on numerous nucleophilic proteins, stimulating inflammatory responses in airway epithelial cells (Wyatt et al., 2005). Acetaldehyde adducts to cilia dynein ATPase and slows CBF (Sisson et al., 1991). MDA damages macromolecules via direct adduction or indirect lipid peroxidation (Munnia et al., 2006). Formation of the malondialdehyde–acetaldehyde complex, only observed in the lungs of smokers with AUD, is stable and resists rapid degradation (McCaskill et al., 2011), leading to a protein kinase C-mediated inflammatory response (Wyatt et al., 1999). Acrolein, an aldehyde detected in cigarette smoke, increases mucin production and regulation of lung matrix metalloproteinase 9 (MMP-9), which decreases lung function in COPD patients (Deshmukh et al., 2008). It irreversibly modifies GSH stores, contributing further to oxidative stress (Yang et al., 2004). While we acknowledge this data is not explicitly testing EC and alcohol use, understanding how cigarette and alcohol use affects the lungs is important for determining testable hypotheses for potential tissue damage related to EC and alcohol use.

HYPOTHESIZED EC AND ALCOHOL-MEDIATED LUNG INJURY

The use of EC has potentially pathogenic health effects on the lungs. Cell types, including human fibroblasts, neutrophils, airway epithelial cells, human embryonic stem cells, and mouse neural stem cells, demonstrate morphological changes, even displaying cytotoxicity in stem cells and fibroblasts when exposed to undiluted e-liquid and flavor aldehydes, respectively (Lerner et al., 2015). EC use impairs physical barriers in the innate immune system of the lungs. EC decrease cilia, potentially as a result of ROS production (Garcia-Arcos et al., 2016; Muthumalage et al., 2019). Similarly, excessive amounts of alcohol blunt cilia responses (Sisson, 2007). In combination, EC with AUD may further slow CBF similar to that of cigarettes and alcohol (Wyatt et al., 2012), leading to an impaired ability to clear pathogens and debris from the airway. In terms of the cellular response of the innate immune system, EC vapor decreases the normal function of macrophages and neutrophils and reduces their ability to phagocytose pathogens and virus-infected cells (Sussan et al., 2015). Lipid-laden macrophages, discovered in bronchioalveolar lavage fluid (BALF) from EC users, suggest users are at risk for lipid-mediated lung injury and interfered pathogen clearance via macrophages (Eissenberg and Maziak, 2020). Lipid concentrations may be related to vaporization of propylene glycol (PG) and vegetable glycerin (VG) solvents in e-liquid. EC vapor exposure to normal human bronchial epithelium and airway epithelial cells exhibited increases in IL-6 and IL-8 production, leading to an inflammatory response and neutrophil recruitment to the lungs (Lerner et al., 2015; Garcia-Arcos et al., 2016). Neutrophils, in response to EC vapor exposure, increased the expression of proteins related to neutrophil extracellular traps (NETs), a process by which the cells excrete a net-like matrix to encompass and kill pathogens (Reidel et al., 2018). However,

an increase in NET release and cell count was not observed. This may suggest that neutrophil activity is hindered by EC exposure. Pulmonary bacterial and viral clearance diminishes after exposure to EC vapor in mice (Sussan et al., 2015). Mice exposed to either EC vapor or air were exposed to *Streptococcus pneumoniae*, with EC vapor-exposed mice demonstrating a significant decrease in pulmonary bacterial clearance. After infection with influenza A, EC-exposed mice exhibited higher viral titers and enhanced viral-induced illness and mortality. Similarly, mice exposed to EC vapor increased human rhinovirus loads (Wu et al., 2014). It is widely accepted that people with AUD have higher pneumonia rates than people without AUD. In combination, EC use and AUD might demonstrate a higher rate of pneumonia than single-substance users due to significant impairment to physical and cellular defenses of lung innate immunity.

EC use is reported to cause COPD-like illnesses (Sisson, 2007; Reidel et al., 2018; Osei et al., 2020). A significant increase in the COPD-associated proteins elastase and MMP-9 was found in EC users when compared with non-users (Reidel et al., 2018). Excessive alcohol consumption is also associated with increased sputum production and cough (Sisson, 2007). Current EC use was associated with 75% higher odds of chronic bronchitis, emphysema, or COPD compared with never users (Osei et al., 2020) as well as a marked increase in mucin production (Gundavarapu et al., 2012; Garcia-Arcos et al., 2016; Javed et al., 2017). Interestingly, mucin secretion was independent of IL-13, a cytokine important in mucin regulation and appeared to be induced via nicotine receptor activation (Gundavarapu et al., 2012; Javed et al., 2017). Upregulation of CD11b and CD66b, proteins involved in the cellular adhesion and migration of macrophages and neutrophils, was detected (Higham et al., 2016). P38 MAPK, a protein kinase activated during stress and cytokine release, was also elevated (Higham et al., 2016). In addition, increased airway hyperactivity with peripheral airway flow resistance and distal airspace enlargement was detected in EC users (Lerner et al., 2015; Garcia-Arcos et al., 2016; Reidel et al., 2018). Distal airspace enlargement, often seen with the destruction of alveoli, and peripheral airway resistance may contribute to breathing difficulty and reliance on supplemental oxygen as seen in COPD patients. Damage caused by EC use may interfere with gas exchange across alveolar membranes, similar to that seen in COPD patients, leading to oxygen dependence. EC use was correlated with higher risk of emphysema and bronchitis, two conditions commonly associated with COPD (Centers for Disease Control and Prevention, 2018). In relation to alcohol, more research is needed to better understand the relationship between AUD and COPD. In moderation, alcohol functions somewhat as a bronchodilator due to the smooth muscle relaxing capabilities of nitric oxide (Sisson, 2007). Under heavy alcohol exposure, however, alcohol likely exacerbates COPD symptoms (Sisson, 2007). While it has not been investigated, AUD may exacerbate the COPD-related symptoms seen in EC users.

EC use was attributed to epithelial barrier damage in capillary endothelial cells and lung epithelial cells (Javed et al., 2017; Muthumalage et al., 2019). This damage caused leaky capillaries in the lungs, allowing for fluid accumulation unrelated to cardiac status. Epithelial damage may be the result of accumulation of ROS or particulate damage from metals found in the vapor. EC use resulted in detectable levels of ROS and free radicals, probably created during the vaporization of the e-liquid (Muthumalage et al., 2019). Similarly, EC reduced glutathione levels in cells exposed to EC vapor (Lerner et al., 2015). Glutathione plays a significant part in reducing cellular ROS and oxidative stress in cells. Decreases in glutathione levels were noted with significant increases in aldehyde detoxification proteins and oxidative stress proteins (Reidel et al., 2018).

The production of ROS by EC, paired with the reduction of cellular glutathione levels, suggests that dual users may be subjected to higher levels of oxidative stress than single-substance users. Further work is needed to determine the exact exposure levels in dual users. During alcohol metabolism by CYP450 enzymes, ROS are directly generated in the lungs. For users of EC who have an AUD, they may experience significantly higher levels of oxidative stress and damage to lung tissue when compared with single-substance users alone. For individuals who consume alcohol, a certain amount of ethanol exits the bloodstream and is exhaled. Exhaled ethanol condenses on epithelial cells, resulting in higher concentrations of ethanol in the conducting airways (Sisson, 2007). This exposure has the potential to damage cells and cause fluid to leak through tight junctions (Sisson, 2007). As a result, individuals with AUD are at an increased risk for injury-induced fluid accumulation in the lungs. In dual users, epithelial barrier damage may be further compromised, allow more fluid to accumulate in the lungs, interfere with normal gas exchange in alveoli, and contribute to an increased risk of pneumonia and ARDS.

Overall, we hypothesize that dual use of EC and alcohol may interfere with normal lung function, may contribute to the pathogenesis of COPD-like illnesses greater than single-substance users, and leave the dual user more susceptible to bacterial and viral infection. Normal innate immune responses in the lungs are altered, particularly macrophage function, decreased ciliary beating, and impaired pulmonary clearance to bacterial and viral infections. Physical barriers such as microcapillary endothelial cells and epithelial barrier cells are disrupted, causing fluid accumulation in the lungs. Nonfunctioning physical barriers and fluid accumulation in the lungs may further increase the risk of infection, decrease gas exchange, and exacerbate COPD symptoms. However, further study is required to determine the extent of the additive effects to the dual user.

VITAMIN E ACETATE LUNG DAMAGE

In 2019, an outbreak of over 2,000 cases of e-cigarette, or vaping, product use-associated lung injury (EVALI) occurred across the United States, leading to 42 deaths and approximately 1,906 hospitalizations (Chatham-Stephens et al., 2019). Of those affected by EVALI, 85% reported the use of tetrahydrocannabinol (THC)-containing products within the past 3 months (Chatham-Stephens et al., 2019). A test from the CDC evaluated 29 EVALI patients and detected vitamin E acetate in BALF,

providing evidence of a potential cause of injury. Vitamin E acetate is a lipid oil additive in some vaping products, particularly those containing THC, as a thickener (Wu and O'Shea, 2020). No alcohol consumption data was collected or has been reported to date on EVALI subjects. While the vaping of THC products poses a significant public health risk, it is critical to distinguish this short-term outbreak from the long-term injuries sustained by the chronic, daily use of nicotine in ECs.

FUTURE AREAS OF INTEREST

The relative newness of ECs as a nicotine delivery system has prevented the long-term epidemiological studies cigarettes have been subjected to. With more time, we will gain a better understanding of the addictiveness, harmful effects, and adverse health conditions that will arise as a result of chronic EC and alcohol dual use. Future study will also answer questions related to second-hand vapor exposure and the chemical composition of ECs.

Second-hand smoke is a significant public health concern related to cigarette smoking. Often, smoking and EC use occur in the same place where alcohol is consumed. This suggests second-hand vapor exposure needs to be investigated to determine the extent of unwanted exposure to bystanders in public locations. EC manufacturers claim vapor released by ECs is water vapor, thus creating no public health concern for environmental exposure. Four studies were identified that examined indoor air quality of ECs. Particulate matter (PM), particulate number count (PNC), volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), carbonyls, and metals were found where EC use occurs (Schober et al., 2014). One study measured VOCs, nicotine, low molecular weight carbonyls, PAHs, tobacco-specific nitrosamines, and metals (O'Connell et al., 2015). Another study measured saliva and urine cotinine levels from those living in EC user homes and compared them with smoking and control homes (Ballbè et al., 2014). Nicotine deposition was measured on 10 cm^2 areas throughout the EC and cigarette user home (Bush and Goniewicz, 2015). Overall, detectable levels of PM, PNC, 1,2-propanediol, nicotine, CO_2, glycerin, PAHs, VOCs, and formaldehyde were present in the indoor household environment of EC users (Schober et al., 2014; O'Connell et al., 2015). Detectable levels were higher than those in control homes, yet lower than those found in the homes of smokers (Bush and Goniewicz, 2015). Levels of airborne nicotine and levels of salivary and urinary cotinine were present in individuals who did not use EC, but lived in households where EC were used, and were found to be at lower concentrations than in non-smoking individuals living in smoker households (Ballbè et al., 2014). This suggests that the vapor from ECs is not water vapor, but includes numerous chemicals found at lower levels than traditional cigarettes. Future study is required to determine if chemical concentration in second-hand vapor is biologically relevant to harm individuals consuming alcohol, or is harmful for at-risk populations, such as the elderly, immunocompromised, and pregnant women.

With a wide variety of EC devices and liquid composition, standardization and control between experiments makes comparison between studies impossible. The chemical composition of EC, both in the liquid and vapor, varied significantly depending on the EC device or liquid used (Varlet et al., 2015). PG and VG typical comprised the base ingredients, with nicotine and a variety of flavor aldehydes added as well (Varlet et al., 2015). Given its massive popularity, JUUL devices may represent an ideal model for standardization for experimentation. However, it is important to acknowledge modifications that can be added to JUUL pods, such as "hacked" pods and pods compatible with JUUL devices that contain liquid not produced by the company (LaVito, 2019). In addition, some EC liquids contained alcohol. E-liquids containing alcohol may decrease the user's psychomotor performance and produce detectable levels of alcohol metabolites in the urine (Valentine et al., 2016). Further study is needed, however, to determine if this impairment is greater in the dual user. Flavor aldehydes represented the biggest variation in chemical composition, with numerous flavor aldehydes present such as vanillin, ethyl vanillin, benzaldehyde, cinnamaldehyde, and citral, some of which have been previously linked to lung tissue damage (Garcia-Arcos et al., 2016). While many of these aldehydes have been tested and deemed safe for oral ingestion, the effects and safety of inhalation have yet to be determined. Interestingly, one study demonstrated that PG and VG reacts with flavor aldehydes in the liquid and vapor to produce PG–aldehyde and VG–aldehyde adducts in both JUUL and EC products (Erythropel et al., 2019). These acetals had a high carryover rate from liquid to vapor during use and remained stable in physiologic pH in the lungs with a 36-h half-life. Such adducts activate cough receptors in the lungs at lower concentrations than the aldehyde alone (Erythropel et al., 2019). This study represents an important finding: reactions between ingredients in EC liquids may create stable adducts and interfere with normal lung function, demonstrating the danger of the formations of new compounds in unstable e-liquids at high temperatures found in ECs and the risk they pose to the body. Unique adduct formation, such as the formation of the malondialdehyde–acetaldehyde complex seen only in smokers with AUD (Sapkota et al., 2017), may also occur during the dual use of EC and alcohol. Future study is warranted to determine the unintended adducts formed within the dual user.

EC + Alcohol and Coronavirus Disease 2019

Coronavirus disease 2019 (COVID-19), caused by infection of the virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), originated in Wuhan, China in 2019 (Wu et al., 2020). This disease quickly spread around the world, leading the World Organization (WHO) to declare COVID-19 a pandemic on March 11, 2020 (Ducharme, 2020). SARS-CoV-2 has been shown to invade cells via binding to the extracellular domain of angiotensin-converting enzyme 2 (ACE2) (Li et al., 2003; Zhou et al., 2020). Inhalation of nicotine from cigarettes has been demonstrated to increase ACE2 expression in human bronchial

epithelial cells via α-7-subtype of the nicotine receptors (α7-nAChR), with a similar increase in ACE2 expression measured in patients with COPD (Brake et al., 2020; Leung et al., 2020). Studies regarding the relationship between ACE2 expression in EC-specific users are yet to be conducted, let alone studies pertaining to the effects of EC and alcohol dual use on SARS-CoV-2. Single-substance studies are starting to be published, showing higher risk of COVID-19 diagnosis in EC users; however, polysubstance use has yet to be examined (Gaiha et al., 2020). This identifies a large area of study regarding the novel coronavirus and polysubstance use. With the given aforementioned studies pertaining to individual effects of EC and alcohol on the effectiveness of lung innate immune system (Wyatt et al., 2012; Wu et al., 2014; Sussan et al., 2015), we hypothesize that dual users of EC and alcohol may be at higher risk to complications of COVID-19. However, future study regarding EC and alcohol's effect on ACE2 when exposed to SARS-CoV-2 and comparison of symptoms between the dual user, single user, and never user are required to better understand how polysubstance use may affect an individual with COVID-19.

CONCLUSION

EC and alcohol dual use is rising, with EC users more likely to participate in hazardous binge drinking, placing such users at a higher risk of AUD than non-users. With a limited body of literature compared with traditional cigarettes, a significant volume of research is still needed to better understand the long-term risks of EC and alcohol dual use (Chudomelka and Wyatt, 2020). Second-hand vapor exposure, while containing fewer chemicals than traditional cigarettes, has the potential to be harmful to vulnerable populations and requires additional study in combination with alcohol consumption. With the variety of devices and liquids available to the user, standardization for experimental testing is important. JUUL devices and liquids, given their popularity, may be the right device for such standardization. However, they too contribute challenges for research. Flavor aldehydes, nicotine, and additional chemicals formed during vaping suggest a wide variety of toxicological responses that may occur in conjunction with alcohol use that need to be investigated. The long history of comorbidity between nicotine and alcohol continues, with EC and alcohol use presenting an additional method of dual substance use yet to be explored and clearly outlines a public health issue that will not likely be diminished in the near future.

AUTHOR CONTRIBUTIONS

TJW and TAW agreed upon the subject matter of this review. TJW collected the review material, drafted the manuscript, and approved the final version. TAW assisted with drafting, editing, and approving the final version. Both authors contributed to the article and approved the submitted version.

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Physical Exercise–Mediated Changes in Redox Profile Contribute to Muscle Remodeling After Passive Hand-Rolled Cornhusk Cigarette Smoke Exposure

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Consumption of non-traditional cigarettes has increased considerably worldwide, and they can induce skeletal muscle dysfunction. Physical exercise has been demonstrated to be important for prevention and treatment of smoking-related diseases. Therfore, the aim of this study was to investigate the effects of combined physical exercise (aerobic plus resistance exercise) on muscle histoarchitecture and oxidative stress in the animals exposed chronically to smoke from hand-rolled cornhusk cigarette (HRCC). Male Swiss mice were exposed to ambient air or passively to the smoke of 12 cigarettes over three daily sessions (four cigarettes per session) for 30 consecutive days with or without combined physical training. 48 h after the last training session, total leukocyte count was measured in bronchoalveolar lavage fluid (BALF), and the quadriceps were removed for histological/immunohistochemical analysis and measurement of oxidative stress parameters. The effects of HRCC on the number of leukocytes in BALF, muscle fiber diameter, central nuclei, and nuclear factor kappa B (NF-κB) were reverted after combined physical training. In addition, increased myogenic factor 5, tumor necrosis factor alpha (TNFα), reduced transforming growth factor beta (TGF-β), and nitrate levels were observed after physical training. However, the reduction in superoxide dismutase and glutathione/glutathione oxidized ratio induced by HRCC was not affected by the training program. These results suggest the important changes in the skeletal muscle brought about by HRCC-induced alteration in the muscle redox profile. In addition, combined physical exercise contributes to remodeling without disrupting muscle morphology.

Keywords: hand-rolled cornhusk cigarette, cigarette smoke, combined physical exercise, oxidative stress, muscle

INTRODUCTION

Any form of tobacco-containing cigarettes such as industrialized cigarette and handmade cigarette is a complex mixture of toxicants which can pose several risks, such as redox profile alteration and resulting cellular structure deformation in the biological systems (Valença et al., 2005; Gochman et al., 2007; Bhalla et al., 2009; Menegali et al., 2009; Saito et al., 2012; Madani et al., 2018). However, the consumption of other forms of cigarettes, especially handmade or natural cigarettes, which do not undergo industrial processing, has increased considerably worldwide. These cigarettes are erroneously considered healthier than industrialized cigarettes, but the consequences of cigarette smoking are not yet sufficiently well known to generate concern among the public. The use of hand-rolled cornhusk cigarettes (HRCC) has increased in Brazil (Levy et al., 2012), and it has generated intense concern among the public with respect to its effects on health.

Hand-rolled cornhusk cigarette consists of tobacco macerated and rolled up in a corn husk. The effect of this HRCC-exposed smoke is similar to the effect of other types of cigarettes (Valença et al., 2004; Lanzetti et al., 2011; Nesi et al., 2016). However, HRCC smoke can be more severe than other forms of cigarettes because the smoke contains particulate matter and other chemical agents. In addition, the absence of gunpowder in corn straw induces the smoker to exert a more intense inspiratory flow, which leads to a greater uptake of cigarette smoke by the airways when compared to industrialized cigarettes. In fact, these characteristics may potentiate the harmful effects of tobacco and induce alterations in the oxidative stress parameters (Camera et al., 2019). The skeletal muscle is highly susceptible to the effects of cigarette smoke, which can interfere with the quality of muscle structure and function in smokers (Barnes, 2014; Krüger et al., 2018). This cigarette smoke is associated with muscle weakness (Seymour et al., 2010; van den Borst et al., 2011; Barreiro et al., 2012) and reduced muscle mass (Mathur et al., 2014). These effects are attributed to the toxic substances contained in the smoke that stimulates the degradation of muscle proteins and impairs protein synthesis (Rom et al., 2012).

Aerobic physical exercise exerts a protective effect on the oxidative and inflammatory agents present or induced by industrial cigarette smoke (Menegali et al., 2009; Nesi et al., 2016; Madani et al., 2018). Moreover, resistance exercise can also be helpful by increasing muscle tone and density (Saito et al., 2012), by assisting the recovery of respiratory function (Singh et al., 2011), and by stimulating the antioxidant and anti-inflammatory systems (Souza et al., 2017; Vilela et al., 2018). However, the effects of the association of both types of exercise (combined exercise) are still under investigation. Thus, the objective of the present study was to investigate the effects of combined physical exercise on muscle histoarchitecture and oxidative stress parameters in mice exposed to HRCC smoke.

MATERIALS AND METHODS

Animals

Male 3 to 4-month-old Swiss mice (30–35 g) were randomly assigned into four groups (n = 9): Ambient air (AA), HRCC, AA-plus combined exercise, and HRCC plus combined exercise. The training protocol and cigarette exposure were performed simultaneously for 4 weeks of experiments. Food and water were available *ad libitum*, and the room temperature was maintained at 20 ± 2°C, with 70% humidity under a 12-h light/dark cycle. The Institutional Committee for Animal Care at Universidade do Extremo Sul Catarinense approved all the procedures under protocol number 087/2015-1.

HRCC Exposure

Cornhusk cigarettes were purchased from farmers in the city of Severino, RS, Brazil. The tobacco leaves were stripped, macerated, and indirectly exposed to the sun for 48 h. To prepare HRCC, 0.8 g of dry tobacco was uniformly wrapped in a cornhusk. The amount of tobacco used in each cigarette was equivalent to that present in one commercial cigarette. Animals were exposed to 12 HRCC smokes per day; the regimen included exposure to four cigarettes, three times a day, 7 days/week over 30 days (Camera et al., 2019). Briefly, animals were placed in an inhalation chamber (40 cm long, 30 cm wide, and 25 cm high), with an exhaust air system. Each cigarette was connected to a plastic 50-mL syringe, and smoke puffs were aspirated and subsequently injected into the exposure chamber. The animals were maintained under this condition for 6 min. After each cigarette use, the box was opened for 1 min to exhaust the air. The animals were kept in this smoke-air condition for 27 min per session.

Training Protocol

The combined-exercise training protocol consisted of 30 min of aerobic training and approximately 30 min of resistance training. The training period lasted 4 weeks, and the training frequency was 3–4 days per week, with 48-h intervals between sessions, for a total of 30 days. For aerobic training, the animals were habituated on a ninechannel motor-driven treadmill at 10 m/min for 10 min/day for 1 week to ease their adaptation to the new environment. The mice did not receive any stimuli to run. The exercise groups performed an incremental running program at progressive levels of intensity (13–17 m/min). Untrained animals were placed on a switched-off treadmill for the same 8 weeks as the exercise-trained groups. For resistance training, animals were familiarized with climbing a ladder (1.1 × 0.18 m, 30.2-cm high steps, 80° slope), as previously described (Souza et al., 2017). A load was secured to the base of the tail using plastic insulation tape. A repetition was deemed successful when the animal climbed from the bottom of the rack to the top. The exercise consisted of climbing the ladder carrying a load corresponding to 25% of the animal body weight; the weight was progressively increased to 50 and 75%, with 8–12 repetitions and a 2-min break between repetitions. When necessary, food was placed at the top of the ladder to encourage animals to perform the exercise.

Bronchoalveolar Lavage (BAL)

The animals were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg), and BAL was performed through a tracheal cannula with 3 × 1 mL phosphate-buffered saline (PBS) lavage. Approximately 1.5 mL (80%) of bronchoalveolar lavage fluid (BALF) was recovered from each mouse examined. A 100 μL aliquot was used for the total cell count, the remainder was immediately centrifuged at 300 × *g* for 10 min, and the cell pellets were washed twice and resuspended. The supernatants of BALF containing total leucocytes were measured in Neubauer counting chamber, and the remaining sample was stored at −80°C.

Euthanasia and Tissue Preparation

After BAL procedures, all the animals were euthanized by cervical displacement. The right quadriceps (central portion) from three animals from each group were fixed with 4% paraformaldehyde and processed for histology. The remaining samples were aliquoted and stored at −80°C for future biochemical analysis.

Histological and Morphometrical Analyses

Material cleavage was performed using specific cuts. Formalin fixed paraffin embedded (FFPE) skeletal striated muscle tissue samples were sliced on a microtome to 4-μm-thick histological sections, which were stained with hematoxylin & eosin. For histological analysis, digital images were captured using a slide scanner (AxioScan Zeiss) and diameter was calculated using a software for morphometric analysis (Image Proplus). In each group, 10 photomicrographies were selected in an area of 288815.2563 μm^2, and the smallest diameter of 10 fibers per field was measured. The data are presented as number and average fiber diameter per field. Quantitative determinations of centralized nuclei were also conducted in the same way as histological analyses using image analysis software. The assessment was performed in a single-blind manner (Stewart et al., 2016).

Tissue Microarray (TMA) and Immunohistochemistry (IHC)

Representative areas of the muscle were transferred from the histology block to a recipient tissue microarray (TMA) block. Next, two 4-μm-thick paraffin-embedded sections of the TMA blocks were transferred to electrically charged Star Frost™ (Braunschweig, Germany) slides and incubated with a primary anti-nuclear factor kappa B (NF-κB) p105/50 (ab797; 1:200; Abcam, Cambridge, United Kingdom) and Tumor necrosis factor alpha (TNFα) (ab6671; 1:100; Abcam) overnight in a humidified chamber at a temperature between 2 and 8°C. The slides were incubated with the secondary antibody for 30 min at room temperature, using the Reveal Polyvalent horseradish peroxidase (HRP)–Diaminobenzidine (DAB) kit (Detection System-Spring Bioscience™, Pleasanton, CA, United States). The immunoreactivity was developed by adding DAB chromogen/substrate solution (Spring) to the slides. Harris hematoxylin was used for counterstaining. Positive and negative controls were run in parallel with all reactions. The slides were scanned using the Axio Scan.Z1 scanner (Carl Zeiss, Germany). The files generated were fragmented into single images, and approximately 25 images were selected for analysis. The areas of immunopositive markings for the anti-NF-κB p105/50 and TNFα antibodies were quantified using Image-Pro Plus software version 4.5 (Media Cybernetics, United States). The immunopositive objects were selected using a "mask" for standardizing and automating the process. The numerical data of the immunopositive marking area were generated and subsequently exported to an Excel spreadsheet.

Biochemical Assays

The levels of oxidized intracellular 2′,7′-dichlorofluorescein (DCF) were monitored in samples incubated with 2′,7′-dichlorodihydrofluorescein (DCFH). The formation of the oxidized fluorescent derivative was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using fluorescence spectrophotometer instruments (LeBel et al., 1992). To evaluate the indirect nitric oxide (NO) production, the levels of nitrate were measured from of reduction of nitrate by vanadium (III) combined with detection by the acidic Griess reaction (Miranda et al., 2001). Superoxide dismutase (SOD) activity was determined spectrophotometrically at 480 nm and estimated by adrenaline autooxidation inhibition and expressed as U/mg of protein (McCord and Fridovich, 1969). The total glutathione (GSH) levels were based on the reaction of GSH with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman's reagent), which forms an oxidized glutathione (GSSG)–2-nitro-5-thiobenzoic acid (TNB) product that is later reduced by glutathione reductase in the presence of NADPH with the consequent synthesis of GSH. The total GSH concentration was determined using a regression curve that was plotted using various GSH standards. The GSSG level was measured from the recycling of GSSG by the spectrophotometric monitoring of NADPH in the presence of 2-vinylpyridine. The total GSH and GSSG concentrations were determined using a regression curve plotted using various GSH standards (Rahman et al., 2006). The myogenic factor 5 (Myf5) level was measured by western blot. The samples were homogenized in lysis buffer supplemented with protease and phosphatase inhibitors for future total protein extraction. The protein extracts were separated by SDS–PAGE and processed for western blot analysis using antibodies against Myf5 (Santa Cruz Biotechnology, Inc.) according to the standard procedure. The values obtained were normalized to those of β-actin (Santa Cruz Biotechnology). Transforming growth factor beta (TGF-β) tissue concentrations were determined using commercial ELISA kits as recommended by the manufacturer (R&D systems®, United States; ALPCO®, United States; Labtest®, Brazil).

Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM), and differences between groups were subjected to one-way analysis of variance (ANOVA) followed by the Newman–Keuls *post hoc* test when appropriate. Differences with $p < 0.05$

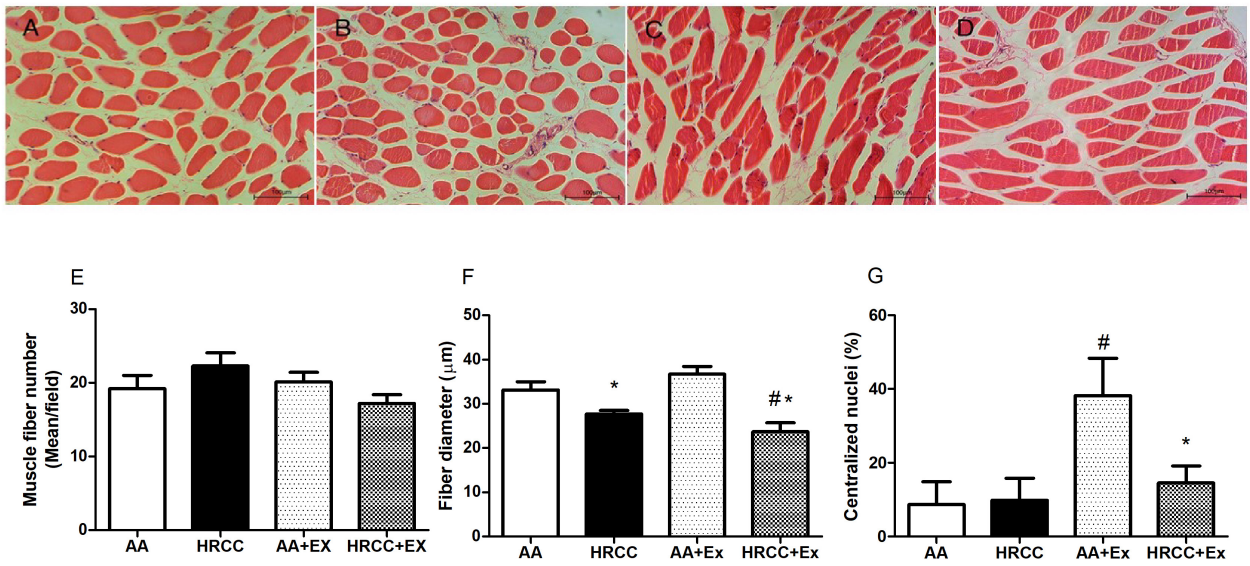

FIGURE 1 | Histology of the quadriceps of mice chronically exposed to hand-rolled cornhusk cigarette (HRCC) smoke and combined exercise. Panels **(A–D)** are photomicrographs stained with H & E: **(A)** ambient air (AA), **(B)** HRCC, **(C)** AA-plus combined exercise, **(D)** HRCC plus combined exercise. Panel **(E)** represents the number of fiber per field, panel **(F)** represents the mean of smallest diameter of 10 fibers per field and panel **(G)** represents the percentage of nuclei centralized. The data are expressed as mean and standard error of the mean and were analyzed statistically using two-way ANOVA, followed by the Newman–Keuls test. The groups were considered different when $p \leq 0.05^*$ (difference in relation relation to the ambient air, # difference in relation to HRCC). Images under 20× objective.

were considered statistically significant. All statistical analyses were performed using GraphPad Prism 6 software.

RESULTS

HRCC Induces Morphological Alterations in Quadriceps

Figure 1 shows representative images of cross-sectional histological sections of the quadriceps muscle. Control animals presented a normal morphology and peripheral nuclei muscle fibers (**Figure 1A**). Animals untrained exposed to HRCC (**Figure 1B**) exhibited decreased muscle fiber diameter compared to the control animals. In **Figure 1C**, trained animals showed increased muscle fiber diameter and the presence of the central nuclei. **Figure 1D** shows a decrease in muscle fiber diameter and the presence of peripheral nuclei on animals exposed to cigarette smoke and combined exercise. These data are represented graphically in **Figure 1E** (fibers number), **Figure 1F** (fiber diameter), and **Figure 1G** (centralized nuclei).

HRCC Smoke-Induces Myf-5 While Suppressing TGF-β Level

Combined exercise with HRCC-exposure-induced changes in muscle remodeling. We observed that Myf-5 level was increased in combined training with HRCC-exposed animals when compared to HRCC (**Figure 2A**), whereas TGF-β level was decreased with combined exercise plus HRCC-exposed animals when compared to HRCC (**Figure 2B**).

Combined Physical Exercise Regulates HRCC-Induced Inflammatory Parameters

Figure 3 inflammatory parameters in BALF and quadriceps. The number of total leukocytes in BALF was used as an inflammatory indicator. The results showed an increased number of leukocytes in the animals exposed to HRCC smoke while there was a decrease after physical exercise in the groups of AA+Ex and HRCC+EX (**Figure 3A**). Quantitative values of immunoexpression of NF-κB p105/50 and TNFα was analyzed and are represented in **Figures 3B,C**. The immunoexpression of NF-κB p105/50 and TNF (within the muscle fiber) increased in the muscles of animals exposed to HRCC, and it was significantly reduced after physical training. TNFα (in connective tissue) immunoexpression did not show significant changes with HRCC. However, it was significantly increased with exercise. Immunostaining data and representative graphic of these molecules are presented in **Figure 3D**.

Combined Exercise Promotes Change in the Oxidative Stress Parameters After HRCC Exposure

Levels of DCF and nitrate were used as indicators of cellular oxidants, while the activity of SOD, total GSH, GSSG and the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) were used as indicators of antioxidant defense systems. The results depicted in **Figure 4A** show a significant increase in nitrate levels in animals exposed to HRCC smoke and a reduction in this level after combined physical training.

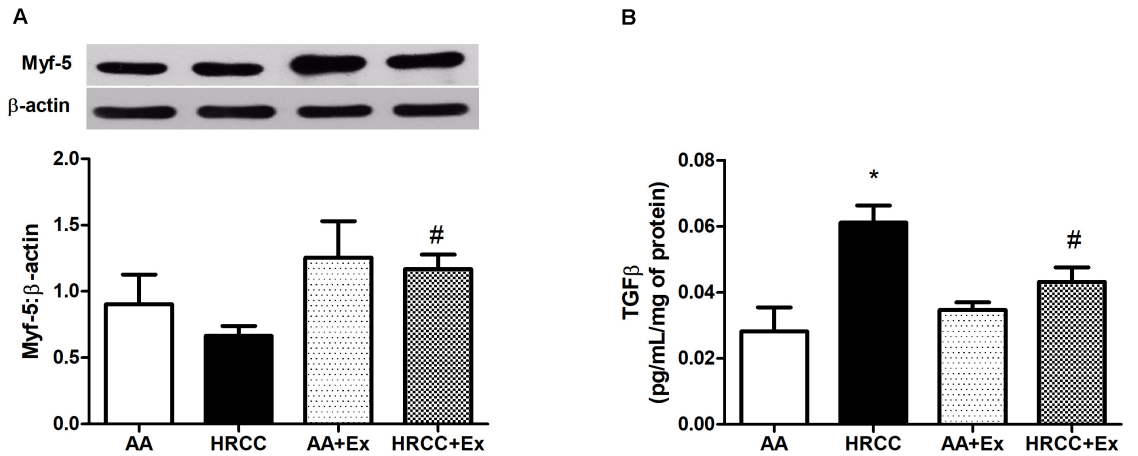

FIGURE 2 | Effect of combined exercise on parameters of muscle remodeling in quadriceps of mice exposed to hand-rolled cornhusk cigarette (HRCC) smoke. The content of myogenic factor 5 (Myf5) **(A)** was normalized to β-actin, and the production of transforming growth factor beta (TGFβ) **(B)** was normalized to the total amount of protein. Data are expressed as mean and standard error of the mean and were analyzed statistically using two-way ANOVA, followed by the Newman–Keuls test. The groups were considered different when $p \leq 0.05$* (difference in relation to the ambient air, # difference in relation to HRCC).

However, no significant changes were observed in DCF levels (**Figure 4B**). SOD activity was significantly reduced with HRCC smoking, but the level of SOD in HRCC with combined exercise program did not have a significant effect (**Figure 5A**). Total GSH was significantly decreased after HRCC exposure (**Figure 5B**), while animals of HRCC plus exercise presented increased levels of GSSG when compared to respective controls (**Figure 5C**). GSH/GSSG ratio was significanlty decreased with HRCC exposure, and these values remained reduced after combined physical training (**Figure 5D**).

DISCUSSION

Physical exercise has been widely used to minimize the cigarette-induced damages (Pinho et al., 2007; Menegali et al., 2009; Koubaa et al., 2015; Nesi et al., 2016). Several studies have demonstrated that smokers have muscle abnormalities, which lead to a reduction in exercise performance (Barnes, 2014; Gea et al., 2015). However, studies conducted on cigarette-exposure-induced abnormalities in the skeletal muscle results are inconclusive because of the characteristics of physical exercise and the type of cigarette promote different responses. The effects of exercise are directly dependent on the frequency, intensity, duration, and period of training (Pinho et al., 2006), whereas the type of cigarette and the form of consumption lead possibly to different changes in cardiorespiratory and muscular functions (Camera et al., 2019). In this scenario, this is the first study to show the effects of combined exercise (aerobic plus resistance exercise) on animals' skeletal muscle exposed to HRCC smoke.

This study showed a decrease in muscle fiber diameter and the presence of peripheral nuclei on animals exposed to HRCC smoke and combined exercise. Previous studies have already demonstrated that chronic smoking induces negative impacts on skeletal muscle structure (Morse et al., 2007; Nogueira et al., 2018), and functions (Degens et al., 2015), while other studies showed improvement in muscle structure in physical exercise (Krüger et al., 2018). When exposed to cigarette smoke, the muscle is more susceptible to atrophy in response to differentiation, proliferation, and remodeling mechanisms (Chan et al., 2020). The muscle morphology alterations induced by HRCC smoke was reverted by combined exercise but, in terms of muscle adaptation, HRCC+exercise group depicts lower fiber diameter and nuclei centralization is not different from HRCC. These results suggest a possible inability of muscle adaptation to combined exercise. This non-adaptive response may be associated with insufficient stimulus (intensity and training period) to induce muscle adaptation mediated by combined exercise. Under these conditions, we observed decreased muscle differentiation by increasing TGFβ expression and inhibiting Myf5 expression. TGFβ is an important marker of tissue remodeling and negatively affects skeletal muscle regeneration by inhibiting satellite cell proliferation and expression of some muscle-specific genes such as Myf-5. Besides, TGFβ is associated with an increase in inflammatory markers (Böhm et al., 2016), and it induces ubiquitin-proteasome protein degradation (Waning et al., 2015). TGFβ reduction by combined exercise may be one of the important mechanisms that contribute to tissue regeneration in the skeletal muscle, especially by stimulating the Akt-mTOR pathway as already demonstrated in smooth muscle cell (Latres et al., 2005; Suwanabol et al., 2012) via IGF-1 signaling, which is stimulated, particularly, through muscle strength exercises. The relationship between TGFβ and Akt-mTOR in the skeletal muscle needs to be better investigated under the effect of cigarette smoking and physical exercise.

Systemic inflammation and oxidative stress are important injury mechanisms that induce independent respiratory and skeletal muscle effects (Cielen et al., 2016). An increase in the recruitment of inflammatory cells to the lung interstitium is observed in smokers (Kennedy-Feitosa et al., 2014), and it

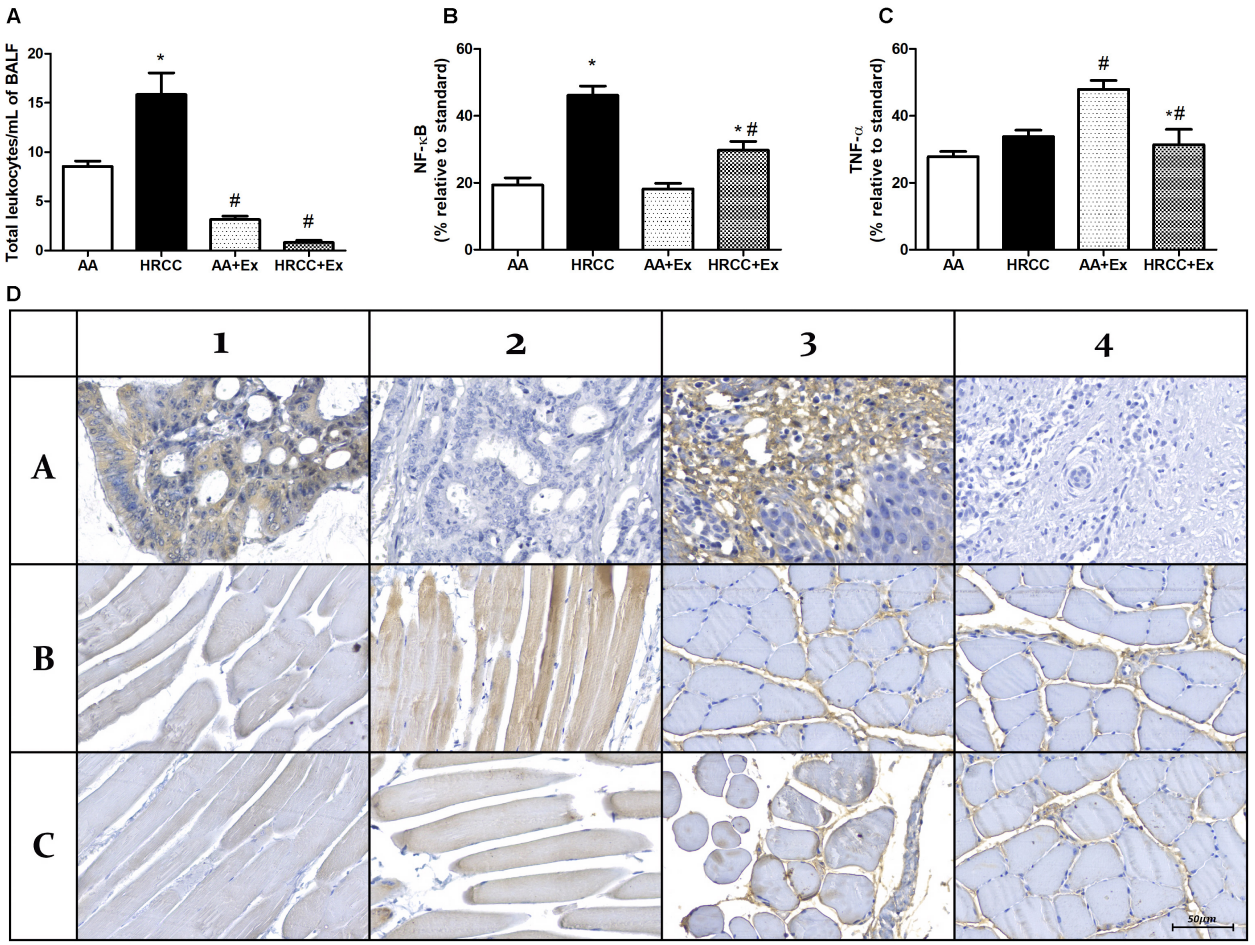

FIGURE 3 | Effect of combined exercise on inflammatory parâmeters of mice exposed to hand-rolled cornhusk cigarette (HRCC) smoke. The number of total leukocytes in Lavage fluid (BALF) **(A)** are expressed as mean and standard error of the mean and were analyzed statistically using two-way ANOVA, followed by the followed by the Newman–Keuls test. The groups were considered different when $p \leq 0.05$* (difference in relation to the ambient air, # difference in relation to HRCC). Percentage of inflammatory proteins immunoexpression in quadriceps of animals exposed to HRCC and combined exercise were selected from 25 images of four animals per group. The areas of immunopositivity for NF-κB p105/50 **(B)** and TNFα **(C)** were expressed as percentage average relative to standard molecule and analyzed statistically using one-way ANOVA, followed by the Newman–Keuls test. The groups were considered different when the value of p was ≤ 0.05* (difference in relation to the ambient air, # difference in relation to HRCC). Representative images of immunohistochemical analysis of quadriceps of animals exposed to HRCC and combined exercise **(D)**. NF-κB positive control (A1), NF-κB negative control (A2), NF-κB Ambient air (B1), NF-κB HRCC (B2), NF-κB AA+exercise (C1) NF-κB HRCC+exercise (C2), (A3), TNFα negative control (A4), TNFα Ambient air (B4), TNFα HRCC (B4), TNFα AA+exercise (C3) TNFα HRCC+exercise (C4).

presents a risk of tissue damage through the release of toxic mediators, including cytokines, proteolytic enzymes, and ROS (Bhalla et al., 2009). Besides, systemic inflammation is associated with reduced protein synthesis and enhanced protein breakdown, accounting for muscle mass loss and function (Costamagna et al., 2015). In this scenario, our results did not demonstrate alterations in the immunoexpression of TNF-α in muscle tissue after exposure to HRCC. However, exercised animals presented elevated levels of TNF-α, and these results may be related to the intensity of exercise and a possible non-adaptation of the animals to the training protocol. There is no evidence to support the understanding that muscle cells secrete or express TNF-α *in vivo*. Most of the evidence for cytokine expression in the skeletal muscle is derived from the analysis of isolated RNA or protein extracts from the homogenized muscle (Egan and Zierath, 2013; Peake et al., 2015). Therefore, our results revealed immunodetection only in the perisimal area. The elevated level of TNF-α is not correlated only to the grade of muscle inflammation, but also to cellular events during the muscle regeneration in response to injury or non-adaptive processes (Li, 2003).

As presented in this study, previous work has shown that cigarette smoke stimulates the NF-κB activation (Kaisari et al., 2013), and its inhibition prevents muscle degeneration, protein breakdown, and myofiber death (Ahn and Aggarwal, 2005). Although the precise mechanism by which inflammation is involved in protein breakdown/turnover rates is still poorly investigated, Krüger et al. (2018) have recently suggested that a decrease in systemic inflammation and inflammatory mediators in the muscle can indirectly reduce the activation

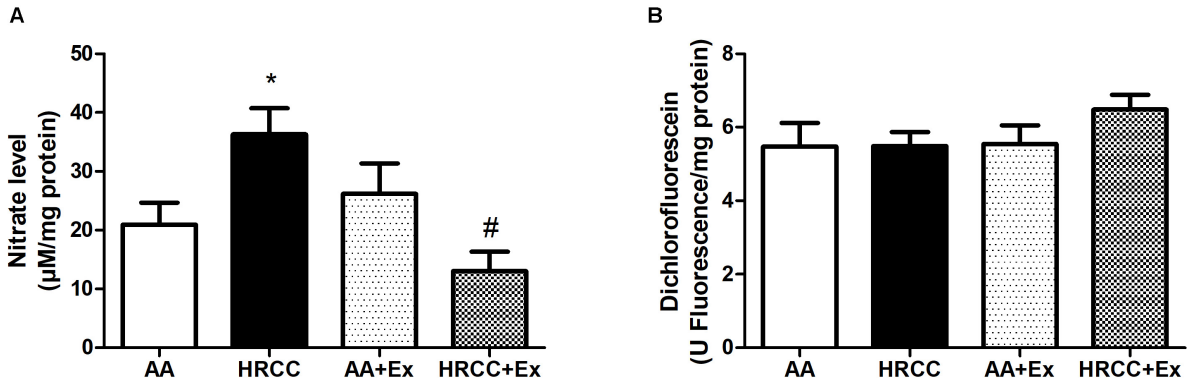

FIGURE 4 | Effect of combined exercise on oxidative stress parameters in quadriceps of mice exposed to hand-rolled cornhusk cigarette (HRCC) smoke. The values of nitric oxide **(A)**, 2′,7′-dichlorofluorescein **(B)** are shown as the mean and standard error of the mean and were analyzed statistically using two-way ANOVA, followed by the Newman–Keuls test. The groups were considered different when $p \leq 0.05$* (difference in relation to the ambient air, # difference in relation to HRCC).

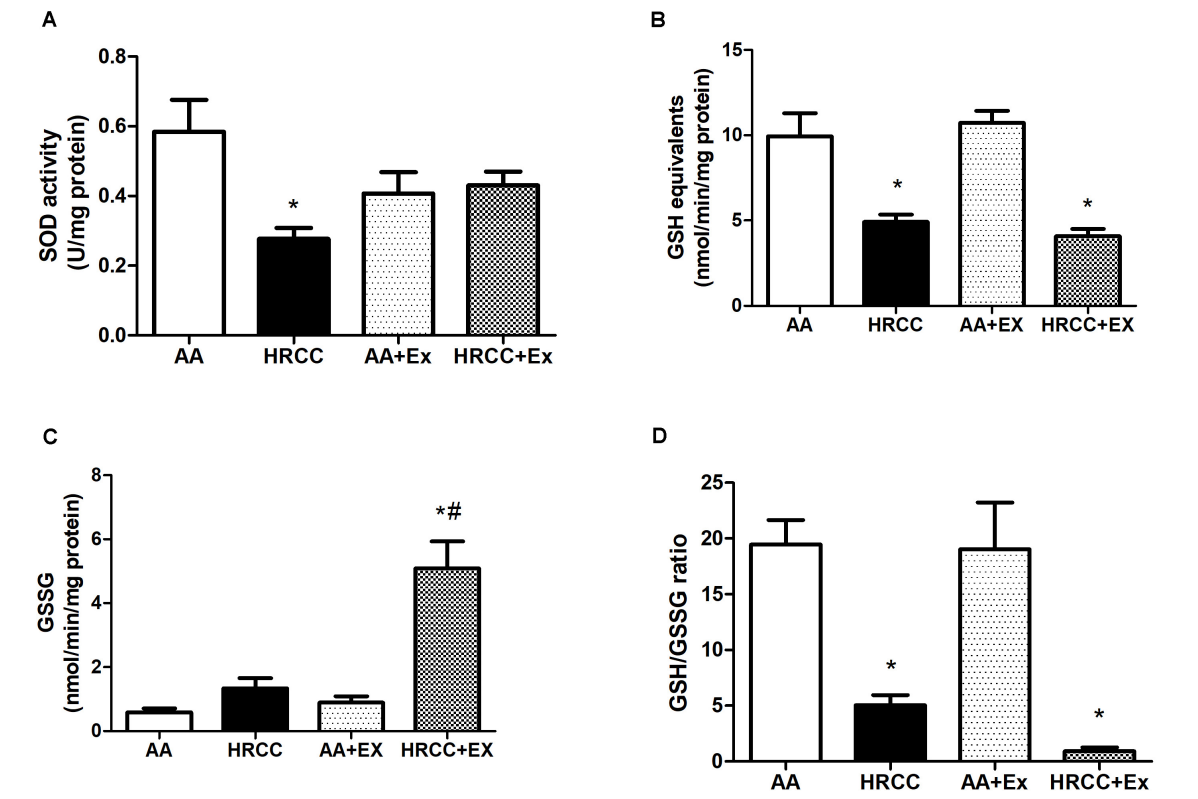

FIGURE 5 | Effect of combined exercise on antioxidants in quadriceps of mice exposed to hand-rolled cornhusk cigarette (HRCC) smoke. Superoxide dismutase activity (SOD) **(A)**, Total GSH activity **(B)**, GSSG level **(C)**, and glutathione (GSH)/oxidized glutathione (GSSG) ratio **(D)** are shown as the mean and standard error of the mean and were analyzed statistically using two-way ANOVA, followed by the Newman–Keuls test. The groups were considered different when $p \leq 0.05$* (difference in relation to the ambient air, # difference in relation to HRCC).

of catabolic pathways via ubiquitin-proteasome proteolytic pathway (Costamagna et al., 2015) and increase anabolic signals (Krüger et al., 2018). Although the contractile muscle activity during exercise also contributes to enhancing the NF-κB levels (Kramer and Goodyear, 2007), moderate exercise training decreases NF-κB activation (Liu and Chang, 2018). Reduced NF-κB levels after exposure to cigarette smoke by exercise may be related to different effects of anti-inflammatory stimulus-induced by moderate exercise, including secretion of anti-inflammatory myokine IL-6, increase in systemic levels of the anti-inflammatory cytokines such as IL-10 and IL-1RA, downregulation of Toll-like receptor expression. Regular exercise

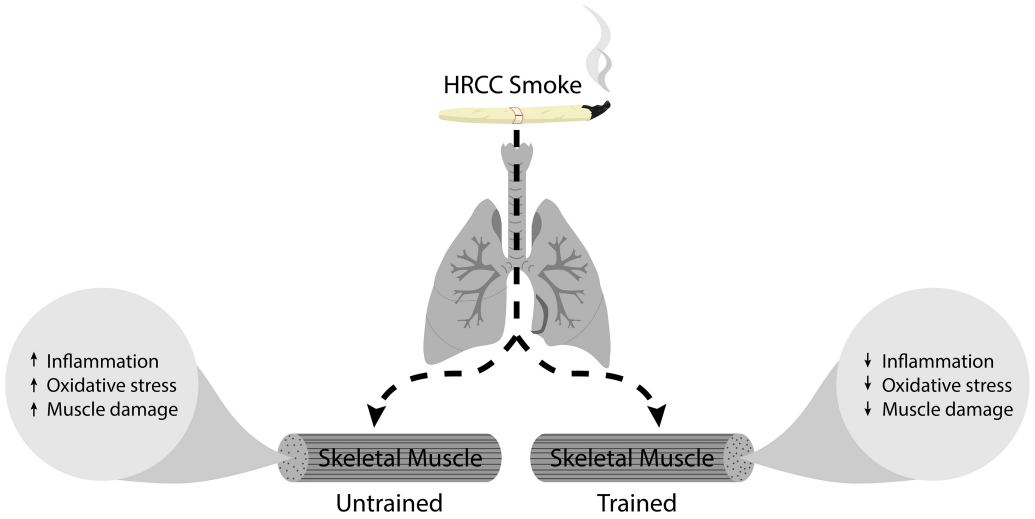

FIGURE 6 | Represents the schematic diagram of HRCC smoke effect on trained and untrained skeletal muscle.

lowers circulating numbers of pro-inflammatory monocytes, inhibits monocyte and or macrophage infiltration, and increases Treg cell numbers in circulation (Gleeson et al., 2011; Wang et al., 2020).

The toxic substances contained in HRCC smoke stimulate the inflammatory response and induce an altered redox system (**Figure 6**). The skeletal muscle is extremely responsive to cigarette smoke (Barnes, 2014; Krüger et al., 2018), favoring ROS production. Here, no significant alteration in the DCF production was observed in the different experimental groups, However, nitrate levels were significantly affected by both HRCC smoke and combined exercise. The results about DCF are apparently surprising because previous studies have already demonstrated that cigarette smoke induces elevated levels of DCF in the lung that are reduced after physical training (Menegali et al., 2009; Nesi et al., 2016). However, the skeletal muscle seems to respond differently to cigarette stimulus than the lung. Observed changes in nitrate levels from HRCC exposure, suggest a possible participation of reactive species of nitrogen on the redox system of the skeletal muscle. This can be reinforced by the low activity of SOD, and the concomitant increase in nitrate levels suggests an alternative pathway to dismutation of superoxide leading to the formation of peroxynitrite (Jourd'heuil et al., 2001). After a combined physical training program, a reduced level of nitrate was observed as well as an increase in SOD activity without changing the DCF levels. This response from exercise is possibly associated with the effects of exercise on the activity of enzymes that catalyze hydrogen peroxide (Pinho et al., 2006; Scheffer et al., 2012; Tromm et al., 2016).

Cigarette-induced glutathione system depletion has been revealed in previous studies (Raza et al., 2013; Gould et al., 2015). Our results show that after exposure to HRCC smoke presented a lower level of total GSH and GSH/GSSG ratio than in control, and this effect was more accentuated with physical exercise. Previous studies have already shown that under the influence of cigarette smoke, the GSH/GSSG ratio is reduced in plasma (Moriarty et al., 2003), heart, and liver (Montiel-Duarte et al., 2004), and other studies indicate a reduced level of GSH in lung (Camera et al., 2019). In skeletal muscle, GSH levels vary depending on the metabolic profile of the tissue (Sen, 1998) and the state of physical exercise (Ji et al., 1992). Notably, the results of this study show that physical training further decreases the cigarette-induced GSH/GSSG ratio. This may be associated with increased detoxification induced by physical exercise. Physical exercise increases the expression and activity of glutathione peroxidase (GPx) in muscle (Lambertucci et al., 2007; Nguyen et al., 2016). GPx catalyzes the reduction of organic hydroperoxides using GSH as a reductant (Brigelius-Flohé and Maiorino, 2013). If the resynthesis of GSH is impaired or if the oxidation rate is greater than resynthesis under the effect of HRCC cigarettes, the level of GSSG remains high, reducing the GSH/GSSG ratio (Lambertucci et al., 2007).

LIMITATION OF THE STUDY

This study selected only male mice to investigate the effect of combined physical exercise that were exposed to AA or passively to the smoke HRCC. This could limit the outcome of precise prediction of sex differences in the redox profile that contribute muscle remodeling after the smoke exposure. Although, studies have shown that female gender have type II fiber atrophy and greater loss of muscle strength when compared to male gender, indicating that intrinsic properties of muscle are prone to altered in female smokers, and this effect is more related to their amount of physical activity (Ausín et al., 2017). Regarding muscle structure with cigarette smoking people, percentage of type II fibers lower in female than male even in similar nutritional status, systemic inflammation, and physical activity (Gosker et al., 2007). Ausín et al. (2017) showed that female with cigarette smoking history had higher level of muscle damage than male. But males

showed improved signs of early steps of muscle regeneration than cigarette smoking females. However, these findings suggest that different susceptibility of patients of both genders to injury may be linked with different stimuli such as tobacco and/or amount of physical activity. Furthermore, the earlier muscle regeneration capacity with male cigarette smoking does not necessarily means that it can complete its reparative process will continue correctly until the end (Thériault et al., 2014). Other factors like vitamin D deficiency is positively influenced with the muscle mass, and female with smoking history is more pronounced with vitamin D deficiency (Dawson-Hughes, 2012). In contrast, Sharanya et al. (2019), observed that female gender who have smoking history and longer times spent standing have reduced muscle fiber size, muscle strength and peak workload. However, this sex difference occurred due to increased circulating pro-inflammatory cytokines in female compared to male. However, this study did not find direct correlations between increased markers of systemic inflammation and fiber atrophy, nor indeed between physical activity levels and fiber atrophy in either sex. This suggests that any contribution of systemic inflammation or physical activity to fiber atrophy is moderated by other influences. Moreover, diffusion abnormalities develop earlier in female than male that causes muscle atrophy and weakness, and this may be due to differences in aetiological factors and downstream signaling pathways that affect the skeletal muscle structure and function (Sharanya et al., 2019). Regarding aging, increasing age is not able to maintain organ integrity. Consequently, less protective against oxidative damage. Recent report have shown that maintenance of peripheral muscle mass in cigarette smoking people is compromised with accelerated aging, and the accelerated aging phenotype is a result of DNA repair impairment and dysregulation of cellular homeostasis in the muscle of cigarette smoking people (Lakhdar et al., 2018). Further, studies have revealed that men and women with cigarette smoking have structural and functional differences in the peripheral muscles (Ausín et al., 2017). Taken together, these studies suggest that females who have smoking history have greater prevalence of muscle damage and weakness than males, suggesting that sex and age influence muscle phenotype and function in smoking people.

CONCLUSION

These results suggest that HRCC smoke induces important morphological changes in the skeletal muscle by altering the muscle redox profile, and it may impair muscle function. In contrast, combined exercise plays an important role in the remodeling process, but its effect is dependent on the response of the redox system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The Institutional Committee for Animal Care at Universidade do Extremo Sul Catarinense approved all the procedures under protocol number 087/2015-1.

AUTHOR CONTRIBUTIONS

AT, SS, PS, LM, FV, RN, and SN conceived the presented idea, developed the framework, and wrote the manuscript. AT, EC, LN, PCS, YG, and RP provided critical feedback and contributed to the final version. All authors were involved in the final direction of the manuscript, contributed to the final version of the manuscript, and have read and agreed to the published version of the manuscript.

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Influence of Cigarettes and Alcohol on the Severity and Death of COVID-19: A Multicenter Retrospective Study in Wuhan, China

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Background: The recent emergence and rapid global spread of coronavirus disease 2019 (COVID-19) is leading to public health crises worldwide. Alcohol consumption and cigarette smoking (CS) are two known risk factors in many diseases including respiratory infections.

Methods: We performed a multi-center study in the four largest hospitals designated for COVID-19 patients in Wuhan. There are totally 1547 patients diagnosed with COVID-19 enrolled in the study, alcohol consumption and CS history were evaluated among these patients. The epidemiology, laboratory findings and outcomes of patients contracted COVID-19 were further studied.

Results: Our findings indicated that COVID-19 patients with a history of CS tend to have more severe outcomes than non-smoking patients. However, alcohol consumption did not reveal significant effects on neither development of severe illness nor death rates in COVID-19 patients.

Conclusion: CS is a risk factor for developing severe illness and increasing mortality during the SARS-CoV-2 infection. We believe that our findings will provide a better understanding on the effects of alcohol intake and CS exposure in COVID-19 patients.

Keywords: cigarette, alcohol, COVID-19, SARS-CoV-2, severity, death

INTRODUCTION

The recent emergence and rapid global spread of Severe Acute Respiratory Syndrome (SARS) Coronavirus 2 (SARS-CoV-2) and the resulting coronavirus disease 2019 (COVID-19) is associated with more than 12,077,210 cases and more than 550,327 deaths worldwide as of July 9, 2020 (COVID-19 Map from Johns Hopkins Coronavirus Resource Center).

Considering the high rate of SARS-CoV-2 transmission and that there is no known cure for this disease at present, identifying vulnerable populations will be crucial for taking measures to protect those who are at increased risk of infection or of severe disease from COVID-19 (Clark et al., 2020).

Alcohol consumption and cigarette smoking (CS) are two known risk factors in many diseases including respiratory infections (Traphagen et al., 2015; Han et al., 2019). Chronic alcohol consumption has been identified as an important risk factor for the development of acute respiratory distress syndrome (ARDS) (Moss et al., 2003), which is one of the most severe complications of COVID-19. CS has been well-recognized as a high-risk factor for respiratory diseases; however, there is still no evidence indicating that it increases the risk of SARS-CoV-2 infection. Even though there are numerous studies focused on the link between smoking and COVID-19, it is still unclear whether CS increases the severity of COVID-19. A meta-analysis conducted by Vardavas and Nikitara (2020) suggested that smoking increases the risk of developing severe COVID-19; however, another meta-analysis demonstrated that active smoking is not associated with enhanced risk in the progressing to severe COVID-19 (Lippi and Henry, 2020). There even are data indicate that smoking might have protective properties against SARS-CoV-2 (Farsalinos et al., 2020b; van Zyl-Smit et al., 2020). The challenge of studies focused on smoking and COVID-19 is that most hospitalized patients have underlying medical conditions such as hypertension, diabetes, cardiovascular disease and chronic obstructive pulmonary disease (COPD).

MATERIALS AND METHODS

Study Design and Participants

Our multi-center study was performed in the four largest hospitals designated for COVID-19 patients in Wuhan. All

TABLE 1 | Characteristics of patients with COVID-19 with and without smoking history and alcohol consumption.

Characteristics	Total (n = 1547)	Smoking history		P-value[a]	Alcohol consumption		P-value[b]
		Yes (n = 145)	No (n = 1429)		Yes (n = 54)	No (n = 1493)	
Age (years)	57.31 ± 16.09	56.57 ± 15.89	57.39 ± 16.11	0.559	60.39 ± 10.68	57.17 ± 16.24	0.037
Sex				<0.001			<0.001
Female	745 (48.16%)	18 (12.41%)	727 (51.85%)		6 (11.11)	739 (49.56)	
Male	802 (51.84%)	127 (87.59%)	675 (48.15%)		48 (88.89)	752 (50.44)	
BMI	24.03 ± 3.22	23.96 ± 2.97	24.04 ± 3.25	0.866	24.12 ± 2.21	24.02 ± 3.27	0.820
Alcohol consumption	54 (3.5)	25 (16.7)	29 (2.1)	<0.001	25 (46.30)	125 (8.38)	<0.001
Comorbidities							
Hypertension	423 (27.34%)	43 (29.66%)	380 (27.10%)	0.512	21 (38.89)	400 (26.83)	0.051
Diabetes	200 (12.93%)	23 (15.86%)	177 (12.62%)	0.269	12 (22.22)	186 (12.47)	0.035
Cardiovascular disease	169 (10.92%)	17 (11.72%)	17 (11.72%)	0.746	8 (14.81)	160 (10.73)	0.344
Cerebrovascular disease	40 (2.59%)	4 (2.76%)	36 (2.57%)	0.890	8 (14.81)	160 (10.73)	0.548
Carcinoma	42 (2.71%)	4 (2.76%)	38 (2.71%)	0.973	2 (3.70)	40 (2.68)	0.650
Chronic lung disease	61 (3.94%)	9 (6.21%)	52 (3.71%)	0.141	2 (3.70)	57 (3.82)	0.964
Signs and symptoms							
Fever	487 (31.48%)	65 (44.83%)	489 (34.20%)	0.256	15 (27.78)	470 (31.52)	0.560
Cough	282 (18.23%)	24 (16.55%)	258 (18.40%)	0.583	5 (9.26)	276 (18.51)	0.083
Expectoration	42 (2.71%)	6 (4.14%)	36 (2.57%)	0.268	1 (1.85)	41 (2.75)	0.690
Myalgia	160 (10.34%)	21 (14.48%)	139 (9.91%)	0.085	6 (11.11)	154 (10.33)	0.853
Diarrhea	115 (7.43%)	12 (8.28%)	103 (7.21%)	0.485	4 (7.41%)	111 (7.43%)	0.872
Shortness of breath	115 (7.43%)	9 (6.21%)	106 (7.56%)	0.554	3 (5.56)	112 (7.51)	0.591
Laboratory Data							
WBC, × 10^9/L	7.02 (2.08,16.78)	7.23 (2.58,16.78)	6.32 (2.08,13.20)	0.133	8.84 (3.88,13.23)	7.02 (2.08,16.78)	0.432
Neutrophil count, × 10^9/L	4.85 (2.89, 5.2)	5.2 (3.29, 5.2)	4.76 (2.86, 5.2)	0.480	4.72 (3.2,5.55)	4.86 (2.88, 5.2)	0.827
Lymphocyte count, × 10^9/L	1.32 (0.85, 1.47)	1.08 (0.68, 1.32)	1.32 (0.88, 1.5)	<0.001	1.06 (0.65,1.32)	1.32 (0.86,1.47)	0.015
Platelet count, × 10^9/L	203.8 (155, 238)	179 (127, 209)	203.8 (160, 242)	<0.001	186.5 (142,241)	203.8 (156,238)	0.196
D-dimer, mg/L	1.26 (0.4, 14.7)	1.26 (0.4, 14.7)	1.26 (0.41, 14.7)	0.967	0.92 (0.49,10.29)	1.27 (0.39, 14.7)	0.684
C-reactive protein, mg/L	9.98 (1.4, 27.59)	23.9 (3.91, 34.3)	8.39 (1.29, 27.59)	0.002	7.18 (2.13,27.59)	10.4 (1.39,27.59)	0.977
ALT, U/L	27 (16, 35.78)	25.9 (17, 40)	27.25 (16, 35.78)	0.603	30.7 (20, 52.6)	27 (16, 35.78)	0.040
AST, U/L	26 (18, 40.05)	30 (20, 43)	25.3 (18, 40.05)	0.003	31 (21, 40.05)	26 (18, 40.05)	0.128
IL-6, pg/mL	28.67 (1.50,798.00)	45.93 (1.50,798.0)	6.52 (1.50, 496.70)	0.503	32.02 (1.50, 798.0)	6.52 (1.50, 374.12)	0.514
Procalcitonin, ng/mL	0.43 (0.02, 57.17)	0.56 (0.05, 57.17)	0.12 (0.02, 28.45)	0.069	0.38 (0.02, 40.08)	0.58 (0.02, 57.17)	0.103

Data are presented as mean ± SD or n (%). Laboratory data on the day of admission were collected. [a]P-values indicate differences between COVID-19 patient with and without smoking history; [b]COVID-19 patient with and without Alcohol consumption; $P < 0.05$ was considered statistically significant. BMI, body mass index; WBC, white blood cell count; ALT, alanine transaminase; AST, aspartate transaminase.

hospitalized patients were confirmed COVID-19 positive according to the interim guidance from the World Health Organization and National Health Commission of China (World Health Organization, 2020), from February 01 to April 10, 2020. The severe illness was defined according to the New Coronavirus Pneumonia Prevention and Control Program (5th edition) issued by the National Health Commission of China (**Supplementary Methods**). The study was approved by the institutional ethics review board and the need for informed consent was waived.

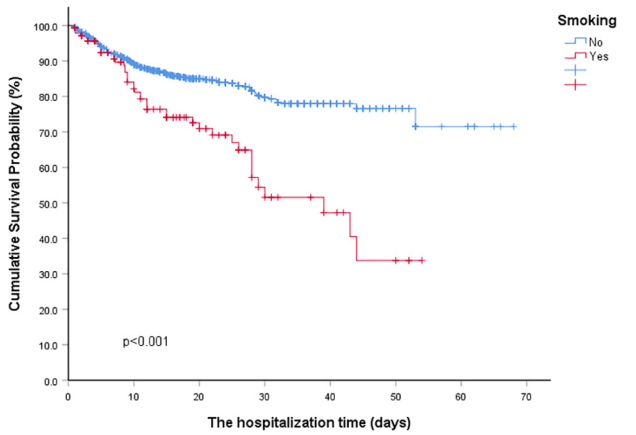

FIGURE 1 | Kaplan-Meier survival curves for COVID-19 patients with and without Smoking history. *$P < 0.01$ for smoking group vs. non-smoking group.

Data Collection

Clinical course, laboratory findings and outcomes were obtained and reviewed. The medical records were analyzed by three investigators independently. Information included demographic characteristics, symptoms, signs, underlying comorbid conditions and treatments were collected from the medical records. Laboratory data on the day of admission were collected. Smoking history was defined according to the WHO guidelines, including smoking for more than 6 months, or smoking more than 5 or more cigarettes per day (US Department of Health and Human Services, 2014); alcohol consumption was defined as an average of more than 7 standard drinks per week or more than 3 standard drinks per day (1 standard drink = 14 g of ethanol) (Burton and Sherons, 2018).

Statistical Analysis

Categorical variables were described as frequency rates and percentages. Continuous variables were presented as mean ± standard deviation (SD) or mean, and interquartile range (IQR) as appropriately. We used the Mann–Whitney *U* test, x^2 test, or Student's *t*-test to compare differences between two different groups where appropriate. Univariable and multivariable logistic regression analyses were used to identify any association between serious illness and smoking history. Univariable and multivariable cox regression analyses were used to identify any association between death and smoking history. Variables with a *P*-value less than 0.05 at univariable analysis were subjected to multivariable analysis, and odds ratios with 95% confidence intervals (CIs) were reported. The areas under the receiver operating characteristic curve were used to evaluate

TABLE 2 | Risk factors associated with severe illness by logistic regression.

	Univariable analysis			Multivariable analysis		
	cOR	**95% CI**	***p***	**aOR**	**95% CI**	***P***
Age	1.052	1.043–1.062	<0.001	1.026	1.015–1.038	<0.001
Gender	1.936	1.528–2.453	<0.001			
BMI	0.992	0.923–1.084	0.992			
Smoking	1.955	1.375–2.779	<0.001	1.910	1.203–3.033	0.001
Alcohol consumption	1.639	0.926–2.900	0.090			
Diabetes	4.313	3.168–5.871	<0.001	3.713	2.432–5.668	<0.001
Hypertension	3.151	2.468–4.024	<0.001	1.644	1.154–2.342	0.006
Chronic lung disease	3.012	1.784–5.087	<0.001	3.114	1.370–7.078	0.007
Cerebrovascular disease	4.256	2.211–8.190	<0.001			
Cardiovascular disease	2.719	1.956–3.779	<0.001			
Carcinoma	3.393	1.831–6.287	0.001			
Lymphocyte count	0.993	0.991–0.994	<0.001	1.192	1.123–1.264	<0.001
Neutrophil count	1.379	1.316–1.446	<0.001			
C-reactive protein	1.036	1.030–1.041	<0.001	1.021	1.015–1.026	<0.001
ALT	1.010	1.006–1.014	<0.001			
AST	1.027	1.021–1.032	<0.001	1.011	1.005–1.017	<0.001
D-dimer	1.000	0.999–1.001	0.964			

Data in parentheses are 95% confidence intervals. P < 0.05 was considered statistically significant; ALT, alanine transaminase; AST, aspartate transaminase. In the univariate model, risk ratios were not adjusted for any confounders. In multivariate model, risk ratios were additionally adjusted for confounders with p < 0.05 in the univariate model, and these independent variables are corrected for each other, thus obtaining the corrected partial regression coefficient (aOR/aHR).

TABLE 3 | Risk factors associated with mortality by cox regression.

	Univariable analysis			Multivariable analysis		
	cHR	**95% CI**	***p***	**aHR**	**95% CI**	***P***
Age	1.054	1.044–1.064	<0.001	1.031	1.019–1.041	<0.001
Gender	2.216	1.638–2.759	<0.001			
BMI	0.984	0.912–1.061	0.671			
Smoking	2.265	1.627–3.153	<0.001	1.825	1.275–2.613	0.001
Alcohol consumption	1.200	0.656–2.196	0.554			
Diabetes	2.280	1.721–3.019	<0.001	1.453	1.063–1.985	0.019
Hypertension	2.365	1.852–3.018	<0.001			
Chronic lung disease	2.899	1.918–4.38	<0.001			
Cerebrovascular disease	3.464	2.194–5.47	<0.001			
Cardiovascular disease	2.295	1.716–3.07	<0.001	1.485	1.078–2.045	0.015
Carcinoma	2.435	1.445–4.102	0.001			
Neutrophil count	1.101	1.091–1.111	<0.001	1.071	1.054–1.089	<0.001
Lymphocyte count	0.309	0.238–0.400	<0.001			
C-reactive protein	1.008	1.007–1.009	<0.001	1.007	1.006–1.009	<0.001
ALT	1.002	1.001–1.003	<0.001	1.001	1.000–1.002	0.010
AST	1.001	1.001–1.001	<0.001			
D-dimer	1.001	1.000–1.001	0.263			

Data in parentheses are 95% confidence intervals. P < 0.05 was considered statistically significant; ALT, alanine transaminase; AST, aspartate transaminase. In the univariate model, risk ratios were not adjusted for any confounders. In multivariate model, risk ratios were additionally adjusted for confounders with p < 0.05 in the univariate model, and these independent variables are corrected for each other, thus obtaining the corrected partial regression coefficient (aOR/aHR).

the discriminative abilities of the disease severity status. *P*-values less than 0.05 were considered to indicate statistical significance. Statistical analyses were performed by using statistical software (SPSS version 26.0).

RESULTS

A total of 1547 subjects, including 802 male patients (51.8%) and 745 female patients (48.2%), were enrolled in the study. According to past medical history, the most common concurrent diseases were hypertension (27.34%), diabetes (12.93%) and cardiovascular disease (10.92%) (**Supplementary Table 1**). Among 1547 patients, there are 390 severe patients (25.21%), and 257 death patients (16.61%).

Characteristics of COVID-19 Patients With and Without Smoking History

Firstly, the population was divided into sub-groups according to whether they had a history of smoking. In the smoking group, the proportion of males was higher than that in the non-smoking group (87.59% vs. 48.15%, $p < 0.001$). There was no statistically significant difference in age, BMI, comorbidities and symptoms between the smoking group and the non-smoking group. However, in terms of biochemical indicators, the counts of lymphocyte and thrombocyte of the smoking group were lower than that of the non-smoking group, while C-reactive protein and AST were higher than that of the non-smoking group. The difference was statistically significant (all *p*-values < 0.05, **Table 1**). To further describe the effects of smoking, we conducted a Kaplan-Meier survival curves for COVID-19 patients. The results revealed that the smoking groups had greater deteriorated outcomes than the non-smoking groups (**Figure 1**).

Characteristics of COVID-19 Patients With and Without Alcohol Consumption

Within the same population, two groups were divided according to whether they had a history of alcohol consumption. The proportion of males in the alcohol group was higher than that in the non-alcohol group (88.89% vs. 50.44%, $p < 0.001$). There was no statistically significant difference in age and BMI. Concerning comorbidities and symptoms upon admission, COVID-19 patients with and without alcohol consumption were similar except for a higher proportion of diabetes (22.22% vs. 12.47%, $p = 0.035$). In terms of laboratory indicators, the counts of lymphocyte from the alcohol group were lower than that of the non-alcohol group, while ALT was higher than that of the non-alcohol group. The difference was statistically significant ($p < 0.05$, **Table 1**). The Odds Ratio (OR) for the effect of CS on severe illness was 1.910 (95% CI = 1.203–3.033, $p = 0.001$), indicating that the risk of severe illness in patients with previous CS history was 1.91 times higher than that in non-smokers.

Risk Factors Associated With Severe Illness and Mortality

However, in both univariable and multivariable analyses, alcohol consumption did not reveal any significant effect on developing severe illness in COVID-19 patients (**Table 2**). Among them, CS had a Hazard Ratios (HR) = 1.825 (95% CI = 1.275-2.613, $p = 0.001$), indicating that the risk of death in smokers was 1.825 times higher than that in non-smokers. Also, lymphocyte count, C-reactive protein, and ALT were risk factors for death. However,

alcohol consumption did not show a significant effect on death rates of COVID-19 patients in both univariable and multivariable analyses (**Table 3**). In this study, we analyzed the association between alcohol consumption, CS, and the risk of COVID-19. Our findings indicated that COVID-19 patients with a history of CS tend to have more severe outcomes than non-smoking patients. However, alcohol consumption did not reveal significant effects on neither development of severe illness nor death rates in COVID-19 patients.

DISCUSSION

Alcohol is the most commonly abused drug in the world. It affects almost every organ of our body. At this time, patients with a history of alcohol consumption did not develop more severe outcomes compared to patients without. That might be because the time we collect our data is still in the early stage of the pandemic. With the SARS-CoV-2 rapidly spreading, governments across the world have issued stay-at-home and face covering orders, which resulted in trillions of people being isolated from each other for long periods of time. Combining with the stress of rising unemployment, excessive use of alcohol consumption becomes a public health crisis (Clay and Parker, 2020). Several meta-analyses suggested that there is a significant association between COVID-19 and CS; however, they also admitted their analyses were limited by sample size and number of primary researches (Farsalinos et al., 2020a). In our study, the counts of lymphocyte of the smoking group were lower than that of the non-smoking group, which may contribute to the deteriorated outcomes. Immunosuppression caused by smoking inhibits the effective activation of T cells, which also inhibit B cells to proliferate and produce antibodies, thus making humoral immunity incapable (McBride et al., 2003). C-reactive protein is positively correlated with smoking. Increased inflammatory in COVID-19 patients with smoking history could contribute to the worse outcome in this subpopulation (Chen et al., 2020). Studies have shown that smoking may increase multiple enzymes in the human liver. Cirrhosis of the liver has a recognized immune dysfunction status that includes immunodeficiency and systemic inflammation, making it reasonable for those patients to be more susceptible to SARS-CoV-2 infection (Velarde-Ruiz Velasco et al., 2020). In this study, the data we collected containing a total of 1,547 patients with 150 smokers, which will provide a better understanding of the effects of CS in COVID-19. Based on our analysis, COVID-19 patients with a history of CS had more severe outcomes when compared to the population without a CS history.

In conclusion, CS is a risk factor for developing severe illness and increasing mortality during the SARS-CoV-2 infection. We believe that our findings will provide a better understanding on the effects of alcohol intake and CS exposure in COVID-19 patients.

AUTHOR CONTRIBUTIONS

ML designed the research. MD and ZC were in charge of data collection and manuscript writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Which Factors, Smoking, Drinking Alcohol, Betel Quid Chewing, or Underlying Diseases, Are More Likely to Influence the Severity of COVID-19?

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The global outbreak of the coronavirus disease 2019 (COVID-19) pandemic occurred in late 2019 and early 2020. The factors that influence disease severity should be of clinical concern. Existing findings on the effects of smoking on COVID-19 are also controversial and need to be confirmed by further research. In addition, the effects of alcohol consumption and betel quid (BQ) chewing on COVID-19 are unclear. The aim of this study was to examine the demographic characteristics of COVID-19 patients and the effects of smoking, drinking, BQ chewing, and underlying diseases on the severity of COVID-19. A retrospective study was conducted on 91 patients with confirmed cases of COVID-19 hospitalized in Yueyang, Hunan Province, China from 21 January to 8 March, 2020. Patient demographic data, and information on smoking, drinking and BQ chewing, and underlying diseases were extracted from the patient electronic medical records (EMR) and telephone interviews. The chi-square test was used to conduct a univariate analysis of the factors influencing the severity of COVID-19, and ordinal logistic regression analysis was used to identify the factors related to the severity of COVID-19. The results showed that the rates of smoking, drinking and BQ chewing were 15.4, 26.4, and 7.1%, respectively, there was no significant relationship between these lifestyle factors and the severity of COVID-19 ($P > 0.05$). However, underlying diseases such as diabetes [odds ratio (OR) = 7.740, 95% confidence interval (CI):1.000–60.740, $P = 0.050$], source of infection (OR = 0.180, 95% CI: 0.030–0.980, $P = 0.049$), and employment status (retired/unemployed vs. employed: OR = 29.430, 95% CI, 1.050 – 822.330, $P = 0.047$) were significant independent predictors of severe COVID-19 infection. These individuals should be informed of methods to increase personal protection, and doctors should prevent these individuals from developing serious diseases. It is important to pay attention to the source of infection and timely medical treatment. This study showed that the clinical classification of COVID-19 was associated with patients with diabetes, source of infection, and retired/unemployed. Therefore in the clinical practice of COVID-19 should be more concern these factors.

Although no statistical significance was found in smoking, drinking alcohol, BQ chewing, and severity of COVID-19 patients, more studies have confirmed that are harmful and risk factors for underlying diseases in the population. Health authorities should formulate policies to publicize the harmful effects of smoking, drinking, and betel nut chewing and promote a healthy lifestyle.

Keywords: SARS-cov-2, smoking, alcohol, betel quid chewing, underlying diseases, route of transmission, diabetes mellitus, COVID-19

INTRODUCTION

Since the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak began in December 2019, it has rapidly swept around the world. The resultant disease, coronavirus disease 2019 (COVID-19),was officially declared a pandemic by the World Health Organization on 11 March 2020 (Huang C. L. et al., 2020; Tian et al., 2020). Whether the source of infection of COVID-19 is related to the severity of the disease is rarely reported. The source of infection is mainly patients displaying symptoms of COVID-19, although asymptomatic infected persons and patients in the incubation period are also contagious to some extent (Special Expert Group for Control of the Epidemic of Novel Coronavirus Pneumonia of the Chinese Preventive Medicine Association, 2020). SARS-CoV-2 is mainly transmitted through droplets and close contact and is also spread by aerosols (Morawska and Milton, 2020; National Health Commission and National Administration of Traditional Chinese Medicine, 2020; Setti et al., 2020). Aerosol transmission needs to be in a relatively closed space. When the aerosol containing the virus reaches a certain concentration, it is likely to cause infection if inhaled by healthy people.

Factors contributing to the severity of the disease have attracted attention. Age, underlying diseases, employment are considered important risk factors that are relevant to disease severity in COVID-19 patients (Chen N. et al., 2020; Huang C. L. et al., 2020; Nandy et al., 2020; Wang D. et al., 2020; Wang X. et al., 2020). Chen R. C. et al. (2020) studied 1,590 COVID-19 patients in China and found that in patients aged 65 years or older, coronary heart disease, cerebrovascular disease, dyspnea and other independent risk factors were related to fatal outcomes. Guan et al. (2020a) reported that compared with patients without underlying diseases, COVID-19 patients with more types of underlying diseases had a worse prognosis. Several Studies have reported that, 20–51% of COVID-19 patients have at least one underlying disease, among which diabetes (10–20%) is the most common, followed by hypertension (10–15%) and other cardiovascular and cerebrovascular diseases (7–40%) (Chen N. et al., 2020; Huang C. L. et al., 2020; Liu K. et al., 2020). Aggarwal et al. (2020) conducted a meta-analysis of 12 studies, involving a total of 2,564 patients, of whom 265 (10.3%) had a history of diabetes exacerbating the clinical progression of COVID-19. Yu et al. (2020) conducted a retrospective study of 1,463 COVID-19 patients and found that age, male sex, and a history of diabetes were independent risk factors for COVID-19 mortality. Liu J. et al. (2020) conducted a retrospective analysis of 48,814 confirmed cases of COVID-19 in the Hubei Province, and found that employment status (retired/working at home) is also an independent risk factor for the severity of COVID-19. However, these findings need to be confirmed by further cohort or prospective studies.

Mao et al. (2020) analyzed the epidemiology of 67 COVID-19 clusters (including 226 confirmed patients) in Sichuan Province, China, and found that families (68.66%) and living with family (60.87%) were the main exposure. However, whether the cause of exposure is related to the severity of the disease is rarely reported.

The relationship between smoking and COVID 19 is controversial. Many studies have shown that the smoking rate of COVID-19 patients is lower than that of the overall smoking rate of the population (Guan et al., 2020b; Zhang J. J. et al., 2020). According to the results of the 2018 Global Adult Tobacco Survey, the smoking rate of Chinese adults is 26.6%, and the average daily smoking amount is 16.0 cigarettes (Global Adult Tobacco Survey [GATS], 2020). Systematic reviews (Farsalinos et al., 2020a; Patanavanich and Glantz, 2020) have shown that the current smoking rate of 5,960 COVID-19 inpatients in China was 1.4–14.6%.

Angiotensin-converting enzyme 2 (ACE 2) is the main receptor of SARS-CoV-2 entering human cells. Studies have shown that smoking can cause high expression of ACE2, which may lead to poor prognosis of COVID-19 (Brake et al., 2020; Leung et al., 2020). Systematic reviews by Vardavas and Nikitara (2020) suggest that smoking is most likely associated with rapid progression and adverse outcomes of COVID-19. Patanavanich and Glantz (2020) showed that smokers were 1.91 times more likely to develop severe COVID-19 than non-smokers.

However, some scholars have proposed that ACE 2 upregulation does not necessarily equate to increased susceptibility or disease severity, and nicotine in tobacco may even have a therapeutic effect (Farsalinos et al., 2020a,b). Guan et al. (2020b) studied 1,099 hospitalized COVID-19 patients in China and found that 16.9, 5.2, and 77.9 the patients with severe COVID-19were current smokers, former smokers, and never smoked respectively. Univariate analysis was statistically significant ($P < 0.001$). Simons et al. (2020) included a total of 233 studies in the systematic review and found that current smokers appeared to have a reduced risk of SARS-CoV-2 infection compared with those who had never smoked, whereas former smokers appeared to have an increased risk of hospitalization for COVID-19, increased disease severity, and death, although these associations were causally unconfirmed. Although the relationship between smoking and COVID-19 severity is uncertain, there is growing evidence that smokers have a higher risk of serious illness and death with COVID-19 (World

Health Organization [WHO], 2020). Although the prevalence of smoking among patients is low, the relationship between smoking and COVID-19 is still worth exploring, and several studies have confirmed that smoking exacerbates COVID-19.

BQ chewing, a centuries-old practice in China, especially in the southern province of Hunan, is as much a stress reliever, social activity or hobby for locals as smoking and drinking. The latest literature reports that there are approximately 0.6–1.2 billion people chewing areca worldwide, with no limitations for children (Gupta and Warnakulasuriya, 2002). Studies have shown (Garg et al., 2014; Mehrtash et al., 2017) that BQ affects almost all organs of the human body, including the brain, heart, lung, gastrointestinal tract and reproductive organs. It also affects the immune system, such as inhibiting T cell activity and reducing cytokine release.

Too much alcohol consumption can also be harmful. The World Health Organization (World Health Organization Regional Office for Europe, 2020) stresses that every drink, whether beer, liquor, or other alcoholic beverage, is harmful. Heavy drinking, in particular, weakens the body's immune system, making it less able to cope with infectious diseases. In Hunan, China, more than 10% of people over 15 chew BQ regularly, and most also smoke and drink alcohol. The latest research shows that the smoking rate of adults aged 15 and older in the Hunan Province is 28.3%, and that of men is 54.4% (Qi et al., 2020). Currently, there are few studies on the effects of alcohol consumption and BQ chewing on COVID-19, which is also a focus of attention. Therefore, this study analyzed the factors influencing the severity of COVID-19 among patients hospitalized in a hospital in Hunan, China, to provide a reference for the prevention and treatment of COVID-19.

MATERIALS AND METHODS

Study Setting and Participants

The study was conducted in a tertiary hospital in Hunan Province, Yueyang City, which is qualified to receive and treat COVID-19 patients.

All participants with COVID-19 received treatment at The First People's Hospital of Yueyang from January 21 to March 8, 2020. Inclusion criteria: COVID-19 was diagnosed. Exclusion criteria: Deaths due to non-COVID-19.

Participants' Material Collection

The researchers used medical records and telephone interviews to collect the data. The survey mainly included demographic data of the subjects, clinical classification of COVID-19, smoking, drinking, and BQ chewing behaviors. Patient data were extracted from the EMR system of the hospital by professionally trained researchers. If the data were not entered, the researchers would conduct a phone interview to supplement the accurate information. Any problems found were corrected in a timely manner, and supplementary investigations were conducted on non-conforming items. The input data were doubled, carefully checked, and then analyzed.

Variable Definitions and Criteria

(1) Disease severity classification criteria for COVID-19: Clinical typing. According to the Diagnosis and Treatment Guidelines by the National Health Commission, the patients were divided into mild type, common type, severe type, and critical type.

Mild Type: Clinical symptoms were mild, and no pneumonia was found on imaging.

Common type: With fever, respiratory symptoms, etc., and imaging findings of pneumonia.

Severe type: Adults meet any of the following criteria: A. Onset of shortness of breath, RR $\geq$ 30 times/min; B. In the resting state, oxygen saturation $\leq$93% when inhaling air; C. Partial arterial oxygen pressure (Pa02)/oxygen absorption concentration [(Fi02) $\leq$ 300 mmHg, 1 mmHg = 0.133 kPa].

Critical type: Participants NA.

(2) Smoker is defined as those who smoke $\geq$1 cigarette/d for more than half a year. Questions included whether smoking, starting age, and the average number of cigarettes consumed per day.

(3) Drinking alcohol was defined as drinking white wine $\geq$once per week for more than 1 year (The amount of alcohol consumed in alcohol is 52 degrees of high-alcohol liquor, and 75 ml of low-alcohol liquor is equivalent to 50 ml of white wine; 750 ml beer is equivalent to 75 ml white wine; 200 ml wine or yellow rice wine equals 50 ml white wine). Questions included whether drinking alcohol, starting age, what wine consumed and the average amount of alcohol consumed per day.

(4) BQ chewing is defined as BQ chewing $\geq$once a day for more than 3 months. Questions included whether chewing betel quid, starting age, and the average number of BQ chewing per day.

(5) Underlying diseases: According to the diagnosis and confirmation of the patient's medical records, information on six common underlying diseases was collected: hypertension, diabetes mellitus, coronary heart disease, cerebral infarction, hyperlipidemia, and renal insufficiency.

Statistical Analysis

Data were entered and analyzed using SPSS version 18.0 (SPSS, Chicago, IL, United States). Descriptive statistics were calculated for general information, smoking, drinking alcohol, BQ chewing. We performed univariate analyses with chi-square tests between the clinical typing of COVID-19. Ordered logistic regression analysis was used to identify the factors related to clinical typing of COVID-19. Odds ratios (OR's) and 95% confidence intervals (CI's) were evaluated in multivariable analysis. A P-value $\leq$ 0.05 denoted statistical significance.

RESULTS

Demographic Information and Characteristics of the Cases

A total of 91 COVID-19 patients who met the diagnostic criteria were enrolled in this study. There were 46 men (50.5%) and 45 women (49.5%), the minimum age was 3 months, the maximum

age was 76 years, and the mean age was 47.3 ± 16.7 years. 72 (79.1%) were married, 19 (20.9%) were single. The employment results showed that the service category, 24 (26.3%) and the retired/unemployed, 22 (24.2%) were in the majority. The distribution of education level was primary school and below, 36 (39.6%), junior high school, 31 (34.1%), senior high school, 18 (19.8%), and junior college and above, 6 (6.5%). Urban residents accounted for 65 (71.4%), while rural residents accounted for 26 (28.6%) (**Figure 1A**). The maximum number of days of virus negative conversion was 42 days, and the minimum was 4 days. The average was 13.8 ± 7.8 days (**Figure 1B**). The patients had a history of living or traveling in Hubei or had contact with COVID-19 patients in Hubei 38 (41.8%) and with COVID-19 infection due to a local or family gathering 53 (58.2%). The classification of 91 COVID-19 cases were 6 (6.6%) mild, 77 (84.6%) common, and 8 (8.8%) severe, respectively (**Figure 1C**). The longest hospital stay was 43 days, and the shortest was 5 days. The average length of hospital stay was 16.1 ± 7.6 days (**Figure 1D** and **Table 1**).

The Subjects' Behaviors of Smoking, Alcohol, and BQ Chewing

Of the 91 COVID-19 patients, 17 were smokers (15.4%); Age at which to start smoking the youngest and the oldest were 14 and 46 years old, respectively. The smoking duration was 22.9 ± 14.6 years, and a smoking duration of more than 10 years accounted for 64.7%. The average daily smoking amount was 15.7 ± 11.4 cigarettes, and 41.2% of those had a daily smoking amount of greater than 10 cigarettes. Twenty-four patients were drinkers (26.4%), 4 in 24 drinkers were often drinkers, and the rest were social drinkers. The average duration of drinking was 19.4 ± 13.5 years, and for 58.3%, the duration was longer than 10 years. Difference between urban and rural drinking duration (year), Monte Carlo $P = 0.028$, urban COVID-19 drinkers (80%) had been drinking for more than 10 years. Rural COVID-19 drinkers (77.8%) had been drinking for less than 10 years. The average consumption of alcohol was 147.9 ± 75.9 ml per time, and approximately 58.3% of those consumed more than 100 ml per time. Of the 91 COVID-19 patients, 6 patients were BQ chewers (7.1%), all in the common type group. The average duration of BQ consumption was 5.5 ± 4.0 years, and the average daily consumption of BQ was 8.7 ± 5.9. The results showed that there were no statistically significant differences in smoking, drinking, and BQ chewing characteristics with COVID-19 clinical typing ($P > 0.05$).

Relationship Between Clinical Typing of COVID-19 and Demographic Factors

Among the 91 COVID-19 patients, the clinical classifications were 6.6, 84.6, and 8.8% for mild, common, and severe types,

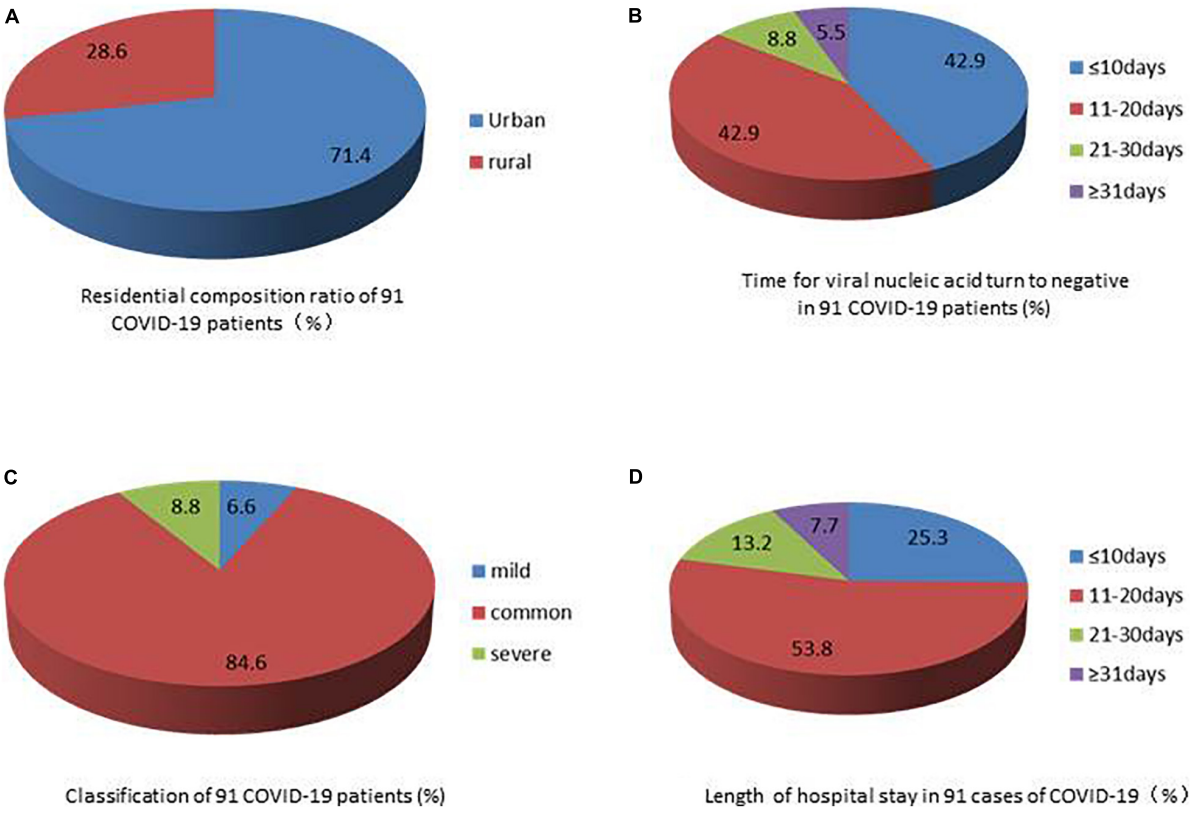

FIGURE 1 | (A) Residential composition ration of 91 COVID-19 patients (%). **(B)**Time for viral nucleic acid turn to negative in 91 COVID-19 patients (%). **(C)** Classification of 91 COVID-19 patients (%).**(D)** Length of hospital stay in 91 cases of COVID-19 (%).

TABLE 1 | Relationship between patient characteristics and disease severity of COVID-19.

Variables	Total *n* = 91(%)	Mild *n* = 6(%)	Common *n* = 77(%)	Severe *n* = 8(%)	*P**
Gender					**0.155**
Male	46 (50.5)	5 (10.9)	36 (78.2)	5 (10.9)	
Female	45 (49.5)	1 (2.2)	41 (91.1)	3 (6.7)	
Age (years)	**47.3 ± 16.7**				**0.001**
≤30	16 (17.6)	5 (31.2)	11 (68.8)	0 (0.0)	
31–64	60 (65.9)	1 (1.7)	53 (88.3)	6 (10.0)	
≥65	15 (16.5)	0 (0.0)	13 (86.7)	2 (13.3)	
Habitation					**0.432**
City	65 (71.4)	3 (4.6)	57 (87.7)	5 (7.7)	
Rural	26 (28.6)	3 (11.5)	20 (77.0)	3 (11.5)	
Marriage					**0.001**
Single	19 (20.9)	5 (26.3)	14 (73.7)	0 (0.0)	
Married	72 (79.1)	1 (1.4)	63 (87.5)	8 (11.1)	
Education					**0.019**
Primary school or less	36 (39.6)	5 (13.9)	24 (66.7)	7 (19.4)	
Middle school	31 (34.1)	0 (0.0)	31 (100.0)	0 (0.0)	
High school	18 (19.8)	1 (5.6)	16 (88.8)	1 (5.6)	
College school or above	6 (6.5)	0 (0.0)	6 (100.0)	0 (0.0)	
Occupation					**0.000**
Retired/unemployed	22 (24.2)	0 (0.0)	18 (81.8)	4 (18.2)	
Service personnel	24 (26.3)	0 (0.0)	22 (91.7)	2 (8.3)	
Other workers	38 (41.8)	2 (5.3)	34 (89.4)	2 (5.3)	
Student	7 (7.7)	4 (57.1)	3 (42.9)	0 (0.0)	
Underlying disease					**0.017**
Yes	26 (28.6)	1 (3.8)	19 (73.1)	6 (23.1)	
No	65 (71.4)	5 (7.7)	58 (89.2)	2 (3.1)	
Infection source					**0.019**
Hubei related infection	38 (41.8)	0 (0.0)	32 (84.2)	6 (15.8)	
Local infection	53 (58.2)	6 (11.3)	45 (84.9)	2 (3.8)	
Length of stay	**16.1 ± 7.6**				**0.231**
≤10	23 (25.3)	1 (4.3)	22 (95.7)	0 (0.0)	
11–20	49 (53.8)	3 (6.1)	39 (79.6)	7 (14.3)	
21–30	12 (13.2)	2 (16.7)	10 (83.3)	0 (0.0)	
≥31	7 (7.7)	0 (0.0)	6 (85.7)	1 (14.3)	
Virus negative days	**13.8 ± 7.8**				**0.087**
≤10	39 (42.9)	1 (2.6)	37 (94.8)	1 (2.6)	
11–20	39 (42.9)	3 (7.7)	30 (76.9)	6 (15.4)	
21–30	8 (8.8)	2 (25.0)	6 (75.0)	0 (0.0)	
≥31	5 (5.4)	0 (0.0)	4 (80.0)	1 (20.0)	
Smoking					**1.000**
Yes	17 (15.4)	1 (5.9)	14 (82.3)	2 (11.8)	
No	74 (84.6)	5 (6.8)	63 (85.1)	6 (8.1)	
Smoking daily (cigarette)	**15.7 ± 11.4**				**0.207**
≤10	10 (58.8)	0 (0.0)	8 (80.0)	2 (20.0)	
11–20	4 (23.5)	0 (0.0)	4 (100.0)	0 (0.0)	
21–30	1 (5.9)	0 (0.0)	1 (100.0)	0 (0.0)	
≥31	2 (11.8)	1 (50.0)	1 (50.0)	0 (0.0)	
Smoking duration (years)	**22.9 ± 14.6**				**0.491**
≤10	6 (35.3)	0 (0.0)	6 (100.0)	0 (0.0)	
11–20	1 (5.9)	0 (0.0)	1 (100.0)	0 (0.0)	
21–30	6 (35.3)	0 (0.0)	5 (83.3)	1 (16.7)	
≥31	4 (23.5)	1 (25.0)	2 (50.0)	1 (25.0)	

(Continued)

TABLE 1 | Continued

Variables	Total *n* = 91(%)	Mild *n* = 6(%)	Common *n* = 77(%)	Severe *n* = 8(%)	*P**
Alcohol					**0.547**
Yes	24 (26.4)	1 (4.2)	22 (91.6)	1 (4.2)	
No	67 (73.6)	5 (7.5)	55 (82.1)	7 (10.4)	
Amount of drinking (ml)	**147.9 ± 75.9**				**0.516**
≤100	10 (41.7)	0 (0.0)	10 (100.0)	0 (0.0)	
101–200	9 (37.5)	1 (11.1)	7 (77.8)	1 (11.1)	
≥201	5 (20.8)	0 (0.0)	5 (100.0)	0 (0.0)	
Drinking duration (years)	**19.4 ± 13.5**				**0.261**
≤10	10 (41.7)	0 (0.0)	9 (90.0)	1 (10.0)	
11–20	7 (29.2)	0 (0.0)	7 (100.0)	0 (0.0)	
21–30	3 (12.5)	1 (33.3)	2 (66.7)	0 (0.0)	
≥31	4 (16.6)	0 (0.0)	4 (100.0)	0 (0.0)	
Chewing betel nut (nut)	**8.7 ± 5.9**				**0.765**
Yes	6 (7.1)	0 (0.0)	6 (100.0)	0 (0.0)	
No	85 (92.9)	6 (7.1)	71 (83.5)	8 (9.4)	
Chewing betel nut duration (years)	**5.5 ± 4.0**				

**Monte Carlo P < 0.05.*

respectively. There were no critically ill patients, and all patients recovered and were discharged in good condition. The chi-square test for univariate analysis showed that there were significant differences between age, marital status, education level, occupation, presence or absence of underlying diseases and source of infection and patients' clinical typing of COVID-19 ($P \leq 0.05$). The most severe cases of COVID-19 occurred in people aged 65 or older, married people, those with primary school education or below, and those with a history of associated Hubei Province. Severe type COVID-19 was more common in patients with underlying diseases.

The Relationship Between the Underlying Disease and the Clinical Classification of COVID-19

Of the 91 COVID-19 patients, 26 (28.6%) had underlying disease, of which hypertension 12 (13.2%) was the most common, followed by diabetes 8 (8.8%). The patients with hypertension, coronary heart disease, hyperlipidemia, diabetes, cerebral infarction and renal insufficiency accounted for 13.2, 3.3, 5.5, 8.8, 6.6, and 6.6%, respectively. A total of 12.1% of patients had 2 or more underlying diseases. The results showed that there were significant differences between the clinical types of COVID-19 associated with underlying disease ($P < 0.05$) (**Table 2**).

Prediction of Influencing Factors for Clinical Typing of COVID-19 Patients

The influencing factors of clinical typing of COVID-19 patients are discussed in depth. The dependent variables were determined as clinical typing of COVID-19 patients (1 = severe type, 2 = common type, and 3 = mild type), the independent variables were locked as statistically significant factors in univariate analysis, and an ordinal logistic regression model was carried out ($\alpha_{in} = 0.05$, $\alpha_{out} = 0.10$). After adjusting for occupation and marriage in the ordinal regression model, it was found that patients with underlying diseases such as diabetes (OR = 7.740, 95% CI: 1.000–60.740, $P = 0.050$), source of infection (OR = 0.180, 95% CI: 0.030–0.980, $P = 0.048$), and retired/unemployed (OR = 29.430, 95% CI: 1.050–822.330, $P = 0.047$) had more severe COVID-19 infection (**Table 3**).

DISCUSSION

Influence of Factors on the Severity of COVID-19

In this study, age, marriage, education, employment status, underlying diseases and source of infection were significantly different in patients with different COVID-19 severities. However, the final multifactor ordered regression results showed that the underlying diseases of diabetes, source of infection, and employment status (retired/unemployed) were independent influencing factors.

Age was a non-independent influencing factor of multifactor regression analysis, which is inconsistent with the results of other studies (Chen R. C. et al., 2020), we still found out retired/unemployed group was affected with more severe COVID-19 who usually were elder. This may be because the incidence of underlying diseases increases with age, which increases the risk of severe COVID-19 in the elderly. The study also indicated that SARS-CoV-2 infection in infants and young adults is associated with mild and common type illness and these patients generally do not have underlying diseases. The protection babies received may be due to the mother's antibodies and the antiviral proteins in milk, such as lactoferrin, which are known to prevent coronavirus infection (Root-Bernstein, 2020). Few other studies have reported relationships between COVID-19 and marriage, or education which require further study.

TABLE 2 | Relationship between patient underlying disease and disease severity of COVID-19.

Underlying disease	Total *n* = 91(%)	Mild *n* = 6(%)	Common *n* = 77(%)	Severe *n* = 8(%)	*P**
Hypertension					**0.658**
No	79 (86.8)	5 (6.3)	68 (86.1)	6 (7.6)	
Yes	12 (13.2)	1 (8.3)	9 (16.7)	2 (16.7)	
Diabetes					**0.029**
No	83 (91.2)	6 (7.2)	72 (86.7)	5 (6.0)	
Yes	8 (8.8)	0 (0.0)	5 (62.5)	3 (37.5)	
Cerebral infarction					**0.135**
No	85 (93.4)	6 (7.1)	73 (85.9)	6 (7.1)	
Yes	6 (6.6)	0 (0.0)	4 (66.7)	2 (33.3)	
Coronary heart disease (CHD)					**1.000**
No	88 (96.7)	6 (6.8)	74 (84.1)	8 (9.1)	
Yes	3 (3.3)	0 (0.0)	3 (100.0)	0 (0.0)	
Hyperlipidemia					
No	86 (94.5)	6 (7.0)	73 (84.9)	7 (8.1)	0.573
Yes	5 (5.5)	0 (0.0)	4 (80.0)	1 (20.0)	
Renal insufficiency					0.135
No	85 (93.4)	6 (7.1)	73 (85.9)	6 (7.1)	
Yes	6 (6.6)	0 (0.0)	4 (66.7)	2 (33.3)	
Number of underlying diseases					**0.015**
No	65 (71.4)	5 (7.7)	58 (89.2)	2 (3.1)	
1	15 (16.5)	1 (6.7)	12 (80.0)	2 (13.3)	
≥2	11 (12.1)	0 (0.0)	7 (63.6)	4 (36.4)	

**Monte Carlo P < 0.05.*

TABLE 3 | Potential multi-factor ordered regression analysis for predicting disease severity of COVID-19.

Variable	b	S_b	Waldχ^2	*P*	Estimated odds ratio	OR 95% CI
(Covid-19 severity = severe)	−7.591	2.932	6.701	0.010		
(Covid-19 severity = common)	0.247	2.645	0.009	0.926		
Marriage	1.996	1.336	2.230	0.135	7.360	0.540–100.900
Education	0.377	0.377	1.000	0.317	1.460	0.700–3.050
Age	0.054	0.806	0.005	0.946	1.060	0.220–5.120
Comorbidity diabetes mellitus	2.047	1.051	3.794	0.050	7.740	1.000–60.740
Source of infection	−1.733	0.875	3.925	0.048	0.180	0.030–0.980
Occupation (retired/unemployed)	3.382	1.699	3.961	0.047	29.430	1.050–822.330
Occupation (service personnel)	2.980	1.560	3.649	0.056	19.690	0.930–418.970
Occupation (other worker)	1.648	1.342	1.508	0.219	5.200	0.370–72.120
Occupation (student)	0[a]					

Marital status (married/single). Source of infection (local infection/Hubei related infection). [a]*Referent.*

There were 46 men (50.5%) and 45 women (49.5%) in this study, with men being slightly more common than females. This is consistent with the results of Abate et al. (2020) systematic reviews have also indicated that COVID-19 is more common in men than in women.

Diabetes Is Associated With the Severity of COVID-19

Of the six underlying diseases, we examined (diabetes, hypertension, coronary heart disease, cerebral infarction, renal insufficiency, and hyperlipidemia) diabetes was the only independent factor affecting the severity of COVID-19, which is consistent with the findings of Guo et al. (2020). This may be because people with diabetes have increased serum inflammatory-related biomarkers, such as IL-6, c-reactive protein, and serum ferritin, blood coagulation indexes, and D-dimer levels, Therefore people with diabetes have a potential proinflammatory environment, which can lead rapid deterioration of COVID-19. Other studies have shown that patients with diabetes have a higher risk of respiratory infection due to an impaired immune system, especially reduced innate immunity (Ma and Holt, 2020; Pal and Bhansali, 2020). Even transient hyperglycemia may temporarily affect the innate immune response to infection (Jafar et al., 2016). Huang I. et al. (2020) conducted a meta-analysis of 30 studies including

6,452 patients, and found that diabetes was associated with severity, disease progression, mortality, and ARDS in COVID-19 patients. In addition, ACE2 is highly expressed in pancreatic islets; therefore, the virus may cause sharp fluctuations in the blood glucose levels of patients by destroying the islets, thus affecting prognosis (Yang et al., 2010). Previous studies have shown a strong correlation between higher blood glucose levels and the risk of death from COVID-19. Hyperglycemia is an important independent predictor of mortality, and controlling hyperglycemia during the entirety of hospitalization may reduce the risk of serious illness or death (Coppelli et al., 2020; Lampasona et al., 2020). Zhu et al. (2020) reported in their study that the mortality rate of COVID-19 patients with good blood glucose control (blood glucose fluctuation within 3.9–10.0 mmol/L) was significantly lower than that of patients with poor blood glucose control. Sardu et al. (2020) found that patients with hyperglycemia and COVID-19 who received insulin injection had a lower risk of developing severe disease than those who did not receive insulin injection. Therefore, COVID-19 patients with diabetes should receive careful attention, and their blood sugar levels should be closely monitored and strictly controlled due to the risk of rapid deterioration. It is also recommended that patients with underlying diseases take additional precautions to minimize the risk of infection. Doctors should closely monitor such patients for signs of disease progression.

Retirement/Unemployment and COVID-19 Severity

Our results revealed that the retirement/unemployment was an independent risk factor for the severity of COVID-19, which is consistent with the findings of Liu et al. (Liu J. et al., 2020). This may be related to the older age of retirees and their underlying diseases, which are associated with poor prognosis (Peykari et al., 2015). Many unemployed individuals are women engaged in domestic work, and have a low education level, poor information access, weak awareness of epidemic prevention and control, and no stable economic source, which leads to delayed treatment of the disease. Therefore, the unemployed and retirees should be provided updated epidemic information and COVID-19 knowledge. This will allow them to detect symptoms in a timely manner, seek medical treatment as soon as possible, and prevent their illness from getting worse.

Transmission Characteristics and Implications for Prevention of COVID-19

The source of infection was an independent factor affecting the severity of COVID-19 in our study. Here, 15.8 and 3.8% of patients with severe disease were from the Hubei Province and were from local residents respectively. Notably, in the early stage of the epidemic, the Hubei Province lacked medical resources and many patients could not receive timely treatment, leading to treatment delays (Zhang Z. Q. et al., 2020). We also found that the main sources of infection were family intimacy and close social contact (58.2%), followed by being in the epidemic area or having human contact history in Hubei Province (and 41.8%). The wave of outbreaks in China late 2019 and early 2020 began in the Hubei Province, especially in the city of Wuhan. Workers in the Hubei Province returned to their hometowns to celebrate the lunar new year, causing family infections. Because the Hubei residents were traveling or because of the COVID-19 outbreak, some non-Hubei residents who were in areas with shortages of local medical resources returned home for medical treatment, causing transmission during travel.

The results of this study are consistent with those of other studies showing that the main routes of transmission were close contact *via* respiratory droplets and close contact. However, contact or aerosol transmission could also be caused by exposure to virus-contaminated objects and environments under certain conditions (Kutti-Sridharan et al., 2020; Setti et al., 2020; Zhang R. et al., 2020).

When there is a COVID-19 epidemic in the community, the main measures to prevent the spread are to track and isolate close contacts of every COVID-19 patient and to diagnose or exclude COVID-19 infection through nucleic acid testing. Emphasis is placed on masks, personal hand hygiene, ventilation in the home and maintaining social distance as much as possible (Cowling et al., 2020).

Smoking and COVID-19

The smoking, drinking, and BQ chewing rates in our study were 15.4, 26.4, and 7.1%, respectively. The chi-square test showed no statistical significance with the classification of COVID-19. The smoking rate of COVID-19 patients was lower than that the general population. No relationship was found between severity of COVID-19 and smoking, drinking alcohol, and BQ chewing in our study. Two studies (Guan et al., 2020b; Zhang J. J. et al., 2020) in China on COVID-19 and smoking showed that only 1 and 12.6% of patients were smokers, respectively, both lower than the smoking rate in our study (15.4%). Additionally, all of these rates were lower than the proportion of male smokers in China (>50%) (Stone et al., 2016; Qi et al., 2020). Therefore, it is not safe to speculate that whether smoking causes susceptibility to severe COVID-19. It should be also emphasize that due to the small sample size of our study, this conclusions need to be further confirmed by more sample size and multi-center studies.

The idea that smoking and alcohol consumption prevent COVID-19 has appeared in the media during the COVID-19 pandemic. One study reported (Luk et al., 2020) that 19.0% of respondents said they had seen claims in the media that smoking and alcohol consumption prevents COVID-19. There are even studies claiming that nicotine has a therapeutic effect on COVID-19. Nicotine is thought to have an anti-inflammatory effect, thereby regulating the body's immune response and inhibiting the release of pro-inflammatory cytokines rather than anti-inflammatory cytokines such as IL-10 (Wang et al., 2003).

However, the World Health Organization (World Health Organization [WHO], 2020) states that smokers have a higher risk of developing severe COVID-19 and dying from COVID-19 and that there is not enough information to confirm any link between tobacco, nicotine, or alcohol consumption for the prevention or treatment of COVID-19. The WHO emphasizes

that smokers' hand-to-mouth behavior and smoke-induced lung diseases may increase their susceptibility to COVID-19 (World Health Organization [WHO], 2020).

Most studies have shown that smoking is related to the severity of COVID-19. Some (de Groot et al., 2019) have shown that smoking causes inflammation of the respiratory mucosa and lungs, and the chronic smoke stimulation of the respiratory tract causes COPD. These authors confirmed that smokers, including e-cigarette smokers, had higher levels of serum ACE2 expression (Brake et al., 2020) and increased susceptibility to COVID-19 caused by the upregulation of ACE2 (Li et al., 2020).

Every smoker should be encouraged to quit, and offered advice, support, and medication to help them quit. Times of crisis often provide the motivation to quit. We should make greater efforts to correct the erroneous view that smoking and alcohol consumption does not prevent COVID-19 from correcting erroneous views. Studies of smoking and COVID-19 require large samples or case-control studies to be able to identify confounding factors.

Alcohol Consumption and COVID-19 Severity

In our study, all the drinkers except 4 frequent drinkers were social drinkers. Social alcohol consumption increases the possibility of gathering and spreading infectious diseases. We are cautious about alcohol consumption during the COVID-19 epidemic. Studies at the start of the COVID-19 outbreak showed that alcohol consumption could prevent COVID-19 from becoming an epidemic (Chick, 2020). Unfortunately, 180 Iranians drank methanol contaminated alcohol and died because they believed drinking could prevent COVID-19 (Delirrad and Mohammadi, 2020). Additionally, during the COVID-19 outbreak, people tend to drink because of increased stress caused by isolation and temporary absence from work. Close conversations while drinking, gathering in crowds, and even physical contact increase the spread of infectious diseases. Based on the prevailing evidence (Mungmungpuntipantip and Wiwanitkit, 2020), the World Health Organization (World Health Organization Regional Office for Europe, 2020) suggests that during the COVID-19 outbreak, only one alcoholic drink should be consumed per day.

Betel Quid Chewing and COVID-19 Severity

At least 10% of the world's population regularly chews BQ, which is the fourth most widely used addictive substance in the world (Sullivan and Hagen, 2002; Secretan et al., 2009). BQ chewing is popular among East Asians and their residents who migrate to other countries (Gupta and Warnakulasuriya, 2002; Changrani and Gany, 2005; Hashim et al., 2019). The Hunan Province in South China has the highest production and popular consumption of BQ, the rate of chewing BQ up to 38.4% (Xiao et al., 2011). Kaur and Rinkoo (2020) also noted that smokeless tobacco, especially betel quid, is popular in China and other parts of Southeast Asia. BQ affects cardiovascular, metabolic, respiratory, and reproductive health (Garg et al., 2014). Studies have also linked smokeless tobacco use, such as BQ chewing, to an increased risk of respiratory disease (Mehrtash et al., 2017). Alkaloids contained in areca nuts are nitrified to form N-nitrosamines, which may have toxic effects on human cells (No authors listed, 1985). In the Hunan Province China, 42.4% of BQ chewers also smoke (Zhang et al., 2008). Most people, who smoke, chew BQ, and drink alcohol, do not realize that their lifestyle is putting their health at serious risk. Chewing BQ leads to oral mucous membrane fibrosis, hyperkeratosis and ulcers (Chen et al., 2006; Song et al., 2015; Nithya et al., 2019). Research by Reichart et al. (2002) has shown that BQ users have a higher rate of oral lesions, and a high proportion of betel chewer's have betel chewer's mucosa (85.4%). Such oral lesions, cause atrophy of the epithelium and decreased blood vessels (Sharma et al., 2019). This not only directly leads to reduced local oral defense, but also damages a variety of immune populations including CD3 cells, CD4 cells, B lymphocytes, and macrophages, allowing for the invasion of pathogens (Pillai et al., 1990). Previous studies (Chang et al., 2005; Reichart et al., 2005; Singh et al., 2012) have indicated that consumption of BQ is strongly associated with HPV, Candida infection, tuberculosis, dengue fever, malaria, typhoid fever, HIV/AIDS, and other infectious diseases. The association between BQ consumption and pathogens such as SARS-CoV-2, including disease severity, is of concern.

The World Health Organization Framework Convention on Tobacco Control (Yach, 2003) provides evidence-based policies to reduce tobacco use, but a global policy to control BQ use is lacking. There is no smokeless tobacco legislation at the national level, and some developed countries may have overlooked the health risks of BQ chewing by immigrants (Lechner et al., 2019). During the COVID-19 pandemic, BQ chewing has increased opportunities for close interpersonal communication and aggregation. Hand-passing BQ increases the chances of virus contamination and transmission to others. Additionally, the increase in saliva caused by chewing may lead to spitting and saliva transmission. Research into the potential link between smokeless tobacco and COVID-19 must be prioritized for evidence-based policy development.

Smoking, drinking and BQ chewing are population-based disease risk factors, and these behavior increases the population agglomeration. The possibility of contact the pandemic may strengthen the behavior; therefore, the government should formulate policies, warn of the dangers of smoking, drinking and BQ chewing, and help affected individuals correct their behavior.

Limitation

In this single-center retrospective cross-sectional study, COVID-19 patients who smoked, consumed alcohol, and chewed BQ accounted for a relatively small proportion of the total sample, and the relationship of these behaviors with the severity of COVID-19 may be affected by other confounding factors. Although all COVID-19 patients in the hospital were included in this study, the study sample is only taken from one hospital center and is relatively small which may indicate under representation of important factors, therefore the results might not be generalizable to the wider population. How these factors interact with

COVID-19 requires large population and case-control studies for further exploration in the future.

CONCLUSION

This study indicates that there is an increased risk of severe COVID-19 among retired and unemployed individuals. Patients with diabetes and those from Wuhan, or the rest part of the Hubei province in the early stage or who traveled to other areas without timely treatment.

Retired/unemployed individuals and people with underlying diseases should be informed of methods of personal protection, and doctors should prevent these individuals from developing serious diseases. In addition, in clinical practice, it is important to pay attention to the source of infection and timely medical treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Hunan Cancer Hospital (Approval Number: SBQLL-2020-094). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RZ and LC wrote this article. WW and YZ supervised the entire work and critically revised the manuscript. Data collection and statistics analysis by RZ, QZ, and YQ. All authors read and amended the final manuscript.

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BRIEF RESEARCH REPORT
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COVID-19 Disease Course in Former Smokers, Smokers and COPD Patients

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The severe respiratory and systemic disease named coronavirus disease-2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Currently, the COVID-19 pandemic presents a huge social and health challenge worldwide. Many different risk factors are associated with disease severity, such as systemic arterial hypertension, diabetes mellitus, obesity, older age, and other co-infections. Other respiratory diseases such as chronic obstructive pulmonary disease (COPD) and smoking are common comorbidities worldwide. Previous investigations have identified among COVID-19 patients smokers and COPD patients, but recent investigations have questioned the higher risk among these populations. Nevertheless, previous reports failed to isolate smokers and COPD patients without other comorbidities. We performed a longitudinal evaluation of the disease course of smokers, former smokers, and COPD patients with COVID-19 without other comorbidities, from hospitalization to hospital discharge. Although no difference between groups was observed during hospital admission, smokers and COPD patients presented an increase in COVID-19-associated inflammatory markers during the disease course in comparison to non-smokers and former smokers. Our results demonstrated that smoking and COPD are risk factors for severe COVID-19 with possible implications for the ongoing pandemic.

Keywords: SARS-CoV-2, COVID-19, infection, smoking, chronic obstructive pulmonary disease

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major public health problem, affecting millions worldwide. Common clinical symptoms are dyspnea, cough, and sputum production. The main risk factor for the development of COPD is tobacco smoking, which leads to pulmonary remodeling and inflammation (Marsh et al., 2006). Smoking and COPD are independent risk factors for other diseases such as lung cancer (Biondini et al., 2019) and respiratory infections (Gilca et al., 2011; Aikphaibul et al., 2020; Yoon et al., 2020).

The infection caused by SARS-CoV-2 can lead to the development of a severe pulmonary and systemic disease named COVID-19 (Alberca et al., 2020c). Previous reports have identified that COVID-19 is often more severe in the elderly and individuals with comorbidities such as obesity, hypertension, diabetes Mellitus, other co-infections (Alberca, 2020; Alberca et al., 2020a,d,f; Castelo Branco et al., 2020; Shaw et al., 2020). Those patients generally present an increase in COVID-19-associated inflammatory markers, such as d-dimer, leukocytes count, neutrophil count, neutrophil-to-lymphocyte (NTL) ratio, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and c-reactive protein (CRP) (Wolff et al., 2020). It still not clear the mechanisms that could increase the susceptibility of those groups to a more severe COVID-19, but several mechanisms have been postulated such as dysregulation of the anti-viral immune response (Codo et al., 2020) and increase in the expression of the angiotensin-converting enzyme 2 (ACE2) receptor, SARS-CoV-2 entry receptor (Leung et al., 2020).

Nicotine, a component in tobacco smoke, can suppress antiviral immune responses, via downregulation of Interferon regulatory factor 7 (Han et al., 2019). COPD patients with frequent exacerbations also present a reduction in antiviral immune response, via a reduction in type I and III interferons and interferon-stimulated genes (Singanayagam et al., 2019). In addition, smokers and COPD patients also present an increase in ACE2 receptor expression in the lungs (Leung et al., 2020).

In one of the first reports with COVID-19 patients, 1.1% of the patients had COPD, 12.6% were smokers and 1.9% were former smokers (Guan et al., 2020). COPD and smoking have also been associated with an increased incidence in COVID-19 (Dai et al., 2020; Lian et al., 2020; Zhao et al., 2020) and worst prognoses (Barrasa et al., 2020; Patanavanich and Glantz, 2020).

However, a recent report identified that smoking and pulmonary diseases, such as COPD, were less common in COVID-19 in comparison to influenza patients (Auvinen et al., 2020) and one meta-analyses found no association between COVID-19 prognosis and smoking (Vardavas and Nikitara, 2020).

Nevertheless, to the moment, no study was performed to investigate the difference in the disease course of COVID-19 among smokers, former smokers, and COPD patients without other comorbidities (Dai et al., 2020). Therefore, we aimed to perform an investigation in our cohort to assess if smoking or COPD could influence the COVID-19 disease course.

MATERIALS AND METHODS

Patients were recruited at the university hospital (Hospital das Clinicas da Universidade de São Paulo – HCFMUSP), in a special ward for COVID-19 patients. Inclusion criteria were: (1) All patients have SARS-CoV-2 infection verified by nasopharyngeal detection of SARS-CoV-2 by reverse-transcriptase polymerase chain reaction; (2) COPD patients were selected based on previous COPD diagnoses, all patients in our cohort were considered mild to moderate COPD patients; (3) Smoking habits were self-reported by patients on the hospital's admission. Exclusion criteria were: (1) any other comorbidities other than smoking or COPD; (2) Other co-infection (bacterial or viral). We tracked patients' clinical laboratory data performed from day 1 at the hospital until SARS-CoV-2 clearance and hospital discharge. This study was approved by the Ethics Committee of HCFMUSP (no. 30800520.7.0000.0068-2020).

In our cohort of 318 patients from the university hospital: Six patients were previously diagnosed with COPD (COPD), seven were active smokers (SMOKERS), and 12 have a previous smoking history (with over 10 years absence) without COPD (EX-SMOKERS), and 10 healthy individuals non-smokers (NC). No patients included in this investigation presented any other comorbidities. Data are shown for the longitudinal graph as median values. Data from the first hospitalization day are shown as median and standard error mean (SEM). Statistical analysis for survival curve was performed using Log-rank test, with Log-rank test for trend and Gehan-Breslow-Wilcoxon test to compare all groups, statistical analysis for other data was performed with Kruskal–Wallis test with Dunn's multiple comparisons with GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, United States).

RESULTS AND DISCUSSION

The patients did not present any difference in age and hospitalization time was only increased in EX-SMOKERS in comparison to NC (**Table 1**). On the first hospitalization day, no difference was observed in inflammatory hallmarks of SARS-CoV-2 infection such as lactate dehydrogenase, C-reactive protein, alanine aminotransferase, aspartate aminotransferase, D-dimer, and alkaline phosphatase (**Table 1**; Alberca et al., 2020b). Previous reports have identified models for predicting the disease outcome based on the first day after hospitalization or based on a risk score (Yildiz et al., 2020). Nevertheless, we hypothesize that longitudinal analysis of clinical data is crucial for the understanding of the immune response to SARS-CoV-2, mainly because it is almost impossible to precisely determine the infection day. Therefore, we performed a daily comparison of clinical data for these patients.

During the disease course, no difference in the number of leukocytes was verified among groups (**Figure 1A**). The NTL ratio was increased in the EX-SMOKERS, SMOKERS, and COPD patients in comparison to NC. It is noteworthy that SMOKERS had a further increase in the NTL in relation to EX-SMOKERS and COPD (**Figure 1B**). NTL is a widely used maker for COVID-19 prognoses (Alberca et al., 2020b), but the increase in NTL in the SMOKERS groups was due to both an increase in the neutrophil count (**Figure 1C**) in comparison to EX-SMOKERS and NC and a reduction in the lymphocytes count in relation to EX-SMOKERS and COPD (**Figure 1D**).

Lymphopenia is a described characteristic in smokers and is linked to worst health prognoses (Biondini et al., 2019). Therefore, smoking may generate immunosuppressive effects, with a reduction in the number of lymphocytes (Düvenci Birben et al., 2016; Tulgar et al., 2016), and consequently a reduction in the anti-SARS-CoV-2 immune response.

TABLE 1 | Patients' characteristics by on the hospitalization day.

	NC (*N* = 10)		EX-SMOKERS (*N* = 12)		SMOKERS (*N* = 7)		COPD (*N* = 6)			
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Reference numbers**	***p*-value**
Age (years)	56.87	3.101	58.43	5.327	60.67	2.909	63.33	3.648	–	0.6657
Hospitalization time (days)	18.3*	2.856	37.42*	4.94	32.29	9.411	31.17	5.924	–	0.0303
Lactate dehydrogenase (U/L)	**439**	**50.88**	**482.9**	**59.83**	**580.2**	**116.4**	**522.8**	**89.93**	**135–225**	0.5546
C-reactive protein (mg/L)	**184.5**	**43.14**	**143.7**	**45.9**	**191**	**68.06**	**116.7**	**37.54**	**<5.0**	0.7150
Alanine aminotransferase (U/L)	**69.5**	**24.78**	**54.25**	**14**	35.57	8.893	**47.5**	**13.4**	**<41**	0.4593
Aspartate aminotransferase (U/L)	**67.38**	**20.61**	**52.58**	**11.41**	**45.14**	**10.48**	**76.5**	**26.07**	**<37**	0.9193
D-dimer (ng/mL)	**1364**	**307**	**4745**	**2011**	**6042**	**3195**	**3604**	**2286**	**<500**	0.7710
Alkaline phosphatase (U/L)	**221.9**	**47.04**	**185.3**	**51.18**	128.8	30.97	**202.7**	**42.49**	**40–129**	0.3862

*One-way ANOVA with Dunn's post test. * Difference between those groups. References values from Divisão de Laboratório Central to HC/FMUSP. Bold indicates outside reference interval. SEM, Standard Error of Mean; COPD, patients were previously diagnosed with COPD; SMOKERS, active smokers; EX-SMOKERS, patients with a previous smoking history (with over 10 years absence) without COPD; and NC, healthy individuals non-smokers.*

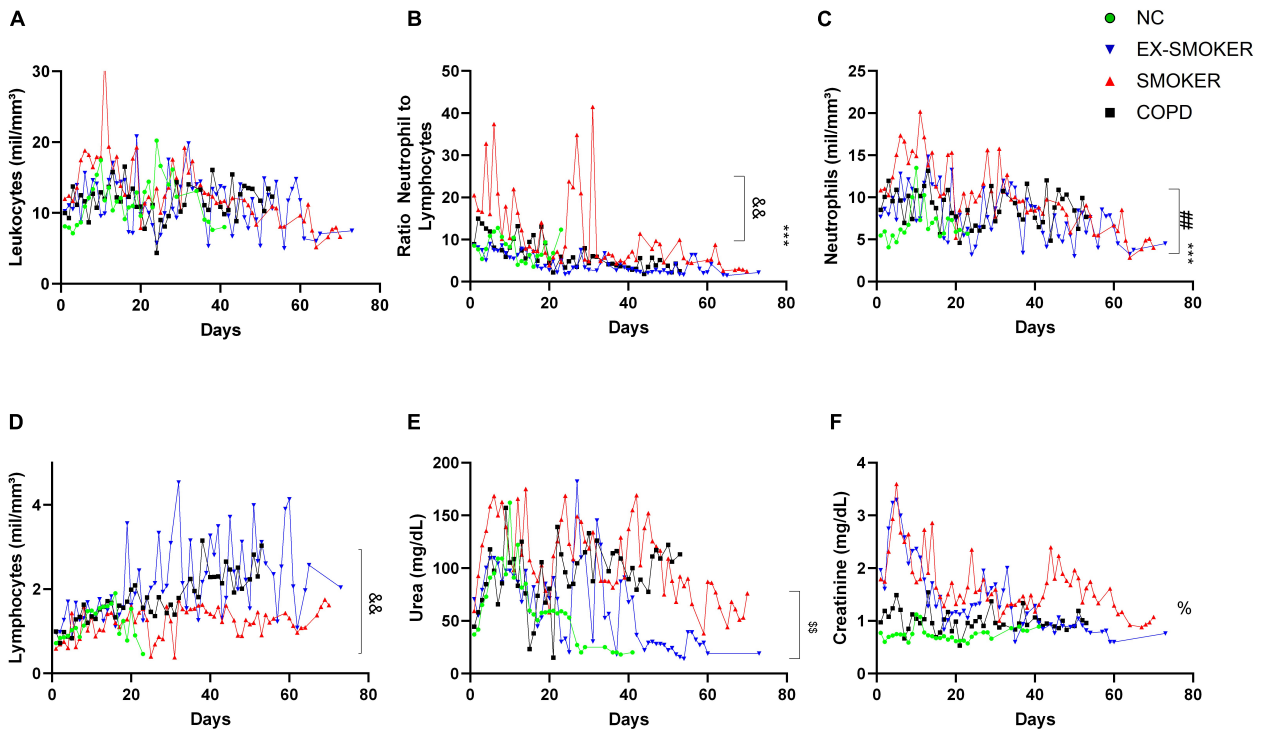

FIGURE 1 | Clinical features of COVID-19 patients. Daily levels of **(A)** Leukocytes, **(B)** Ratio Neutrophil-to-lymphocytes, **(C)** Neutrophils and **(D)** Lymphocytes counts, **(E)** Urea and **(F)** Creatinine levels during the COVID-19 disease course from the first hospitalization day to SARS-CoV-2 clearance and hospital discharge or patients death. NC, No-comorbidities COVID-19 patients; SMOKERS, active smokers by the hospitalization time with COVID-19. EX-SMOKERS, patients that had smoked, but quieted for over 10 years by the hospitalization time with COVID-19; COPD, patients diagnosed with chronic obstructive pulmonary disease prior to COVID-19. *** < 0.001 difference from NC in relation to all other groups. && < 0.01 difference from SMOKER in relation to EX-SMOKERS and COPD. $$ < 0.01 difference from SMOKER and COPD in relation to EX-SMOKERS and NC. ## < 0.01 difference from SMOKER in relation to EX-SMOKERS. % < 0.05 difference among all groups. Kruskal–Wallis test with Dunn's multiple comparisons. Data collected between 1 March 2020 and 30 August 2020.

In our cohort, COPD and SMOKERS presented increased levels of urea in relation to NC and EX-SMOKERS (**Figure 1E**). Creatinine levels were different among all groups, indicating that EX-SMOKERS also presented an increase in this inflammatory marker in relation to NC group (**Figure 1F**). Alterations in the urea and creatinine levels among COPD and Smokers patients could also further indicate an increase in the risk of kidney injury during COVID-19 (Yang et al., 2020).

A report from Wong et al. identified that former smokers present an increased influenza-associated hazard ratio in comparison to non-smokers (Wong et al., 2013). Similarly, in our cohort, the group of EX-SMOKERS presented a difference in the disease course compared to the NC group.

It's noteworthy that during the investigation two patients from the SMOKERS group passed away due to severe respiratory distress, corroborating with previous reports that identified that smokers with COVID-19 possess a higher odd of developing severe COVID-19. All other patients cleared SARS-CoV-2 infection and were discharged from the hospital. Therefore, in our cohort SMOKERS groups presented a statistically significant difference in the survival curve in comparison to all other groups ($p = 0.0349$).

This phenomenon in smokers and COPD patients could be partially explained by the local inflammatory and oxidative response (Tian et al., 2017) and the up-regulation of ACE2 receptor, SARS-CoV-2 entry receptor, in the lungs (Jacobs et al., 2020). In comparison, allergic asthma downregulates ACE2 receptor expression in the lungs (Castelo Branco et al., 2020), possibly being a protective factor in COVID-19 (Alberca et al., 2020e). This still needs to be further explored since the elderly, an established risk group for severe COVID-19, also presents a downregulation in the ACE2 receptor expression in the lungs (Tavares et al., 2020).

Our report highlights the difference in disease course among smokers, ex-smokers, and no smokers (NC group), indicating that ex-smokers do present a better disease course than smokers. Importantly, we demonstrate that COPD and smoking do influence COVID-19 disease course independently of other comorbidities. Is important to highlight that this investigation possesses a small number of patients and should be further expanded to better understand the influence of these comorbidities on COVID-19. A possible synergic effect of smoking and COPD with other comorbidities in COVID-19 needs to be further explored to aid in the development of specific treatments for these populations.

CONCLUSION

In our investigation on the hospitalization day non-smokers, smokers, former smokers, and COPD patients did not present differences in COVID-19 associated inflammatory markers. Nevertheless, a longitudinal investigation demonstrated that smokers and COPD patients, without other comorbidities, present a higher risk for severe COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hospital das Clinicas da Universidade de São Paulo – HCFMUSP (no. 30800520.7.0000.0068-2020). Written informed consent for participation was not required for this study in accordance with the National Legislation and the Institutional Requirements.

AUTHOR CONTRIBUTIONS

RA, GB, AD, and MS: conception, analyze the data, and write and review of the manuscript. JL, EO, SG-S, YR, MA, DB, LO, AB, AP, NP, FT, and IF: data collection, analyze the data, and review of the manuscript. All authors contributed to the article and approved the submitted version.

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Co-inhibition of CD73 and ADORA2B Improves Long-Term Cigarette Smoke Induced Lung Injury

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Adenosine (ADO) involvement in lung injury depends on the activation of its receptors. The ADO A_{2A} receptor (ADORA2A) and A_{2B} receptor (ADORA2B) are best described to have both tissue-protective and tissue-destructive processes. However, no approach has been effective in delineating the mechanism(s) involved with ADO shifting from its tissue-protective to tissue-destructive properties in chronic airway injury. Using cigarette smoke (CS) as our model of injury, we chronically exposed Nuli-1 cells to 5% CS extract (CSE) for 3 years establishing a long-term CSE exposure model (LTC). We found significant morphological changes, decreased proliferation, and migration resulting in impaired airway wound closure in LTC. Further investigations showed that long-term CSE exposure upregulates CD73 and ADORA2B expression, increases ADO production, inhibits PKC alpha activity and p-ERK signaling pathway. Knocking down ADORA2B and/or CD73 in LTC activates PKC alpha and increases p-ERK signaling. Knocking down both showed better improvement in wound repair than either alone. *In vivo* experiments also showed that double knockout CD73 and ADORA2B remarkably improved CS-induced lung injury by activating PKC alpha, reducing the inflammatory cell number in bronchoalveolar lavage fluid and the production of inflammatory mediator IL-6, inhibiting the fibrosis-like lesions and decreasing collagen deposition surrounding bronchioles. Collectively, long-term CSE exposure upregulates CD73 expression and increases ADO production, which promotes low affinity ADORA2B activation and subsequent diminution of PKC alpha activity and ERK signaling pathway, and inhibition of airway wound repair. Moreover, the data suggesting ADORA2B and CD73 as potential therapeutic targets may be more efficacious in improving chronic CS lung diseases and impaired wound repair.

Keywords: CD73, adenosine, cigarette smoke, ECIS, lung

INTRODUCTION

Cigarette smoke (CS) is a major risk factor for several chronic lung diseases including asthma, emphysema, and chronic obstructive pulmonary disease (COPD). COPD is a serious burden throughout the word both economically and socially and is characterized by chronic inflammation and injury of both the airways and the parenchymal structures of the lung. It was recognized as the third leading causes of mortality and morbidity in the United States. An estimated 95% of COPD cases are caused by CS, not only smokers but also those involuntary exposed to second hand smoke (Barnes et al., 2003).

Adenosine (ADO) is a purinergic molecule which modulates tissue damage and repair (Fredholm, 2007). It is best known for promoting anti-inflammatory activities during acute injury, whereas elevations in ADO contribute to destructive tissue remodeling processes in chronic injury (Hasko and Pacher, 2008; Zhou et al., 2009). ADO levels are elevated in the lungs of patients with COPD, where it is believed that the balance between tissue repair and excessive airway remodeling is regulated through its receptors (A_1, A_{2A}, A_{2B}, and A_3) (Zhou et al., 2010). Recent studies have identified the ADORA2B as an important target in the regulation of both acute and chronic lung disease with opposing activities. Several studies have demonstrated a protective role of A_{2B} adenosine receptor (AR) during acute lung injuries (Ahmad et al., 2009; Eckle et al., 2009; Konrad et al., 2017). However, there is a substantial amount of evidence indicating that ADORA2B also has a non-protective role; for instance, smokers with COPD have elevated mRNA levels of ADORA2B as compared to non-smokers and smokers without COPD (Sun et al., 2006; Zhou et al., 2009, 2010; Karmouty-Quintana et al., 2013). No approach has been effective in delineating the mechanism(s) involved with ADO shifting from its tissue-protective to tissue-destructive properties, which may implicate the ADORA2B in contributing the tissue destructive events observed in COPD.

CS can induce release of ATP both *in vitro* and *in vivo* (Lommatzsch et al., 2010); ATP degradation is regulated by ectonucleoside triphosphate disphosphohydrolase (CD39) that hydrolyzes ATP and ADP to AMP and subsequently to ADO by CD73, an ecto-5′nucleotidase (also known as NT5E) that is found in most tissues (Thome et al., 2009). CD73-generated extracellular ADO is known to regulate all four ARs (Burnstock, 2008); however, high concentration levels (in the micromole range) of endogenous ADO is required to activate the low affinity receptor, ADORA2B (Beukers et al., 2000; Hamann et al., 2015). CD73 has been reported that it also has both protective and promoting effects in lung inflammation and fibrosis (Volmer et al., 2006; Ehrentraut et al., 2013; Bou Ghanem et al., 2015; Wirsdorfer et al., 2016; Minor et al., 2019). In a bleomycin induced lung injury model, CD73 knock out (KO) mice exhibited enhanced inflammation and collagen production (Volmer et al., 2006). However, the radiation-induced lung epithelial damage and fibrosis was significantly blunted in CD73 KO mice (Wirsdorfer et al., 2016). There are no studies correlating the effect of CS on the activities of CD73 and subsequent purinergic signaling pathway in airway epithelial cells that may play a role in shifting the tissue protective to tissue destructive properties in ADO-mediated wound repair. We proposed to investigate CD73 role in switching on the adenosinergic signaling by catalyzing the hydrolysis of AMP into ADO, subsequently up-regulating ADORA2B.

MATERIALS AND METHODS

Cell Culture

The Nuli-1 human bronchial epithelial cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured on type VI placenta collagen (Sigma, St. Louis, MA) pre-coated dishes in bronchial epithelial growth media (BEGM; Lonza, Walkersville MD). Cells were maintained in humidified incubator at 37°C in an atmosphere of 5% CO_2 as described previously (Tian et al., 2017). Cell images were taken by EVOS XL imaging system (Life technologies, United States).

Animals

C57BL/6 background CD73 KO mice were purchased from the Jackson Laboratories (Bar Harbor, ME). C57BL/6 background ADORA2B KO mice were a gift from Dr. Michael Blackburn (University of Texas Medical School at Houston, McGovern Medical School, Houston TX). WT and ADORA2B/CD73 double KO (DKO) mice were generated by cross breeding ADORA2B KO and CD73 KO mice. Genotyping was performed by Transnetyx® (Transnetyx, Cordova, TN) using real-time PCR. All mice used for experiments were between 8 and 10 weeks and were maintained under standard housing conditions in the animal care facility at the University of South Florida (USF). All studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health* and were approved by USF Institutional Animal Care and Use Committees.

Generation of Long-Term CS Extract (CSE) Exposed Nuli-1 Cells (LTC-Nuli)

CSE was prepared as previously described using 3R4F reference cigarettes (University of Kentucky, Lexington, KY) (Tian et al., 2017). Nuli-1 cells were treated 1 day in normal media, and then 2 days in media containing 5% CSE; cells (designated LTC) were passaged every 3 days as shown in **Figure 1A**. Control Nuli-1 cell (designated LTM) culture in normal media and passaged at same time as LTC. Both LTM and LTC are cultured for more than 3 years.

Microarray Analysis

RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The Molecular Genomics Core at the H. Lee Moffitt Cancer Center and Research Institute performed the Affymetrix arrays. The data has been uploaded to NCBI, the GEO submission number is GSE111952.

PKC Alpha Activity Assay

PKC activity from Nuli-1 cell was determined using PKC alpha kinase enzyme system (Promega, Madison, WI) according to the

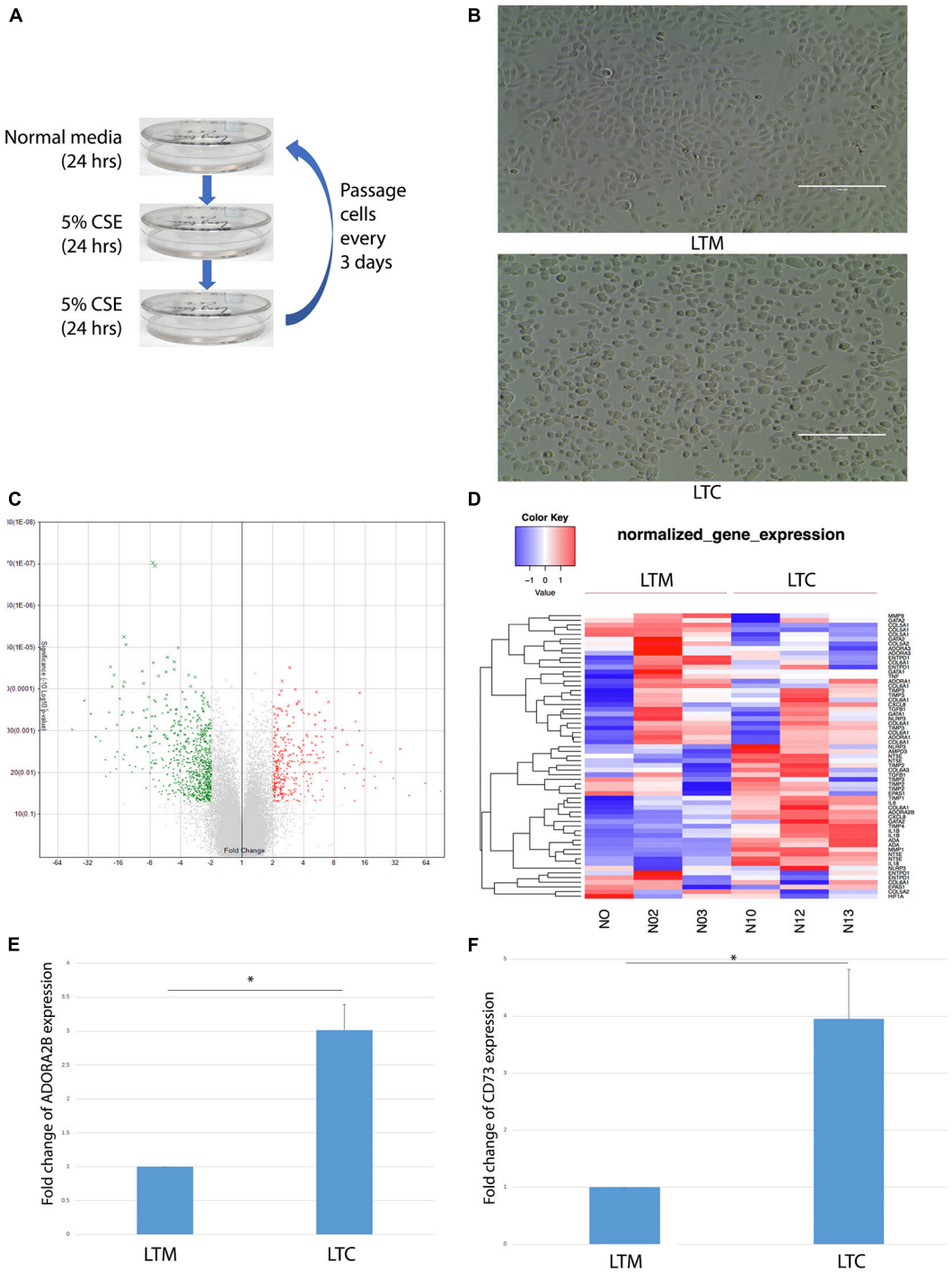

FIGURE 1 | (A) Workflow of long-term CSE exposure. **(B)** Morphological changes observed in LTC. Scale bar, 100 μm. **(C)** Volcano plots showed that over 1,000 genes changed at least twofold after long-term CSE exposure. Red indicates up-regulated and green indicates down-regulated genes. **(D)** Heat map of up- or down- regulated wound repair related genes that are differentially expressed (> twofold) in LTC. Red indicates up-regulated and blue indicates down-regulated genes as shown in the scale bar. **(E)** ADORA2B expression level up-regulated after long-term CSE exposure * indicated $p < 0.05$. **(F)** CD73 expression level up-regulated after long-term CSE exposure * indicated $p < 0.05$.

manufacturer's instructions. Briefly, cells lysates were prepared in RIPA buffer (Cell signaling, Danvers, MA). Equal amounts of protein lysates (5 μl) were co-incubated with PKC alpha mixture containing 1 μl of CREBtide, 2.5 μl of 10× PKC lipid activator and 0.125 μl of 200× ATP in 96 well plate at room temperature (RT) for 60 min. At the end of incubation, 5 μl of ADP-Glo

reagent was added and incubated at RT 40 min followed by 10 μl of kinase detection reagent for a final incubation at RT for 60 min. PKC alpha was measured via luminescence signal using the BioTek H1 plate reader (BioTek Inc., Winooski, Vermont). PKC activity from mice trachea was determined in crude whole-cell fractions of bronchial epithelial cells, using a modification of procedures previously described (Jiang et al., 1992; Allen-Gipson et al., 2013).

Cell Proliferation Assay

8W10E + ECIS culturewares (Applied BioPhysics, Troy, NY) were used for proliferation assay as described before (Tian et al., 2017). Briefly, 8×10^3 cells were seeded per well and cultured in incubator at 37°C in an atmosphere of 5% CO_2. Media were changed next day and the resistances were recorded in real-time at 4,000 Hz using ECIS Zθ instrument (Applied BioPhysics, Troy, NY).

Cell Migration Assay

8W1E ECIS culturewares (Applied BioPhysics, Troy, NY) were used for migration assay as described previously (Tian et al., 2017). Briefly, 8×10^4 cells were seeded each well and cultured in incubator at 37°C in an atmosphere of 5% CO_2. Media were changed next day; and cell monolayer were wounded using an elevated field pulse of 3,000 μA at 80,000 Hz applied for 20 s, producing a uniform circular lesion 250 μm in size. The wounds were tracked in real-time at 4,000 Hz using ECIS Zθ instrument (Applied BioPhysics, Troy, NY).

Cell Barrier Function Assay

8W10E + culturewares were used for barrier function assay, 8×10^4 cells were seeded in each well and the resistance was measured immediately at 2,000 Hz and 64,000 Hz using ECIS Zθ instrument (Applied BioPhysics, Troy, NY).

Taqman Real-Time PCR

RNA was extracted using TRIzol® reagent (Invitrogen, United States) according to the manufacturer's instructions. cDNA was synthesized by using 100 ng of total RNA and TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions were prepared in triplicate using 1× TaqMan Master Mix (Applied Biosystems, Foster City, CA) and primers and probes, as previously described (Tian et al., 2017). TaqMan real-time PCR was performed using an ABI 7,900 Sequence Detection System (Applied Biosystems, Foster City, CA). The relative fold change was calculated by the $2^{-\Delta\Delta Ct}$ method.

Knockdown of Gene Expression by shRNA

MISSION shRNA Lentiviral transduction particles were purchased from Sigma-Aldrich (St. Louis, MO). 2×10^4 LTC cells were plated in a 96-well plate and incubated overnight (ON). Fresh experimental media were added containing 110 μl 0.8 μg Hexadimethrine bromide (Sigma, St. Louis, MO) and 10 μl of lentiviral shRNA particles to each well and incubated ON. Following the transduction, experimental media were replaced with normal media ON, and puromycin (10 μg/ml) was added to remove any non-transduced cells.

Western Blot

Cells lysates were prepared in RIPA buffer (Cell signaling, Danvers, MA) containing PMSF (0.5 mM). Equal amounts of protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene difluoride (PVDF) membranes. Membranes were blocked at RT for 1 h with 5% bovine serum albumin (BSA, Thermo Fisher Scientific, Hampton, NH) followed by exposure to different primary antibody, including anti-ADORA2B (1:200, Abcam, Cambridge, United Kingdom), anti-CD73 (1:1,000, Cell signaling, Danvers, MA), anti-ZO-1 (1:1,000, Cell signaling, Danvers, MA), anti-p-MEK (1:1,000, Cell signaling, Danvers, MA), anti-p-ERK (1:1,000, Cell signaling Danvers MA), anti-p-p90RSK (1:1,000, Cell signaling, Danvers, MA), anti-p-CREB (1:1,000, Cell signaling, Danvers, MA), anti-GAPDH (1:2,000, Cell signaling, Danvers, MA), ON. After washing with Tris-Buffered Saline (TBS) plus 1% Tween-20, membranes were incubated with 1:5,000 diluted goat anti rabbit IgG-HRP secondary antibody for 1 h at RT. Pierce™ Western Blotting Substrate (Thermo Fisher Scientific, Hampton, NH) was used and membranes were imaged via Bio-red Doc. All the WB image we presented in this manuscript is representative of 3 experiments conducted on three separate occasion. Antibody to GAPDH (Cell signaling, Danvers, MA) was used as a loading control. Densitometric analysis of Western blots were analyzed by ImageJ (Public domain, BSD-2, NIH, United States).

High-Performance Liquid Chromatography (HPLC) Measurement of Extracellular ADO

ADO was determined using HPLC as described with modifications (Fu et al., 2000). Briefly, 1 ml of cell culture supernatant was mixed with 50 μl of perchloric acid for 5 min, followed by centrifugation at 8,000 g for 5 min. The supernatant was transferred to a micro-insert tube (placed in a brown vial) for analysis using Agilent 1,260 infinity HPLC system (Agilent Technologier, Santa Clara, CA) with Luna 5 u C18(2) 100 A column (Phenomenex, United States) guarded by a SecurityGuard™ Cartridge System (Torrance, CA). The injection volume was 100 μl and total running time was 20 min. The mobile phase was prepared as follows: 50 mM of sodium perchlorate, 0.1 M of sodium acetate, 2.4 mM of sodium 1-heptanesulfonate, 0.9% acetonitrile and 0.1 M of sodium azide. The flow rate was 2 ml/min and the ADO retention time was 7.7 min.

CD73 Activity Assay

Enzymatic activities for CD73 in Nuli-1 cells were performed using CD73 Activity Assay Kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Briefly, 1×10^6 cells were prepared in 100 μl of Assay Buffer for 10 min at 4°C. The supernatant was collected by centrifuging at

10,000 × g for 10 min at 4°C and 100 μg of the protein sample was added into a 96 well-plate. The sample then incubated with CD73 Reaction Mix at 37°C for 20 min. The incubation reaction stopped by adding 4 μl of Stop Solution and followed by 80 μl of CD73 Developer I and 40 μl of Developer II. Each well incubated at RT for 20 min and the absorbance were recorded at 670 nm using SPECTRA MAX 190 (Analytical instrument brokers, LLC, Golden Valley, MN).

CS and Air Exposure

A total of 10 mice (5 males and 5 females) in each group (WT, ADORA2B KO, CD73 KO, and ADORA2B/CD73 DKO) were passively treated with CS using a Teague-10 Smoking Machine (Teague Enterprises, Davis, CA). Using the Teague device, mice were exposed to smoke from eighty 3R4F reference cigarettes (University of Kentucky, Lexington, KY) per day. Mice receiving CS were gradually brought to their target exposure over a period of 5 days, treated 5 days/week for 3 months. The same amount of control mice from each group were exposed to air in the same manner in a similar apparatus for the same periods of time.

Bronchoalveolar Lavage Fluid (BALF)

Mice were euthanized with a cocktail of xylazine and ketamine (0.1 mL/10 g). BALF were collected as previously described (Tian et al., 2017). Tracheas were exposed and the ends of the tracheas were tied off, a total of 1.0 mL cold sterile PBS (Gibco, Grand Island, NY) was gently flushed into the lungs and recovered. Collected BALF was centrifuged at 300 g for 7 min at 4°C. Pelleted cells were resuspended in 1.0 ml of PBS. Total cells were counted on a hemocytometer, and 1–5 × 10^3 cells were spun onto glass microscope slides (cytospin 3; Shandon Scientific, Cheshire, United Kingdom). Cells were air dried for 24–36 h, fixed, and stained with a HEMA 3 stain set (Thermo Fisher Scientific, Kalamazoo, MI). Differential cell counts of at least 300 cells per slide were made according to morphological criteria. The number of cells recovered was calculated and expressed as absolute cell numbers.

Lung Collection, Histology, and Collagen Staining

Whole lungs were excised and inflated to 10 cm H_2O pressure with 10% formalin (Sigma, St. Louis, MO) to preserve pulmonary architecture. Lungs were embedded in paraffin, and sections were cut (5 μm) and processed for hematoxylin and eosin staining. Rehydrated lung sections were stained with Picro-Sirius Red Solution (Abcam, Cambridge, United Kingdom) to determine bronchial airway collagen deposition according to the manufacturer's instructions. Briefly, the rehydrated lung sections were incubated with Picro-Sirius Red Solution for 60 min at RT, then rinsed twice by 0.5% Acetic Acid Solution (Thermo Fisher Scientific, Rockford, IL) and once by absolute alcohol (Sigma, St. Louis, MO). The slides were cleared and dehydrated in absolute alcohol, mounted with mounting medium (Thermo Fisher Scientific, Rockford, IL) and cover slips. The images were taken by EVOS XL imaging system (Life technologies, United States). For quantitative histology, airways were grouped by size in diameter of 100–200 μm, the percentage of collagen in each image were measured using ImageJ (NIH, United States).

Statistics Analysis

All experiments were conducted in triplicate and results were expressed as mean ± SE. Data were statistically analyzed using Student's paired *t*-test followed by Tukey's multiple-comparison test. Statistical differences among groups were determined using one-way ANOVA followed by Tukey's multiple-comparison test. Significance was assigned at $P < 0.05$.

RESULTS

Microarray Analysis of Gene Expression Profile in Long-Term CSE Exposed Airway Epithelial Cell

To assess the long-term exposure of CSE, Nuli-1 cells were treated in cycles of 24 h in 100% media followed by 48 h of 5% CSE treatment (1 cycle); repeated over the course of 3 years (LTC); the media control Nuli-1 cells were exposed long-term with 100% normal media (LTM) as shown in **Figure 1A**. After 3 years of CSE exposure, LTC showed visually appreciable morphological changes when compared to LTM. LTC took on a more rounded shape with less physical contact with each other (**Figure 1B**). Affymetrix array revealed at least twofold changes in over 1,000 genes in LTC (**Figure 1C**). The heatmap in **Figure 1D** showed that CD73 (NT5E) and ADORA2B were upregulated by CSE exposure (**Figure 1D**). Moreover, the ADORA2B gene revealed a threefold increase in expression (**Figure 1E**) and the CD73 was upregulated by 3.94-fold (**Figure 1F**). Collectively, data revealed the long-term CSE promote physical and gene changes in the Nuli-1, which may contribute to the CS-dysregulation of airway-wound repair.

Long-Term CSE Exposure Impairs Airway Wound Repair

The proliferation, migration, and barrier function are regarded as important repair processes in wound repair; the ECIS was used to assess how long term CSE exposure affects these processes. Consistent with the morphological changes, long-term CSE exposure not only decreased the rate of proliferation (**Figure 2A**), but also reduced the rate of migration (**Figure 2B**). Additionally, we measured the barrier function of the CSE exposed cells using ECIS barrier function assay. Electric current can couple capacitively through cells membranes in high AC frequencies (e.g., 64,000 Hz), while moving through the paracellular passage between the cells (the barrier function) in low AC frequencies (e.g., 2,000 Hz). With the cell attached to the ECIS culture-well, the resistance will increase consistently. The measured resistance at 64,000 Hz were similar, which suggests there were the same number of cells attached to the ECIS culture-well (**Figure 2Ca**); however, the recorded resistance at 2,000 Hz from LTC were decreased when compared to LTM (**Figure 2Cb**), indicating that CSE treatment blunted

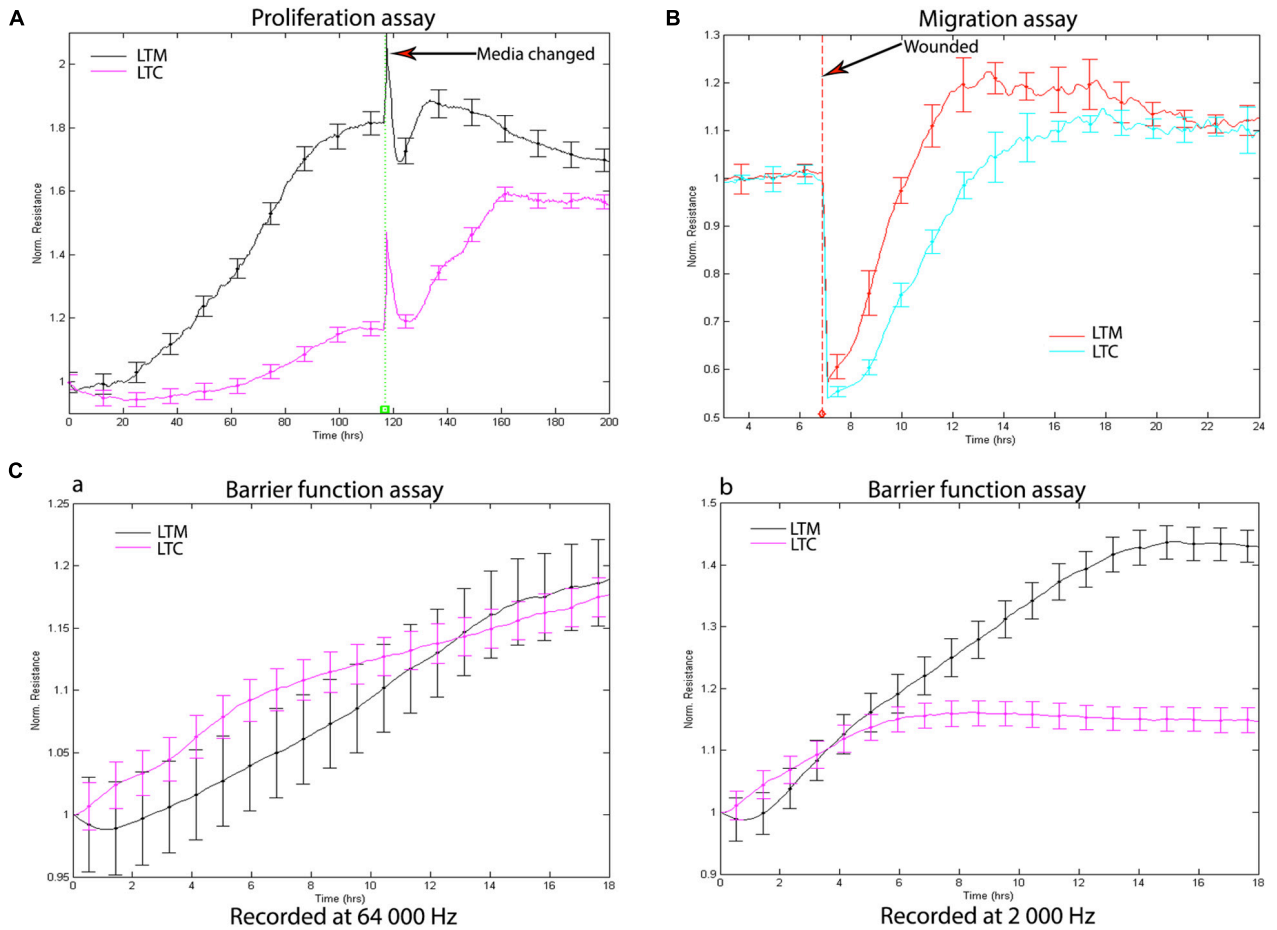

FIGURE 2 | (A) Long-term CSE exposure inhibits Nuli-1 cells proliferation. Media were changed after 118 h. Signals were recorded at 4,000 Hz. Each condition was conducted in triplicate. Statistically differences observed after 24 h ($P < 0.05$) **(B)** Long-term CSE exposure inhibits Nuli-1 cells migration. The resistances were measured at 4,000 Hz. Each condition was conducted in triplicate. Statistically differences observed after 7.5 h ($P < 0.05$) **(C)** Long-term CSE exposure impairs Nuli-1 cells barrier function. a. Resistances were recorded at 64 000 Hz; b. Resistances were recorded at 2,000 Hz. The resistances were measured simultaneously. Each condition was conducted in triplicate. Statistically differences observed after 7 h ($P < 0.05$).

barrier function of LTC. Collectively, the data indicate long-term CSE treatment impaired critical processes necessary for airway wound repair.

Long-Term CSE Exposure Triggers ADORA2B-Mediated Tissue Destructive Sensors Through Inhibition of p-ERK Pathway

As the low-affinity adenosine receptor, the ADORA2B has been implicated in tissue injury as it relates to COPD. Most recently we reported significant increase in transcriptional expression of ADORA2B in CS-treated mice (Tian et al., 2017), however, very little is known regarding long-term CSE exposure on ADORA2B tissue destructive signaling. To track the change of CD73, ADORA2A, and ADORA2B, we analyzed the mRNA expression level in the different time point of exposure. The ADORA2A expression decreased in the early stage of CSE exposure, but it back to normal after 8-month exposure. The expression level of both CD73 and ADORA2B were upregulated (**Figure 3A**). Western Blot also confirmed an increase in the ADORA2B expression as well as CD73 expression (**Figures 3B,C**), which may contribute to the increase in extracellular ADO concentration observed in LTC (**Figure 3D**).

To further understand the mechanisms of ADORA2B-mediated tissue destructive properties, we investigated the involvement of PKC activity and downstream MAPKs signaling pathway. Interestingly, we observed a significant decrease in the tight junction protein ZO-1 in LTC (**Figures 3B,C**), which infers the observed reduced barrier function (**Figure 2C**) may also be contributed to decreased ZO-1. Likewise, luminescence assay revealed that long-term CSE exposure significantly diminished PKC alpha activity in Nuli-1 cells (**Figure 3E**). Moreover, the phosphorylation of ERK/MEK, as well as downstream p90RSK and CREB in LTC were decreased (**Figures 3F,G**), but there were no significant changes observed in phosphorylation of JNK or p38MAPK (data not shown), which indicates long-term CSE may regulate airway wound repair via inhibiting p-ERK pathway.

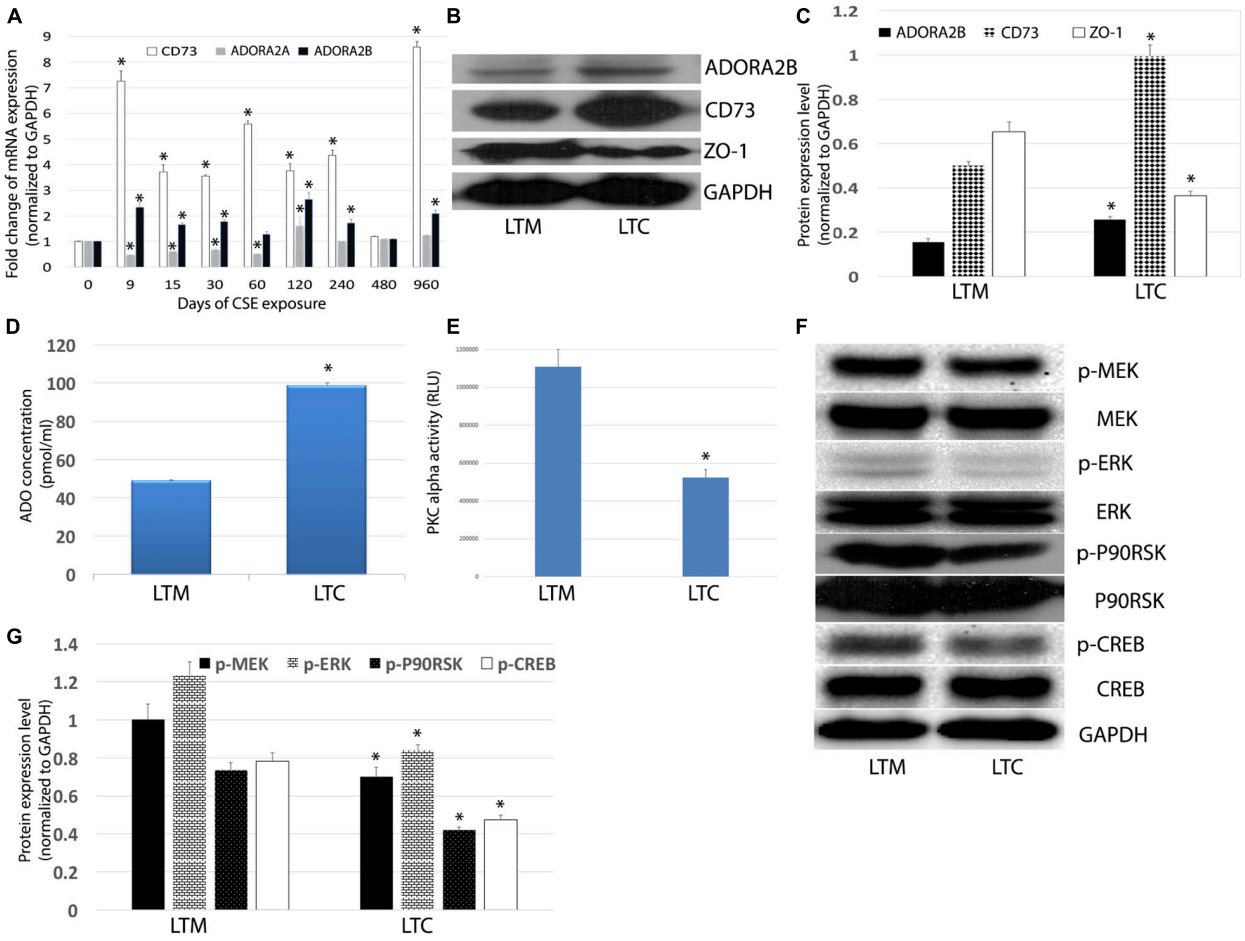

FIGURE 3 | (A) Effects of longterm CSE on transcript levels of ADORA2B. Expression of mRNA in airway epithelial cells treated with CSE for 3 years. Values are mean ± SE, * indicated $p < 0.05$. **(B)** CSE stimulates ADORA2B, CD73, and ZO-1 protein expression. **(C)** Normalized densitometry analysis of ADORA2B, CD73, and ZO-1. * indicated $p < 0.05$. **(D)** Extracellular ADO regulated by long-term CSE exposure. The extracellular ADO concentration was measured using HPLC. Data are mean ± SE, * indicated $p < 0.05$. **(E)** Activation of PKC alpha inhibited by long-term CSE exposure. Data are mean ± SE, * indicated $p < 0.05$. **(F)** Long-term CSE exposure inhibits phosphorylation of MEK, ERK, p90RSK, and CREB. **(G)** Normalized densitometry analysis of p-MEK, p-ERK, p-p90RSK, and p-CREB. Data are mean ± SE, * indicated $p < 0.05$.

Knockdown of ADORA2B and CD73 or Double Knockdown Ameliorates Long-Term CSE Impaired Airway Wound Repair

Our microarray analysis revealed increased gene expression of CD73 and ADORA2B. To determine whether limiting the generation of adenosine and adenosine signaling can affect long-term CSE-impaired airway wound repair, we knocked down CD73 and/or ADORA2B in LTC. We observed when ADORA2B was knocked down, there was a significant increase in CD73 expression (**Figures 4A–C**). Consistent with the changes of CD73 protein and mRNA expression level in each group, the enzyme activities of CD73 also changed at the same manner (**Figure 4D**). Proliferation (**Figure 4E**) and migration (**Figure 4F**) studies also revealed knocking down either CD73 or ADORA2B increased the rate of proliferation and migration while knocking down both CD73 and ADORA2B demonstrated significant improvement than individually knocking down each gene. Furthermore, when we double knocked-down CD73 and ADORA2B, this group showed improvement of barrier function; however, there were no significant changes observed in either shCD73 or shADORA2B cells (**Figure 4G**). Consequently, our data suggest knocking down in combination CD73 and ADORA2B as potential therapeutic strategy to ameliorate long-term CSE impaired airway wound repair.

Targeting CD73 and ADORA2B Improves Chronic CSE Impaired Wound Repair via Activating p-ERK Signaling Pathway

Unlike the acute CSE exposure, long-term CSE exposure inhibited PKC alpha activity in airway epithelial cells and subsequently decreased phosphorylation of ERK. Both shADORA2B and/or shADORA2B + shCD73 combined groups significantly increased PKC alpha activity; however,

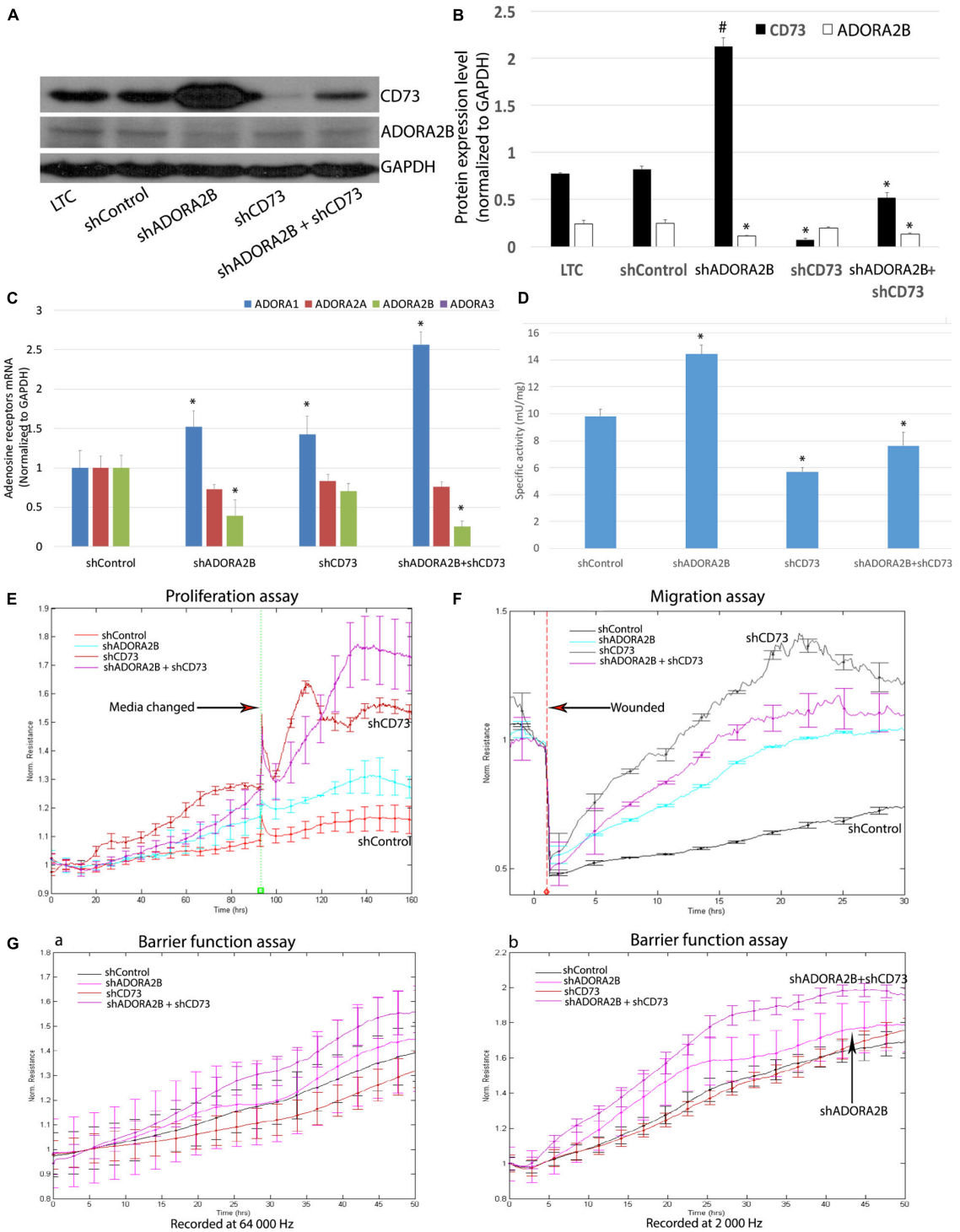

FIGURE 4 | (A) Efficient ADORA2B and/or CD73 knock down in LTC. **(B)** Normalized densitometry analysis of ADORA2B and CD73. Values are mean ± SE, * indicated $p < 0.05$, # indicated that CD73 protein expression level significantly increased, $p < 0.05$. **(C)** Transcript levels of ADORA1, ADORA2A, ADORA2B, and ADORA3 in Nuli-1 cells. * indicates significance value $P < 0.05$ compared with shControl group. The transcript level of ADORA3 was not detected in Nuli-1 cells. **(D)** CD73 enzyme activity in Nuli-1 cells. * indicates significance value $P < 0.05$ compared with shControl group. **(E)** Targeting both ADORA2B and CD73 ameliorates long-term CSE impaired cell proliferation. Media were changed after 96 h. Signals were recorded at 4,000 Hz. Each condition was conducted in triplicate. Statistically differences observed after 80 h ($P < 0.05$). **(F)** Targeting both ADORA2B and CD73 ameliorates long-term CSE impaired cell migration. The resistances were measured at 4,000 Hz. Each condition was conducted in triplicate. Statistically differences observed after 6 h ($P < 0.05$). **(G)** Targeting both ADORA2B and CD73 ameliorates long-term CSE impaired cell barrier function. a. Resistances were recorded at 64,000 Hz; b. Resistances were recorded at 2,000 Hz. The resistances were measured simultaneously. Each condition was conducted in triplicate. Statistically differences observed after 27 h ($P < 0.05$).

there were no significant changes observed in shCD73 group alone (**Figure 5A**). Furthermore, knocking down CD73 and/or ADORA2B significantly increased phosphorylation of MEK and ERK, as well as downstream p90RSK and CREB (**Figures 5B,C**). Collectively, our data suggest long-term CSE activation of CD73 and ADORA2B affect down-stream events that are critical to airway wound repair.

Double Knockout of ADORA2B and CD73 Impairs Chronic CS Induced Lung Injury in Mice

We studied the effects of CS as a model of airway damage to elucidate the mechanisms of co-inhibition of CD73 and ADORA2B improves wound repair in mice exposed to CS for 3 months. WT mice treated with CS increased the total number of BAL cells compared to WT control animals; however, there is a significant decrease in the knockout CD73 and/or ADORA2B mice compared to the CS-treated WT mice (**Figure 6A**). The expression level of pro-inflammatory cytokine interleukin-6 (IL-6) also significantly increased in CS group, and remarkably decreased in CD73/ADORA2B DKO mice while compared with control mice (**Figure 6B**). Consistent with our *in vitro* results, 3 months CS exposure decreased the activity of PKCα in WT mice. Interestingly, CD73/ADORA2B DKO mice have the same activity of PKCα with WT mice in normal circumstance, while significantly increases PKCα activity when treated with CS for 3 months (**Figure 6C**). The qPCR results also supported that expression levels of ADORA2B and CD73 significantly increased in the CS group when comparing to Air group (**Figure 6D**). Lung pathology revealed that epithelial hyperplasia in bronchioles appeared to be present in WT mice after exposure to CS 3 months. The major adverse change was the presence of fibrosis-like lesions consisting of connective tissue components, extracellular matrix and collagen. CD73 or ADORA2B knockout mice have showed some improvement, and CD73/ADORA2B DKO mice showed remarkable improvement as compared to the CS-treated WT mice (**Figure 6E**). Picro-Sirius Red staining of lung sections to detect collagen deposition (red color in **Figure 6F**) showed more abundant collagen accumulated at the sites surrounding bronchioles in CS-treated WT mice while comparing to control group, the CD73/ADORA2B DKO mice significantly reduced the collagen deposition than ADORA2B KO or CD73 KO group (**Figure 6G**). Together, these results demonstrate that double knockout CD73 and ADORA2B remarkably improved CS-induced lung injury by activating PKCα, reducing the inflammatory cell number in BALF, inhibiting the fibrosis-like lesions and decreasing collagen deposition surrounding bronchioles.

DISCUSSION

The epithelium provides the airway a barrier against inhaled environment toxins and airborne pathogens and can initiate a variety of responses when injured, such as rapidly supporting repair processes. Extracellular accumulation of ADO in response to tissue damage is an important indicator for control of wound repair. ADO has been known to have both anti-inflammatory (tissue-protective) and pro-inflammatory (tissue-destructive) properties (Hasko and Cronstein, 2013). The nature of ADO's action depends on the magnitude of changes in extracellular concentrations and the expression levels of each AR subtype. We previously reported that ADO promotes wound repair through ADORA2A signaling pathway in acute injury in bronchial epithelial cells (Allen-Gipson et al., 2006, 2007). Recently, we demonstrated that CS exposure increases ADORA2B expression level in mice (Tian et al., 2017). This study was designed to understand the mechanisms of ADO shifting from its tissue-protective to tissue-destructive features in long-term CS exposure. We found that long-term CS exposure upregulates the transcriptional and protein expression of CD73 and ADORA2B, increases ADO production and decreases PKC alpha activity. This was a marked contrast to our earlier findings where PKC alpha was increased in our 6 weeks *in vivo* model (Tian et al., 2017). Furthermore, our findings revealed long-term CSE inhibits the ERK signaling pathway resulting in the dysregulation of airway wound repair (**Figure 7**).

Airway epithelial cells act differently in different stages of CS exposure. Rubio et al. (2017) reported that CS exposure increases proliferation and migration in epithelial cells28, and other groups demonstrated progressive morphological changes and epithelial-to-mesenchymal (EMT)-like phenotype in normal airway epithelial cell after CS treatment (Wang et al., 2015; Vaz et al., 2017). We also observed that CSE exposure increases proliferation and migration, and EMT-like phenotype in the early stage (data not shown); however, long-term CSE exposure significantly impaired cell proliferation, migration and tight junction integrity after 3 years exposure, consistent with Das's observation that CSE exposure disrupted cell structure and tubulin-microtubule function in lung epithelium cells (Das et al., 2009). Our microarray analysis indicated that long-term CSE exposure upregulated matrix metalloproteinase-1 (MMP1) expression and downregulated Col5A1, Col5A2, and Col6A1 gene expression. These findings suggest CSE plays a tissue destructive role in long-term exposure while it promotes cell activity in the early stage of wound repair.

Mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated protein kinase (ERK), stress-activated protein kinases (SAPK) p38 and SAPK c-jun N-terminus kinase (JNK), are well known to play roles in barrier function, proliferation and migration (Olson et al., 2009; Crosby et al., 2011; Mihai et al., 2012); ADORA2B can affect proliferation and migration through all three pathways (Kuno et al., 2008; Darashchonak et al., 2014; Merighi et al., 2017); however, there is still no research that focuses on how ADORA2Bs regulate the MAPK pathway in airway epithelial cells in wound repair. While several studies have indicated that ADORA2B plays a tissue-protective role in acute lung injury, it may become tissue-destructive in chronic lung injury (Karmouty-Quintana et al., 2012; Hoegl et al., 2015). Sun et al. (2006) reported that chronic lung disease in ADA-deficient mice is partially mediated by ADORA2B10. Other researchers also demonstrated ADORA2B's highly distinct roles in acute and chronic stages of bleomycin-induced lung injury (Zhou et al., 2011). However, the

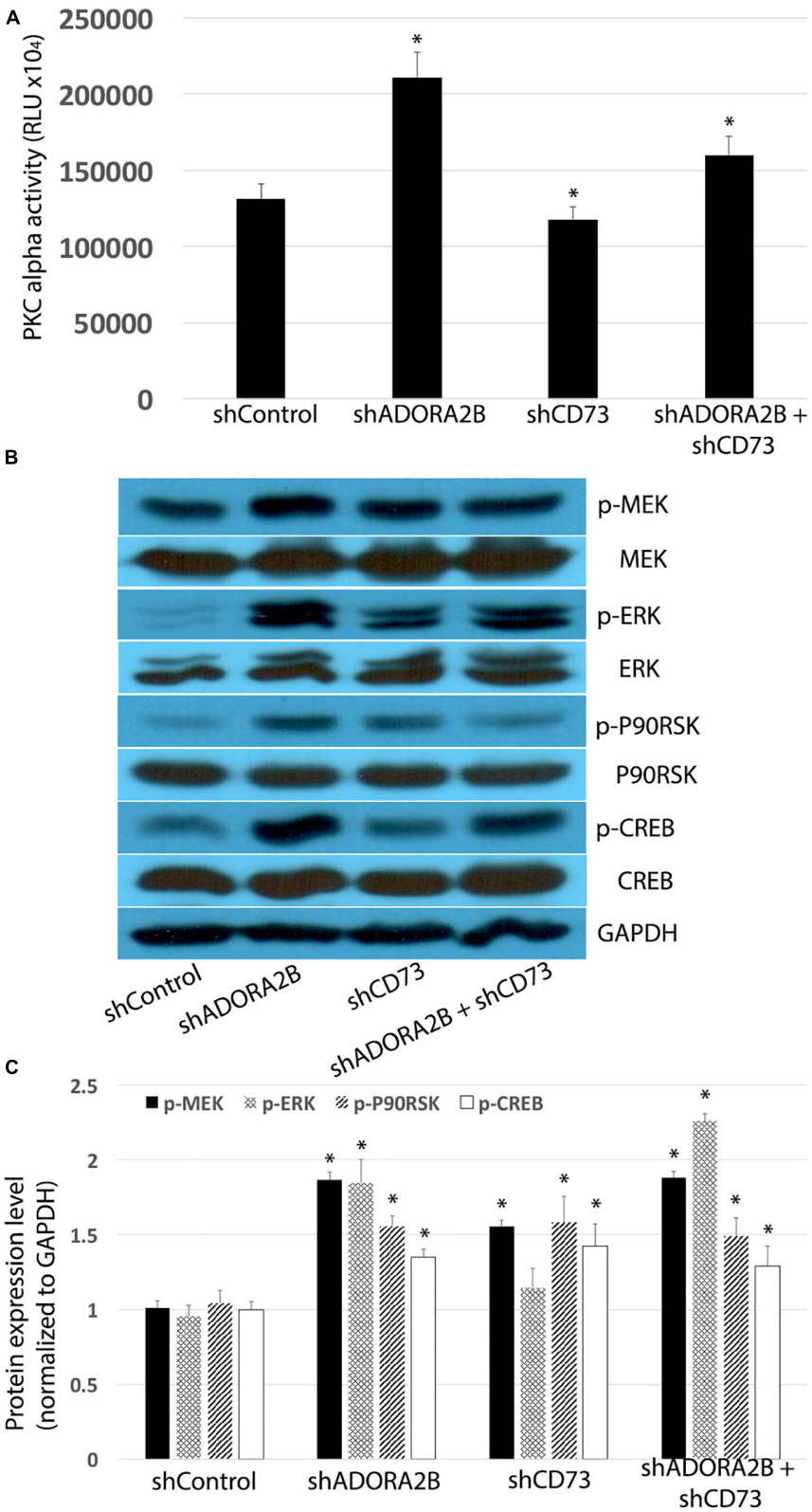

FIGURE 5 | (A) Knocking down ADORA2B alone or both ADORA2B and CD73 activates PKC alpha in LTC. Data are mean ± SE, * indicated $p < 0.05$. **(B)** Knocking down ADORA2B and CD73 activates MEK/ERK/p90RSK/CREB signaling pathway. **(C)** Normalized densitometry analysis of p-MEK, p-ERK, p-p90RSK, and p-CREB. Data are mean ± SE, * indicated $p < 0.05$.

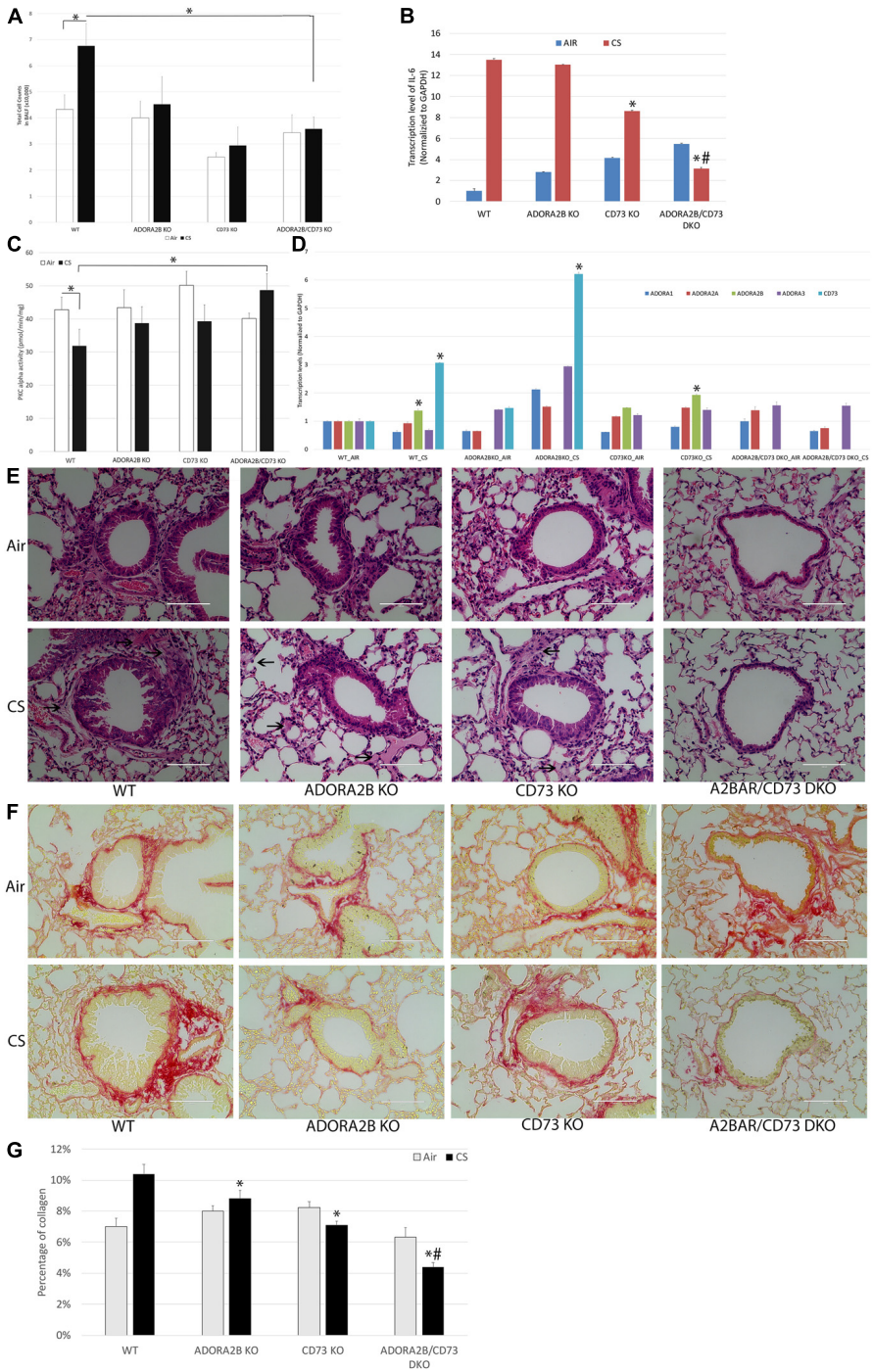

FIGURE 6 | Double knockout CD73 and ADORA2B improves CS-induced lung injury. **(A)** CD73/ADORA2B DKO decreases CS-induced inflammatory cells in BALF. Data are mean ± SE, * indicated $p < 0.05$. $n = 6$. **(B)** Transcript levels of IL-6 in mice. * indicates significance value $P < 0.05$ compared with WT mice. # indicates significance value $P < 0.05$ compared with ADORA2B KO and CD73 KO mice. **(C)** CD73/ADORA2B DKO activates chronic CS treatment inhibited PKC alpha. Data are mean ± SE, * indicated $p < 0.05$. $n = 6$. **(D)** Transcript levels of ADORA1, ADORA2A, ADORA2B, ADORA3, and CD73 in mice. * indicates significance value $P < 0.05$ compared with their AIR control group. **(E)** Photomicrographs of representative histopathological profiles. Sections of mouse lung tissue were paraffin-embedded and stained with hematoxylin and eosin in each cohort. Arrows show fibrosis-like changes comprised of connective tissue and extracellular matrix present in samples. Scale bar, 100 μm. **(F)** Representative micrograph of lung sections from different groups of mice. Sections were stained with Picro-Sirius Red Solution (collagen was highlighted as red color, muscle fibers and cytoplasm were highlighted as yellow). Scale bar, 100 μm. **(G)** Quantitative analysis of lung collagen contents by ImageJ were expressed as Mean ± SD. * indicated $p < 0.05$ compared with WT-CS group. # indicated $p < 0.05$ compared with ADORA2B KO and CD73 KO mice. More abundant collagen is accumulated in CS-treated WT mice as compared to WT control mice, and double knockout ADORA2B and CD73 dramatically reduced collagen accumulation.

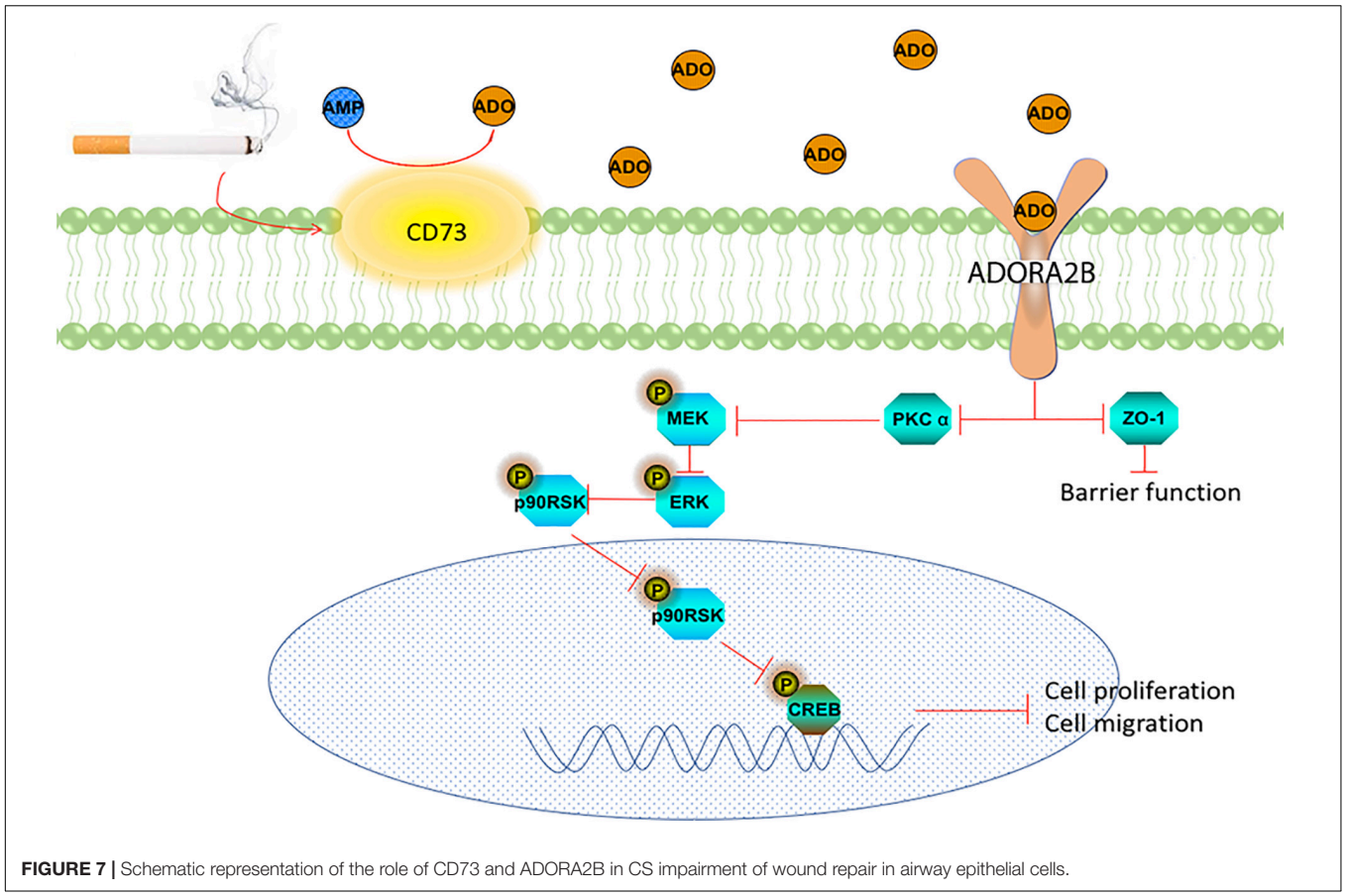

FIGURE 7 | Schematic representation of the role of CD73 and ADORA2B in CS impairment of wound repair in airway epithelial cells.

exact mechanisms involved remain unclear. PKC alpha is well known to promote the phosphorylation of ERK signal (Ueda et al., 1996; Clark et al., 2004; Giacomelli et al., 2018). It is well documented that CS activates PKC alpha, including our previous study (Simet et al., 2010; Tian et al., 2017). However, we observed a decrease in the PKC alpha activity after 3 years CSE treatment in this study. The effects of CS on signaling may depend on the length of exposure of the cells and the concentration of nicotine or other constituents in the CS (Mehta et al., 2008). PKC alpha has been reported to promote cell proliferation and migration (Wu et al., 2008; Gao et al., 2009). In our long-term CSE exposure cell model LTC, cell proliferation and migration have been significantly decreased, which is consistent with the decreasing of PKC alpha activity.

We have also investigated whether targeting ADORA2B or upstream CD73 could improve long-term CSE impaired airway epithelial cell wound repair. Our data suggest that knocking down ADORA2B and/or CD73 could significantly ameliorate airway epithelial cell wound repair via activating PKC alpha/MEK/ERK/p90RSK/CREB pathway. Interestingly, rather than displaying redundancy, knocking down both ADORA2B and CD73 revealed to be more potent than knocking down either alone. Increasing evidence indicates that ADORA2B is a potential therapeutic target in not only lung injury (Huerter et al., 2016; Philip et al., 2017) but also in other diseases (Molck et al., 2016; Alencar et al., 2017; Ballesteros-Yanez et al., 2017; Borea et al., 2018). In this study, we demonstrate that knocking down ADORA2B increased CD73 expression in LTC, which indicates that the lack of ADORA2B creates a negative feedback loop via CD73 upregulation, in its attempt to increase ADO level and subsequently to activate ADORA2B. Similarly, Young et al. (2016) also observed that CD73 expression level was increased in the tumor core of ADORA2A deficient mice54. As a proof of concept for the synergistic interaction of the combination with CD73 and ADORA2B inhibition, the increasing of CD73 caused by ADORA2B inhibition was also decreased when co-inhibiting both CD73 and ADORA2B. Especially, the CD73/ADORA2B DKO mice demonstrated dramatically decreasing in the production of inflammatory mediator IL-6 and collagen deposition surrounding bronchioles than knock out CD73 or ADORA2B alone. This provides an important insight for understanding AR(s) drug targeting, especially when utilizing specific AR antagonist(s) which may promote a high level of CD73-generated ADO. It has been well documented that prolonged increases in ADO levels can promote pulmonary inflammation airway remodeling, and causes pulmonary tissue destruction (Zhou et al., 2009). *In vivo* experiments also confirmed that double knockout CD73 and ADORA2B significantly improves CS-induced lung injury. Collectively, co-targeting AR(s) and upstream CD73 has more therapeutic potential than targeting either individually.

In conclusion, **Figure 7** summarizes our proposed role of ADORA2B on long-term CSE exposure induced wound repair. During long-term exposure, CSE induced overexpression of CD73 increasing ADO production, which subsequently activates ADORA2B. The activation of ADORA2B stimulates PKC alpha activation and inhibits MEK/ERK/p90RSK/CREB signaling pathway, which eventually inhibits airway wound repair. Moreover, co-targeting ADORA2B and CD73 exhibited to be more potent than targeting either individually in improving CS related airway wound repair.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the USF Institutional Animal Care and Use Committees.

AUTHOR CONTRIBUTIONS

ZT, JD, BD, and XG performed experiments presented in the manuscript. FC analyzed microarray data. ZT and DA-G designed experiments presented in the manuscript. ZT, JD, QL, YZ, and DA-G prepared and approved manuscript for submission. All authors contributed to the article and approved the submitted version.

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Smoking Is Correlated With the Prognosis of Coronavirus Disease 2019 (COVID-19) Patients: An Observational Study

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Background: Cigarette smoking has been proven to be a risk factor in the development of many diseases. However, it remains controversial with respect to the relationship of smoking with COVID-19. The purpose of this study was to explore the role of smoking in COVID-19.

Methods: A total of 622 patients with COVID-19 in China were enrolled in the study. Corresponding clinical and laboratory data were collected and analyzed. Meanwhile, Kaplan-Meier curve and Cox regression analysis were employed to analyze the association of smoking with survival in patients with COVID-19.

Results: Smoking was statistically significant comparing non-survivors and survivors of patients with COVID-19 ($P = 0.007$). Males had higher proportion of smoking than females (91.9% vs. 8.1%, $P < 0.001$). Compared with the non-smoker, there was significant statistical difference in the incidence of cerebrovascular disease in smoking patients with COVID-19 (9.7% vs. 3.4%, $P = 0.017$). White blood cell count (6.3 vs. 5.4; $P = 0.037$), hemoglobin level (139.0 vs. 127.0; $P < 0.001$), and creatinine level (77.3 vs. 61.0; $P < 0.001$) were significantly increased in COVID-19 patients who smoked. Moreover, smoking patients showed a worse survival compared with non-smoking patients (Log Rank $P = 0.045$). After adjustment for age, gender and underlying diseases, patients with smoking still had higher risk of mortality than that of non-smoking patients (hazard ratio[HR] 1.897, 95% confidence interval [CI]1.058–3.402, $P = 0.032$).

Conclusion: Smoking was thought to be a risk factor in predicting the prognosis of COVID-19 and smoking patients might have a higher risk of mortality than that of the non-smoking patients.

Keywords: COVID-19, SARS-CoV-2, cigarette, smoking, ACE2, inflammation, prognosis

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) was firstly reported in Wuhan, China in late 2019 and has already become an evolution of pandemic (Cucinotta and Vanelli, 2020). The World Health Organization has declared it as a "Public Health Emergency of International Concern." SARS-CoV-2 belongs to the same

family of RNA virus as SARS and Middle East respiratory syndrome (Zhou et al., 2020) and has a higher risk of human-to-human transmission (Chan et al., 2020). Until now, more than sixty-one million cases of COVID-19 as well as 1440000 deaths have been identified across the world (World Health Organization, 2020).

Smoking history is defined as a history of continuous or cumulative smoking at least 6 months during the whole life (World Health Organisation, 1997), and cigarette smoking is quite prevalent all over the world. It kills approximately 50% of users and 8 million people are died from it every year, 1.2 million of which are exposed to the second-hand smoking (Lippi et al., 2020). The mechanisms of smoking in inducing the occurrence of respiratory diseases are altering airway architecture, enhancing mucosal permeability, disrupting respiratory epithelium and inhibiting ciliary clearance (Arcavi and Benowitz, 2004). It was reported that smoking played an important role in chronic obstructive pulmonary disease (COPD) in developed countries which was the fourth leading cause of death (Agarwal et al., 2020), and smokers were also more likely to have increased incidence of cancer, influenza, tuberculosis and pneumonia relative to non-smokers (Warren et al., 2014; Brake et al., 2020). However, the relationship of smoking and COVID-19 remains controversial. The purpose of this study was to explore the role of smoking in COVID-19.

TABLE 1 | Demographics and baseline characteristics of survivor and non-survivor of COVID-19 patients.

	No. (%) Total (*n* = 622)	Survivor (*n* = 547)	Non-survivor (*n* = 75)	*P* value
Age,%				**<0.001**
≥65 year	212 (34.1)	161 (29.4)	51 (68.0)	
45 ≤ age < 65	231 (37.1)	207 (37.8)	24 (32.0)	
<45 year	179 (28.8)	179 (32.8)	0 (0.0)	
Gender,%				**0.011**
Male	318 (51.1)	269 (49.2)	49 (65.3)	
Female	304 (48.9)	278 (50.8)	26 (34.7)	
Symptoms				
Fever,%	489 (78.6)	426 (77.9)	63 (84.0)	0.226
Cough,%	475 (76.4)	416 (76.1)	59 (78.7)	0.617
Myalgia,%	99 (15.9)	83 (15.2)	16 (21.3)	0.158
Fatigue,%	241 (38.7)	204 (37.3)	37 (49.3)	**0.044**
Headache,%	82 (13.2)	70 (12.8)	12 (16.0)	0.447
Diarrhea,%	149 (24.0)	130 (23.8)	19 (25.3)	0.766
Abdominal pain,%	32 (5.1)	26 (4.8)	6 (8.0)	0.224
Shortness of breath,%	196 (31.5)	166 (30.3)	30 (40.0)	0.077
Chest CT with ground glass change,%	370 (59.5)	351 (64.2)	19 (25.3)	**<0.001**
Comorbidities				
Hypertension,%	176 (28.3)	147 (26.9)	29 (38.7)	**0.034**
Cardiovascular disease,%	51 (8.2)	36 (6.6)	15 (2.0)	**<0.001**
Diabetes,%	104 (16.7)	86 (15.7)	18 (24.0)	0.072
COPD,%	6 (1.0)	4 (0.7)	2 (2.7)	0.108
Chronic bronchitis,%	29 (4.7)	26 (4.8)	3 (4.0)	0.297
Cerebrovascular disease,%	25 (4.0)	20 (3.7)	5 (6.7)	0.061
Cancer,%	21 (3.4)	14 (2.6)	7 (9.3)	**0.002**
Smoking,%	62 (12.1)	48 (8.7)	14 (18.7)	**0.007**

COVID-19, Coronavirus disease 19; CT, computed tomography; COPD, chronic obstructive pulmonary disease. P values indicate differences between survivor and non-survivor of COVID-19 patients. P < 0.05 was considered statistically significant. Statistically significant values are indicated in Bold.

MATERIALS AND METHODS

Study Design and Participants

This case series was subjected to the approval by the institutional ethics board of the Second Xiangya Hospital of Central South University (No. 2020001). The objects of study were laboratory-confirmed adult COVID-19 patients using real-time polymerase chain reaction who were admitted to the Public Health Treatment Center of Changsha and Tongji Medical College of Huazhong University of Science and Technology, China, by March 26th 2020. The patients older than 18 years were included in the study and were divided into two groups according to the survival and smoking statuses, including survivors and non-survivors, as well as the smokers and non-smokers.

Data Collection

Two members of our team carefully collected and reviewed the medical records of enrolled patients individually. The detailed information of those patients were recorded, including the demographic data, underlying diseases, symptoms throughout the course of the disease, blood test parameters, and results of chest computed tomography (CT) scans. The date of disease onset was defined as the day when the symptoms were noticed.

Definition and Study Endpoints

According to the criteria of severe cases of COVID-19 (National health commission and National administration of traditional Chinese medicine, 2020), the following criteria was used to determine severe COVID-19: (1) respiratory rate ≥ 30/min; (2) oxygen saturation ≤ 93%; (3) arterial partial pressure of oxygen(PaO2)/fraction of inspiration oxygen(FiO2) ≤ 300 mmHg; (4) progression of lung lesions progressed >50% within 24–48 h; (5) implementation of mechanical ventilation; (6) shock; and (7) intensive care unit admission. The primary endpoint was the mortality of COVID-19 patients.

Statistical Analysis

All continuous variables were depicted using Median with interquartile range, and Mann-Whitney test was used to analyze all continuous variables because of their non-normal distributions. The χ^2 test or Fisher's exact test was used to analyze the categorical variables. The Kaplan-Meier (KM) curve with Log Rank tests were applied to estimate the survival of

TABLE 2 | Comparison of laboratory parameters between the survivor and non-survivor of COVID-19 patients.

	Normal range	Survivor	Non-survivor	*P* value
White blood cells, $\times 10^9$/L	3.5–9.5	5.2 (3.9–7.0)	10.5 (6.1–13.4)	**<0.001**
Lymphocytes, $\times 10^9$/L	0.8–4.0	1.1 (0.8–1.6)	0.6 (0.4–0.7)	**<0.001**
Neutrophils, $\times 10^9$/L	1.8–6.3	3.3 (2.4–4.9)	8.8 (5.3—12.6)	**<0.001**
Hemoglobin, g/L	115–150	127.0 (116.5–139.0)	129.0 (114.0–144.0)	0.900
Platelets, $\times 10^9$/L	125–350	183.5 (142.3–247.0)	161.0 (97.0–224.0)	**0.001**
ALT, U/L	7–40	20.0 (14.1–30.6)	29.0 (19.0–48.4)	**<0.001**
AST, U/L	13–35	24.9 (19.0–33.0)	43.0 (28.0–67.0)	**<0.001**
Total bilirubin, μmol/L	3.4–17.1	10.0 (7.5–14.9)	13.8 (9.9–19.8)	**<0.001**
Albumin, mg/L	40–55	37.3 (33.9–40.6)	30.8 (27.7–34.2)	**<0.001**
Creatinine, μmol/L	44–133	60.0 (49.0–76.0)	88.0 (66.8–137.0)	**<0.001**
CK, U/L	40–200	69.0 (45.1–120.5)	135.5 (67.8–461.8)	**<0.001**
CK-MB, U/L	0–24	8.6 (2.1–13.1)	5.2 (1.9–9.9)	0.320
PT, sec	10–14	13.0 (11.7–14.0)	11.6 (10.8–11.9)	**<0.001**
APTT, sec	28–45	35.3 (31.4–39.4)	15.3 (14.1–18.1)	**<0.001**
D-dimer, μg/L	0–0.55	0.4 (0.2–0.9)	2.6 (1.3–5.7)	**<0.001**
ESR, mm/h	0–20	39.0 (20.0–65.3)	46.0 (17.0–63.0)	0.925
CRP, mg/L	0–8	13.7 (2.9–40.1)	88.0 (58.7–171.5)	**<0.001**

COVID-19, Coronavirus disease 19; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CK-MB, creatine kinase-MB; PT, prothrombin time; APTT, activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. P values indicate differences between survivor and non-survivor of COVID-19 patients. P < 0.05 was considered statistically significant. Statistically significant values are indicated in Bold.

smoking patients. Finally, the risk of mortality was estimated using Cox regression model with adjustment for the gender, age, and underlying diseases. All analyses were carried out by using IBM SPSS version 26 software.

RESULTS

Baseline characteristics of the included patients grouped according to the survival status(survivors and non-survivors) were summarized in **Table 1**. A total of 622 adult patients with laboratory-confirmed COVID-19 were included in our study, including 547 survivors and 75 non-survivors. Patients of non-survivors were further classified into three groups based on ages, i.e., ≥65 years (68%), 45 years ≤ age < 65 years (32%), <45 years (0%), respectively. Males who smoked cigarettes were found to have a higher rate of mortality than females (65.3% vs. 34.7%, $P = 0.011$). In addition, statistically significant difference were shown in the symptoms of fatigue, ground glass changes indicated by chest CT, comorbidities (hypertension, cardiovascular disease, and cancer), and smoking between non-survivors and survivors with COVID-19 ($P < 0.05$).

White blood cells (10.5 vs. 5.2; $P < 0.001$), neutrophils (8.8 vs. 3.3; $P < 0.001$), alanine aminotransferase (29.0 vs. 20.0; $P < 0.001$), aspartate aminotransferase (43.0 vs. 24.9; $P < 0.001$), total bilirubin (13.8 vs. 10.0; $P < 0.001$), creatinine (88.0 vs. 60.0; $P < 0.001$), creatine kinase (135.5 vs. 69.0; $P < 0.001$), D-Dimer (2.6 vs. 0.4; $P < 0.001$), C-reactive protein (88.0 vs. 13.7; $P < 0.001$) were significantly increased in COVID-19 patients who died; However, lymphocytes (0.6 vs. 1.1; $P < 0.001$), platelets (161.0 vs. 183.5; $P < 0.001$), albumin (30.8 vs. 37.3; $P < 0.001$), prothrombin time (11.6 vs. 13.0; $P < 0.001$), and activated partial thromboplastin time (15.3 vs. 35.3; $P < 0.001$) were significantly decreased in survivors than that of non-survivors (**Table 2**).

Baseline characteristics of the smoking and non-smoking patients were summarized in **Table 3**. There were 560 non-smokers and 62 smokers. The ratios of smokers who aged ≥ 65 years, 45 years ≤ age < 65 years and <45 years were 38.7%, 33.9%, 27.4%, respectively. The proportion of male smokers was higher than that of female smokers (91.9% vs. 8.1%, $P < 0.001$). Besides, there was statistical difference in the incidence of cerebrovascular disease between non-smoking and smoking patients with COVID-19 (9.7% vs. 3.4%, $P = 0.017$). However, the severity of the patients and smoking were not statistically related (40.8% vs. 59.2%, $P = 0.105$), to further confirm the relationship, we did logistic regression and found that smoking was not a risk factor for the severity of COVID-19 (**Supplementary Tables 1**–**4**).

White blood cells (6.3 vs. 5.4; $P = 0.037$), hemoglobin (139.0 vs. 127.0; $P < 0.001$), creatinine (77.3 vs. 61.0; $P < 0.001$), and activated partial thromboplastin time (37.4 vs. 35.4; $P < 0.001$) were significantly increased in COVID-19 patients who smoked, but erythrocyte sedimentation rate (28.0 vs. 40.0; $P = 0.016$) was significantly decreased than that in non-smokers with COVID-19 (**Table 4**).

Moreover, the association between smoking and survival were analyzed by KM curve and Cox regression analysis in non-survivors after admission (**Table 5** and **Figure 1**). Smoking patients showed a worse survival compared with non-smoking patients (Log Rank $P = 0.045$). After adjusting for age, gender and underlying diseases, patients with smoking still had higher risk of mortality than non-smoking patients (hazard ratio [HR] 1.897, 95% confidence interval [CI] 1.058–3.402, $P = 0.032$).

TABLE 3 | Demographics and baseline characteristics of smoking and non-smoking COVID-19 patients.

	No. (%) Total (*n* = 622)	Non-smokers (*n* = 560)	Smokers (*n* = 62)	*P* value
Age, years				0.209
≥65 year	212 (34.1)	187 (33.4)	25 (38.7)	
45 ≤ age < 65	231 (37.1)	211 (37.7)	20 (33.9)	
<45 year	179 (28.8)	162 (28.9)	17 (27.4)	
Gender				**<0.001**
Male	318 (51.1)	261 (46.6)	57 (91.9)	
Female	304 (48.9)	299 (53.4)	5 (8.1)	
Symptoms				
Fever,%	489 (78.6)	445 (79.5)	44 (71.0)	0.122
Cough,%	475 (76.4)	430 (76.8)	45 (72.6)	0.460
Myalgia,%	99 (15.9)	87 (15.5)	12 (19.4)	0.440
Fatigue,%	241 (38.7)	215 (38.4)	26 (41.9)	0.583
Headache,%	82 (13.2)	78 (13.9)	4 (6.5)	0.098
Diarrhea %	149 (24.0)	130 (23.2)	19 (30.6)	0.194
Abdominal pain %	32 (5.1)	28 (5.0)	4 (6.5)	0.627
Shortness of breath %	196 (31.5)	179 (32.0)	17 (27.4)	0.460
Chest CT with ground glass change %	370 (59.5)	339 (60.5)	31 (50.0)	0.105
Comorbidities				
Hypertension %	176 (28.3)	155 (27.6)	21 (33.9)	0.305
Cardiovascular disease %	51 (8.2)	43 (7.7)	8 (12.9)	0.155
Diabetes %	104 (16.7)	89 (15.8)	15 (24.2)	0.097
COPD %	6 (1.0)	6 (1.1)	0 (0.0)	0.413
Chronic bronchitis %	29 (4.7)	27 (4.8)	2 (3.2)	0.715
Cerebrovascular disease %	25 (4.0)	19 (3.4)	6 (9.7)	**0.017**
Cancer %	21 (3.4)	18 (3.2)	3 (4.8)	0.502
Severity %				0.105
Non-severe	254 (40.8)	235 (42.0)	19 (30.6)	
Severe	368 (59.2)	325 (58.0)	43 (69.4)	

COVID-19, Coronavirus disease 19; CT, computed tomography; COPD, chronic obstructive pulmonary disease. P values indicate differences between smoking and non-smoking COVID-19 patients. P < 0.05 was considered statistically significant. Statistically significant values are indicated in Bold.

DISCUSSION

So far, there is still no definitively effective vaccine for COVID-19. Besides, it is still controversial concerning the relationship between smoking and COVID-19. Some findings supported that smoking patients with COVID-19 had greater severity of illness (Ahmed et al., 2020; Brake et al., 2020; Kaur et al., 2020; van Zyl-Smit et al., 2020). However, others suggested that the risk of infection was lower among smokers for the reason of nicotine (Tindle et al., 2020). Farsalinos et al. pointed out that nicotine might have protective effect against acute inflammatory lung injury caused by cholinergic mediated COVID-19 (Panigrahi et al., 2020). Lippi and Henry even claimed that active smoking had no relationship with the severity of COVID-19 (Lippi and Henry, 2020). Subsequently, Silvano et al. argued that there were several mistakes in the study and concluded that smoking did play a role in the severity of COVID-19 (Gallus et al., 2020). In the present study, smoking was thought to have a statistically significant influence in the prognosis of COVID-19, and smoking patients had higher risk of mortality than non-smokers.

Robust evidences supported smoking to be a significant risk factor during the development of human diseases. Smoking is thought to play an important role in the progression of cancers and respiratory distress such as COPD and pulmonary fibrosis (Hikichi et al., 2019). Smoking seriously affects vascular system including fatal cardiovascular diseases and neurological diseases, abnormal brain development, ischemic stroke and Alzheimer's diseases (West, 2017). Smoking can lead to lung injury and structural changes thus develop minimal or no resistance to virus attack (Arcavi and Benowitz, 2004), increase the patients' susceptibility to viral and bacterial infections (Archie and Cucullo, 2020). Besides, the vulnerability to influenza infection increased to a five-hold enhancement in smokers when compared with non-smokers (van Zyl-Smit et al., 2020).

As for prevention of COVID-19, owing to the requirements of social isolation and stay-at-home, the stress on potentially fatal condition, possibility of unemployment and feeling of confinement could stimulate people's desire to smoke (van Zyl-Smit et al., 2020). Smoking implies repeated exposure among fingers, cigarettes shafts and lips, which will in turn increase the risk of COVID-19 transmission (Sherman, 1991; Sabino-Silva et al., 2020). Exhaled smoke, coughing or sneezing caused by tobacco smoking may produce aerosols containing SARS-CoV-2 which can survive for several hours to days in the surroundings and contaminating surfaces. It has also reported that secondhand smoke had the same damage caused by smoking (Moritsugu, 2007) which may suggest that passive smokers are equally possible to suffer from COVID-19 (Benjamin, 2011; Ma et al., 2020). Furthermore, compared with non-smoking, smoking, which can regulate both the immune and adaptive response, weakens the normal defective system of body (Qiu et al., 2017). It was a strange phenomenon that the proportion was extremely low among smokers of hospitalized COVID-19 patients constituting only 6.5% in China and 1.3% in America (Cai, 2020; Lange et al., 2020). Similarly, our investigation suggested that smokers comprised a proportion of 10% in all COVID-19 hospital admissions. Scholars explained that part of older smokers progressed too fast to be sent to hospital for treatment, and hence their death data were not captured (Appleby, 2020; Simons et al., 2020); Moreover, there was no detailed information of patients if they were exposed to second-hand smoke (Silva et al., 2020).

Coronaviruses have large type 1 transmembrane spike (S) glycoproteins, including two quite different functional domains S1 and S2. To be specific, S1 contains binding site for angiotensin-converting enzyme-2 (ACE2) (Li et al., 2005), while S2 promotes viral and host-cell membrane fusion which is necessary for cellular infiltration (Coutard et al., 2020). S proteins can be enzymatically modified and then fusion sites related to cellular adhesion are exposed (Coutard et al., 2020), which play important roles in virus attack and transmission

TABLE 4 | Comparison of laboratory parameters between smoking and non-smoking COVID-19 patients.

	Normal range	Non-smokers	Smokers	*P*-value
White blood cells, $\times 10^9$/L	3.5-9.5	5.4 (3.9-7.3)	6.3 (4.5-8.3)	**0.037**
Lymphocytes, $\times 10^9$/L	0.8-4.0	1.0 (0.7-1.5)	1.0 (0.6-1.7)	0.703
Neutrophils, $\times 10^9$/L	1.8-6.3	3.5 (2.4-5.5)	3.8 (2.8-6.7)	0.163
Hemoglobin, g/L	115-150	127.0 (116.0-138.0)	139.0 (124.5-149.3)	**<0.001**
Platelets, $\times 10^9$/L	125-350	181.0 (138.0-246.0)	183.0 (136.0-248.8)	0.616
ALT, U/L	7-40	20.7 (14.6-32.0)	21.9 (14.9-33.5)	0.738
AST, U/L	13-35	25.3 (19.5-36.0)	24.7 (19.0-37.3)	0.642
Total bilirubin, μmol/L	3.4-17.1	10.2 (7.7-15.3)	11.9 (8.0-16.4)	0.249
Albumin, mg/L	40-55	36.5 (32.9-40.2)	35.9 (31.5-41.5)	0.833
Creatinine, μmol/L	44-133	61.0 (49.0-77.0)	77.3 (61.8-92.3)	**<0.001**
CK, U/L	40-200	71.2 (46.3-132.0)	82.6 (46.5-191.4)	0.332
CK-MB, U/L	0-24	8.2 (2.2-13.0)	6.4 (1.0-12.9)	0.188
PT, sec	10-14	13.2 (11.9-14.3)	13.8 (11.9-14.5)	0.083
APTT, sec	28-45	35.4 (31.5-40.3)	37.4 (33.4-44.2)	**0.010**
D-dimer, μg/L	0-0.55	0.5 (0.2-1.3)	0.4 (0.2-1.3)	0.769
ESR, mm/h	0-20	40.0 (20.0-66.0)	28.0 (9.5-56.0)	**0.016**
CRP, mg/L	0-8	20.0 (4.2-57.9)	7.8 (2.6-58.2)	0.052

COVID-19, Coronavirus disease 19;ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CK-MB, creatine kinase-MB; PT, prothrombin time; APTT, activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. P values indicate differences between smoking and non-smoking COVID-19 patients. P < 0.05 was considered statistically significant. Statistically significant values are indicated in Bold.

(Archie and Cucullo, 2020). ACE2 receptors have been verified to be the site of access for the SARS-CoV2 virus entry. A significantly higher affinity was observed between modified S protein of SARS-CoV-2 and ACE2, almost 10 to 20 folds compared with S protein of the previous SARS-CoV (Di Filippo et al., 2020; Wrapp et al., 2020), which might explain the high susceptibility of human-to-human transmission in the spread of COVID-19. It was reported that ACE2 was upregulated in the resected lung tissue of smokers, but no ACE2 was found in non-smokers, and smokers were more likely to have a significant elevation of ACE2 in respiratory epithelial cells (Brake et al., 2020; Leung et al., 2020). In addition, cumulative cigarette smoke was associated strongly with human ACE2 expression, and subjects with the longest pack-years of smoking had the highest ACE2 levels (Smith et al., 2020) indicating that the older were more vulnerable and easier to have poor outcomes. Besides, ACE2 also was thought to have higher expression in germ cells of males when compared with females (Sama et al., 2020), and smoking males accounted for nearly 50% in rural areas of China while approximately 44.8% overall (Zhi et al., 2019), suggesting possibly that males were more prone than females to be at the risk of COVID-19, which were consistent with our study.

Lung macrophages contribute importantly to the development and resolution of human lung inflammation. Macrophages can exert anti-inflammatory effects, downregulate acquired immune responses and suppress inflammation response under normal physiological conditions. Nevertheless, when encountering the attack of pathogenic fungi, bacteria or viruses, macrophages differentiate into pro-inflammatory phenotype, release a large number of inflammatory cytokines and recruit other types of inflammatory cells to the sites of inflammation simultaneously (Hussell and Bell, 2014; Joshi et al., 2018; Hu and Christman, 2019). It was reported that alveolar cavities were filled with amounts of macrophages, which could express ACE2 receptors and recognize SARS-CoV-2 viruses, resulting in cytokine storm (Kloc et al., 2020). Significantly, cytokine storm signifies the failure of restoring homeostasis due to inflammatory response and has become a well-established phenomenon in the viral and bacterial infections. It may lead to acute lung injury and further develop into acute respiratory distress syndrome (Tisoncik et al., 2012).

The aforementioned interpretation supports the importance of ACE2in the process. While, serine protease TMPRSS2 also acts as a crucial role for S protein priming to promote the entry of SARS-CoV2 (Hoffmann et al., 2020b). It was observed that only a small number of ACE2 + cells express TMPRSS2 by single cell RNA sequencing analyses, demonstrating that there were

TABLE 5 | Univariate and multivariate Cox regression analysis for the mortality of COVID-19 patients.

	Univariate	Multivariate		
Variables	**Log Rank *P* value**	**HR**	**95% CI**	***P* value**
Age	<0.001	0.344	0.227-0.523	<0.001
Gender	0.025	/	/	0.140
Cardiovascular disease	<0.001	/	/	0.073
COPD	0.021	6.796	1.596-28.947	0.010
Cerebrovascular disease	0.048	/	/	0.277
Smoking	0.045	1.897	1.058-3.402	0.032

COVID-19, Coronavirus disease 19; COPD, chronic obstructive pulmonary disease; HR, hazard ratios; P < 0.05 was considered statistically significant.

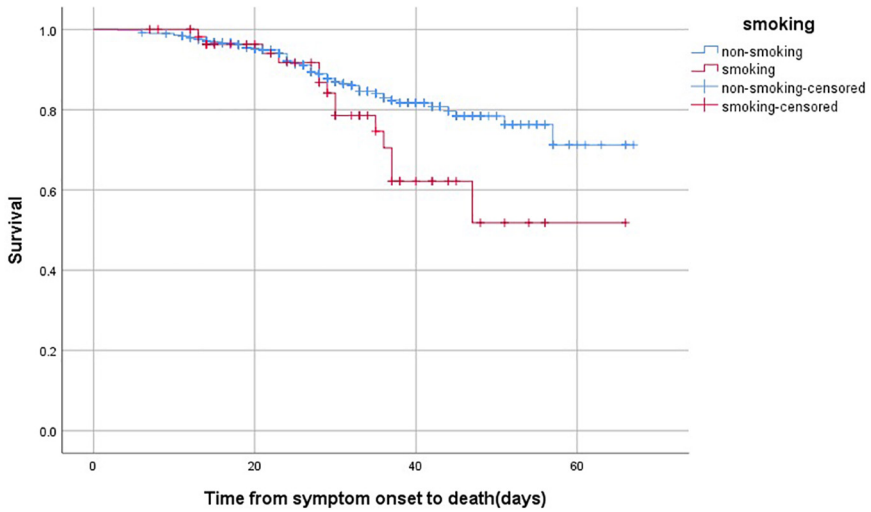

FIGURE 1 | The time-dependent risk of death in COVID-19 patients who smoked using Kaplan-Meier curve. Smoking patients showed a worse survival compared with those with non-smoking (Log Rank $P = 0.045$).

other proteases exerted the same effects (Sungnak et al., 2020). Interesting, through the stimulation of smoking, the level of Cathepsin B was increased and the activity of furin (cleave the spike protein of SARS-CoV-2 on the S1/S2 site) was preserved, which raised the likelihood of COVID-19 infection (Hoffmann et al., 2020a; Kaur et al., 2020).

Besides, in the lung autopsy of COVID-19 patients, there were an infiltration of neutrophil in pulmonary capillaries, along with fibrin deposition and neutrophil extravasation into the alveolar space, which indicated that the formation of Neutrophil Extracellular Traps might lead to organ damage and mortality of COVID-19 patients (Barnes et al., 2020). Evidence suggested that smoking might exert impact on the formation of Neutrophil Extracellular Traps, neutrophil trafficking and mediating both humoral and cell immune responses (Kaur et al., 2020), which was tightly associated with the acute respiratory distress syndrome development or even death (Barnes et al., 2020).

CONCLUSION

In conclusion, smoking might play a significant role in the prognosis of COVID-19, and smoking patients might have a higher risk of mortality than the non-smokers.

This study has some limitations. Firstly, there was no information to determine whether non-smoking patients were in the environment of secondhand smoke before the onset of COVID-19, which might show a presence of observation bias. Secondly, for the reason of the medical records which had no specific distinction if patients were former, active or never smokers, so we couldn't elaborate on the role of smoking status more in detail. Finally, the small total sample size and the number of patients who smoked which might also affect the current results. If it's possible, we would enlarge the sample size in the future to make the results more convincing.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the manuscript/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Second Xiangya Hospital of Central South University (No.2020001). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YZ and SW contributed to the study design, implementation, and critical revision. FP contributed to methodology, software, and writing original draft preparation. SL and QZ collected and interpreted the data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.634842/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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COVID-19 and Smoking: What Evidence Needs Our Attention?

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The current COVID-19 pandemic has caused severe morbidity and mortality worldwide. Although relevant studies show that the smoking rate of COVID-19 patients is relatively low, the current smoking status of people with COVID-19 cannot be accurately measured for reasons. Thus, it is difficult to assess the relationship between smoking and COVID-19. Smoking can increase the risk of severe COVID-19 symptoms and aggravate the condition of patients with COVID-19. Nicotine upregulates the expression of ACE2, which can also increase susceptibility to COVID-19, aggravatiing the disease. Although nicotine has certain anti-inflammatory effects, there is no evidence that it is related to COVID-19 treatment; therefore, smoking cannot be considered a preventative measure. Furthermore, smokers gathering and sharing tobacco may promote the spread of viruses. Despite the COVID-19 epidemic, the findings suggested that COVID-19 has not encouraged smokers to quit. Additionally, there is evidence that isolation at home has contributed to increased smoking behavior and increased quantities. Therefore, it is recommended that governments increase smoking cessation messaging as part of public health measures to contain the COVID-19 pandemic. This review analyzes the existing research on smoking's impact on COVID-19 so that governments and medical institutions can develop evidence-based smoking-related prevention and control measures for COVID-19.

Keywords: COVID-19, smoking, tobacco, electronic cigarette, viral transmission

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by an infection from a new type of coronavirus. The virus was named the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses (ICTV), like SARS-CoV and MERS-CoV, and it belongs to the Betacoronavirus genus (β-COV) (Graham et al., 2013; Song et al., 2019). The genetic sequence of SARS-CoV-2 is 79.5% similar to SARS-CoV, and the receptor used by SARS-CoV-2 to enter the human body is the same as that used by SARS-CoV, namely, the angiotensin-converting enzyme 2 (ACE2) (Lu et al., 2020). However, the binding affinity of the spiked envelope of SARS-CoV-2 to human ACE2 is approximately 10–20 times that of SARS-CoV, with an extremely high rate of human-to-human infection and rate (Wrapp et al., 2020; Yu et al., 2020).

From December 2019, it took only a few months for the new coronavirus, which causes pneumonia, to spread from sporadic regional outbreaks to countries to worldwide. The number of patients affected, the seriousness of the disease, and the high mortality rate are unprecedented. As of February 21, 2021 (Beijing time), the latest report of the World Health Organization (WHO) showed that the number of confirmed cases of new COVID-19 worldwide was more

than 111.69 million people (111,696,136), with an estimated 2.46 million deaths. The United States (with approximately 28.67 million cases) and India with (approximately 10.99 million cases) are reportedly the most severely affected countries. There is still no effective treatment for COVID-19.

At this stage, the clinical characteristics of patients with the new coronavirus are still being explored in-depth; moreover, epidemiological data show higher morbidity and mortality rates from COVID-19 among the older adults and those with lower immunity and prior illnesses (cancer, hypertension, diabetes, and especially respiratory diseases) (Du et al., 2020; Salako et al., 2020). Smoking is a major risk factor for common chronic diseases (Tonnesen et al., 2019), especially those closely related to the occurrence and development of respiratory diseases. During the COVID-19 epidemic, the role of cigarette smoking on COVID-19 has been a controversial issue. This article reviews the latest research and evidence on smoking and COVID-19 to be used as a reference by medical institutions and clinicians.

SMOKING INCREASES THE RISK OF VIRAL RESPIRATORY INFECTIONS

COVID-19 is primarily transmitted through the respiratory tract (saliva), and smokers may be at increased risk of contracting the virus due to reduced lung function, impaired immune systems, cross-infection, and susceptible hygiene habits (Zhou et al., 2016; Ahmed et al., 2020). Cigarette smoking also increases the amount of forced vital capacity (FVC) and stimulates hyperproliferation of the bronchial mucosal glands, resulting in increased mucosal permeability, excessive mucus production and inhibitedclearance of mucosal cilia, reducing the airway purification function and harmful microorganisms screening in the upper respiratory system, leading to potential pulmonary inflammation.

In a prospective study that explored smoking and alcohol consumption's suppression of host resistance to viral infections, 391 participants were exposed to five respiratory viruses (including coronavirus type, respiratory syncytial virus and three rhinovirus types rhinovirue). The results showed that smoking increased the risk of infection (OR = 2.23; 95% CI: 1.03–4.82), as well as the risk of developing clinical symptoms after infection (OR = 1.83; 95% CI: 1.00–3.36) (Cohen et al., 1993). Another cell experimental study showed that when infected by the virus, cigarette smoke extracts preconditioned RSV-infected cells to cause cell necrosis rather than apoptosis, resulting in increased inflammation and increased viral replication (Groskreutz et al., 2009). Thus, smoking can increase the risk of viral respiratory infections.

A systematic review analyzed the current evidence and quantified the risk of influenza infection between smokers and non-smokers. Nine studies with a total of 40,695 participants were included in this review, of which three were laboratory-confirmed case-control studies of influenza showing that current smokers were 5 times more likely to develop influenza than non-smokers (OR = 0.73; 95% CI: 0.73–0.99). In six studies reporting the occurrence of influenza-like illness, current smokers were 34% more likely to have influenza than non-smokers (OR = 1.34; 95% CI: 1.13–1.69) (Lawrence et al., 2019). Jaspers et al. (2010) reported that in the early stage of influenza virus infection, the antiviral defense ability of smokers' nasal epithelial cells was inhibited, namely, the signal transduction of type I interferon (IFN) was inhibited, IFN-α and IRF7 (a key transcription factor controlling the expression of IFN-α) expression was reduced, which would increase smokers' susceptibility to the influenza virus. Meanwhile, Noah et al. (2011) suggested that the inhibition of type 1 IFN signal transduction would facilitate the replication of the influenza virus in smokers and individuals exposed to tobacco smoke. Therefore, it would increase the number of influenza viruses.

THE EPIDEMIOLOGY OF SMOKING AND COVID-19

The WHO stated that 1.4–18.5% of hospitalized COVID-19 adult patients were smokers (World Health Organization, 2020a). Some scholars believe the prevalence of COVID-19 in Chinese men was higher than among women because the smoking rate among Chinese men was much higher than women (Underner et al., 2020). Some studies do not support the above conclusions. Farsalinos et al. (2020) conducted a systematic analysis of 13 studies from China (including 5,960 patients) indicating the current prevalence of smoking among hospitalized patients with COVID-19 was 6.5% (95% CI: 4.9–8.2%) based on a pooled estimate; In the secondary analysis, the unknown data were adjusted (integrating former smokers into the group of current smokers), and the pooled estimate of smoking prevalence was 7.3% (95% CI: 5.7–8.9%), which is still far lower than the prevalence of smoking among Chinese residents (26.6%). Tsigaris and Teixeira da Silva (2020) conducted an ecological study of 38 European countries, and after strictly controlling for confounding factors, smoking prevalence was significantly negatively correlated with COVID-19 prevalence ($P = 0.001$). Furthermore, a meta-analysis of 233 studies showed (Simons et al., 2020), current smokers compared with never smokers were at reduced risk of testing positive for SARS-CoV-2 infection (RR = 0.74; 95% CI: 0.24–0.64); But former smokers compared with never smokers were at increased hospitalization risk (RR = 1.20; 95% CI: 0.06–0.37).

Some researchers suggested that studies on smoking and COVID-19 have similar limitations, namely, they cannot accurately determine people's current smoking status (Emami et al., 2020; Harapan et al., 2020; Guan et al., 2020; Miyara et al., 2020; Petrilli et al., 2020). There were significant differences between these incomplete patient health histories and actual smoking behavior, leading to the underestimation of current smoking rates of COVID-19 patients, which also caused a certain deviation in the early evaluation of the COVID-19 infection rate and smoking status (Polubriaginof et al., 2018; Lippi et al., 2020; Patanavanich and Glantz, 2020). Exposure to SARS-CoV-2 was heterogeneous, with higher infection risk in different subgroups at different stages of the pandemic, and some research

analyses are based on unadjusted ORs (calculated for age and other confounding factors) (Feldman and Anderson, 2013; Leung et al., 2020; Lippi and Henry, 2020; Liu et al., 2020; Zhong et al., 2021), even some peer-reviewed meta-analyses investigating the association between smoking and COVID-19 were based on unadjusted ORs (Lippi and Henry, 2020; Zhao et al., 2020; Zheng et al., 2020). Therefore, the reliability of these studies needs to be confirmed. Also, smokers were more likely to have symptoms similar to COVID-19, such as cough and sputum, making them more likely to accept SARS-CoV-2 testing, even if they may not be infected, this included with the object selection bias would increase the negative samples detection rate, creating a bias (Cole et al., 2010; de Lusignan et al., 2020). Thus, the single smoking rate of patients cannot be used to judge the relationship between smoking and COVID-19, scientists need to be cautious in assessing the impact of smoking on COVID-19.

SMOKING AND COVID-19 DISEASE PROGNOSIS

In April 2020, the WHO announced that smokers were more likely to develop serious illness from COVID-19 than non-smokers (World Health Organization, 2020b). More studies have shown that smoking is associated with the prognosis of COVID-19 infection, and current and former smoking is significantly associated with the risk of serious illness from COVID-19 (Grundy et al., 2020; Liu et al., 2020). During previous influenza outbreaks (MERS-CoV), smokers were twice as likely to be infected with influenza and had more severe symptoms as non-smokers, and had a higher mortality rate (Arcavi and Benowitz, 2004; Park et al., 2018). Vardavas and Nikitara (2020) reported that from the initial original study of COVID-19 in China, smokers were 1.4 times more likely to have severe COVID-19 symptoms than non-smokers (RR = 1.4; 95% CI: 0.98–2.00), and smokers were approximately 2.4 times more likely to be admitted to the intensive care unit (ICU). Smokers may require mechanical ventilation more often than non-smokers or they may be more likely to die. In a single-center study, Zhao et al. (2020) showed that on-going smoking increased the risk of developing severe COVID-19 by approximately 2-fold (OR = 1.98; 95% CI: 1.29–3.05), and COPD patients who developed severe COVID-19 symptoms had a >4-fold increased risk (OR = 4.38; 95% CI: 2.34–8.20). However, the analysis of the subgroup revealed (the largest sample size was excluded for a sensitivity analysis) that the effect of active smoking on COVID-19 severity was no longer significant. In COVID-19 patients with chronic diseases, a history of respiratory and cardiovascular diseases accelerated deterioration, and smoking was closely associated with the development of these diseases (Wang B. et al., 2020; Wang X. et al., 2020).

Most of these studies confirmed that cigarette smoking can aggravate COVID-19 symptoms in patients, which may be caused by a variety of chronic diseases related to smoking, especially because smoking can damage lung function and the immune system, causing a reduced ability to fight COVID-19. Cigarette smoking also causes excessive airway secretions, impaired drainage during expectoration, and impaired ventilation function, all of which increase susceptibility to acute respiratory tract dysfunction due to sudden phlegm asphyxiation. Furthermore, cigarette smoke can activate the autophagy-dependent mechanism mediated by the deacetylase HDAC6, while causes the cilia proteins to be delivered to the lysosome for degradation or recycling, shortening the airway cilia (Lam et al., 2013). The excessive activation of autophagy may eventually lead to programmed epithelial cell death, contributing to impaired mucociliary clearance (Cloonan et al., 2014). It can also reduce the A-kinase anchoring protein expression (AKAP)-9 *in vitro* to cause e-cadherin-mediated barrier function destruction of airway epithelial cells, leading to increased infection risk by viruses and bacteria that exacerbate COPD (Oldenburger et al., 2014). At the same time, cigarette smoking activates MAPKs in the lung, increasing the number of neutrophil granulocytes, lymphocytes, macrophages, and other cells, and inducing cells to release pro-inflammatory factors and chemokines in **Figure 1** (Barnes, 2009; Chung, 2011). This results in acute lung inflammation, such as neutrophil infiltration, the mRNA expression of TNF-α and MIP-2, proteinase expression (MMP-12 mRNA), and oxidative DNA damage, causing respiratory barrier dysfunction and epithelial cell death. It also promotes the development of chronic inflammation in COPD and other related diseases (Marumo et al., 2014; Aghapour et al., 2018).

NICOTINE AFFECTS ACE2 EXPRESSION, INCREASES SUSCEPTIBILITY TO COVID-19, AND CAUSES AGGRAVATION OF THE DISEASE

The angiotensin-converting-enzyme II (ACE2) receptor is the confirmed entry point of the SARS-CoV-2 virus into host cells because the S1 domain of the SARS-CoV-2 virus membrane spike protein has a high affinity with the ACE2 receptor on lung epithelial cells (Kaur et al., 2020). ACE2 expression plays a key role in susceptibility to COVID-19 and is involved in innate and adaptive immune responses, affecting the immune regulation of B cells and cytokine secretion (e.g., IL-1, IL-10, IL-6.). Its high levels of expression may increase viral activity and promote viral replication, transcription, and release (Li G. et al., 2020).

In the process of virus replication and transcription, the combination of SARS-CoV-2 and ACE2 may progressively downregulate the expression of ACE2 protein and reduce the residual ACE2 activity (Dijkman et al., 2012), causing the renin-angiotensin system (RAS) to be unregulated, and then changing the ACE/ACE2 Balance and producing higher ACE and/or lower ACE2, while angiotensin-converting enzyme (ACE)/angiotensin (ANG) II/Ang II type I receptor (AT_1R) axis could causing vasoconstriction, pro-inflammatory and pro-oxidative effects. It could eventually evolve into acute heart failure, ARDS, and renal acute failure (Garami, 2020; Henry et al., 2020).

Studies have confirmed that nicotine can affect the homeostasis of the renin-angiotensin system. It can upregulate

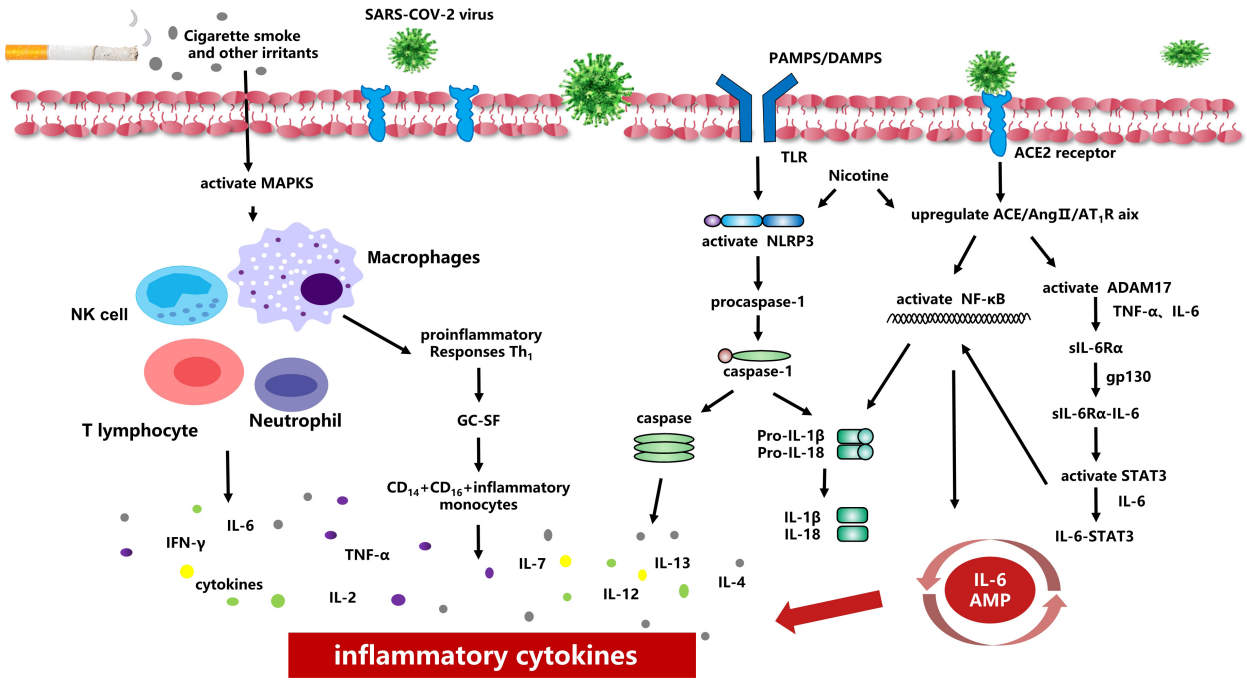

FIGURE 1 | Smoking and inflammatory cytokines Smoking causes activation of MAPKs in the lung, increasing the number of neutrophil granulocytes, lymphocytes, macrophages and other cells, and inducing cells to release pro-inflammatory factors and chemokines. Smoking induces overexpression of ACE2 receptor and increases viral adhesion. When the virus is activated and released, NF-κB-mediated signaling pathways are activated, and thus up-regulated the transcription of inflammasomal-related components. Smoking can upregulate ACE/Ang II/AT1 raxis, activate NF-κB, integral protein and ADAM17, induce TNF-α and IL-6 to the soluble form sIL-6Rα, and finally activate IL-6 AMP to promote the release of inflammatory cytokines.

the ACE/Ang II/AT_1R axis and increase renin expression or activity, ACE and AT_1R, while the ACE2/Ang 1-7/Mas receptor axis can downregulate the expression or activity of ACE2/AT_2R in **Figure 2** (Russo et al., 2020). The imbalance of the RAS system caused by nicotine may promote and aggravate the occurrence and development of cardiovascular, cerebrovascular, and pulmonary diseases.

ACE2 expression is regulated by nicotinic acetylcholine receptors (nAChRs). The nAChRs receptors are widely distributed throughout the central nervous system and activate acetylcholine neurotransmitter signaling pathways, while nAChRs can be easily activated by nicotine (Dani and De Biasi, 2001). When people smoke, nicotine induces overexpression of the ACE2 receptor in pulmonary epithelial cells (e.g., bronchial epithelial cells, type II alveolar epithelial cells.) through nAChRα7 (Biron et al., 1969; Wang Q. et al., 2020), and studies have confirmed that smokers (including e-cigarettes) have higher serum levels of ACE-2 in **Figure 2** (Brake et al., 2020). In healthy human volunteers, serum ACE activity increased significantly immediately after smoking and returned to control levels 20 min after smoking cessation (Kitamura, 1987).

Farsalinos et al. (2020) believe that upregulation of ACE2 expression may be induced as a defense mechanism against the effects of angiotensin II. In animal experiments, ACE2 protected mice from severe acute lung damage (Imai et al., 2005), while tobacco smoke exposure increased lung injury in ACE2 knockout mice (compared with wild-type mice) (Huang et al., 2016), further supporting the upregulation of ACE2 as a beneficial defense mechanism. Smith and Sheltzer (2020) reported that smoking causes a dose-dependent upregulation of ACE2 expression; that is, long-term smoking increases ACE2 expression, while smoking cessation decreases ACE2 levels in the lungs.

Even if upregulation of ACE2 in the initial stage of tobacco exposure helps prevent acute lung injury, there is no doubt that upregulation of ACE2 expression can increase COVID-19 susceptibility, and long-term tobacco exposure can cause an imbalance of the body's RAS system, leading to a series of changes. Thus, it progresses the COVID-19 disease.

NICOTINE, IL-6 AND OTHER PRO-INFLAMMATORY FACTORS

One of the main causes of death from COVID-19 is acute respiratory distress syndrome (ARDS), and the main mechanism of ARDS is the "cytokine storm," which is caused by immune effector cells releasing large amounts of pro-inflammatory cytokines (e.g., TNF-α, IFN-γ, IL-1β, IL-6, IL-12.) and chemokines (e.g., CCL2, CCL3, CCL5) (Li X. et al., 2020). Cytokine storms can activate the immune system's attack on the body, triggering a deadly and uncontrollable systemic inflammatory response that can cause acute respiratory distress and multi-organ failure (Xu et al., 2020).

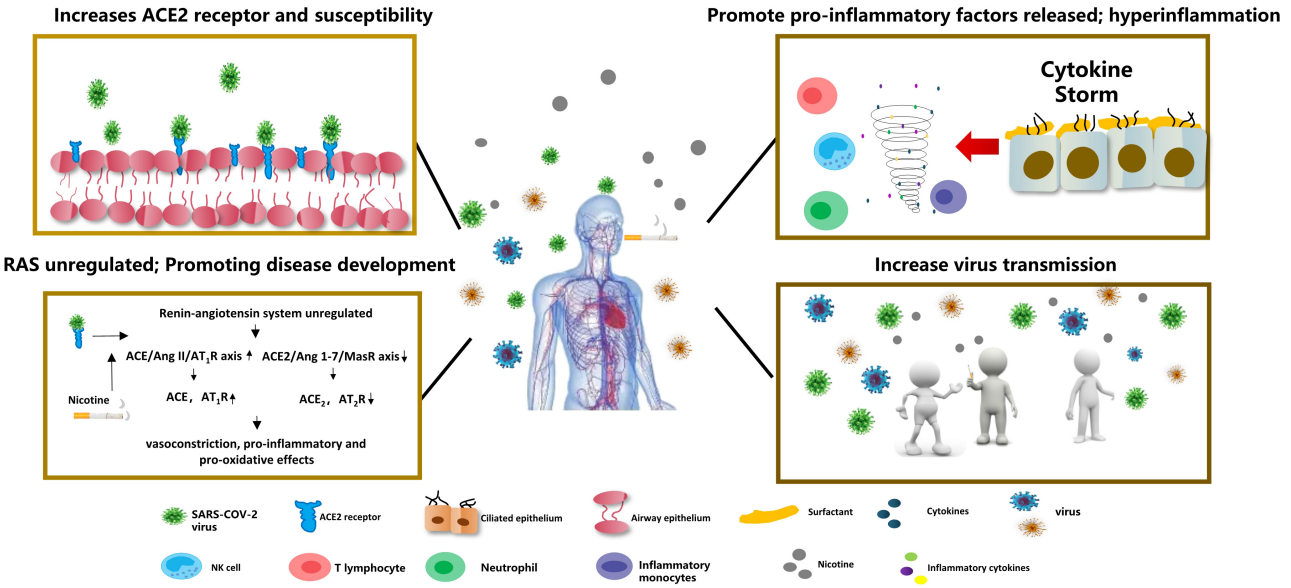

FIGURE 2 | COVID-19 and smoking Nicotine induces overexpression of the ACE2 receptor in lung epithelial cells, increasing SARS-COV-2 virus susceptibility. Nicotine can also deregulate the renin-angiotensin system (RAS), leading to vasoconstriction, proinflammatory, and pro-oxidative reactions. Meanwhile, nicotine can increase the release of pro-inflammatory factors and aggravate the inflammatory response. Smokers' social behaviors for gathering to smoke can increase the transmission of COVID-19.

When the SARS-COV-2 virus enters the body, it can rapidly activated $CD4^+$T lymphocytes to become T helper 1 (Th1) cells to secrete pro-inflammatory factors (e.g., granulocyte-macrophage colony-stimulating factor (GC-SF), IFN-γ, IL-2, IL-7, IL-6.). While the GC-SF further activates $CD14^+CD16^+$ inflammatory monocytes, producing a large number of IL-6, TNF-α and other cytokines in **Figure 1** (Yonggang et al., 2020). The release of pro-inflammatory factors i s consistent with the activation of NLRP3/inflammasomes (Chen et al., 2019). During COVID-19 infection, SARS-CoV proteins E and 3a IC induces $Ca2^+$ efflux and activates NLRP3/inflammasome complex, which in turn, triggers zymogen procaspase-1 to convert into the active caspase-1 and eventually establish the inflammatory caspase (Farag et al., 2020). Inactive pro-IL-1βand pro-IL-18 were converted to active pro-inflammatory cytokines (such as IL-1βand IL-18) (Franchi et al., 2012). Franchi et al. (2014) found that when pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) were recognized by Toll-like receptors (TLRs), activating the nuclear factor kappa B (NF-κB) -mediated signaling pathways, and thus up-regulated the transcription of inflammasomal-related components (Epstein and Dinarello, 1984). Ultimately, it stimulates the release of TNF and IL-6 and increases levels of TNF-α, IL-13, IL-4, and IL-8, contributing to acute pro-inflammatory response in **Figure 1** (Martinon and Tschopp, 2007; Davis et al., 2011; Luigi and Gabriel, 2012; Hirano and Murakami, 2020). Hirano and Murakami (2020) proposed that the AngII pathway caused potential mechanisms for cytokine storms When the SARS-COV-2 virus engulfed ACE2, the content of ACE2 on cells decreased and stimulated the ACE/Ang II/AT1R axis through pattern-recognition receptors to activate NF-κB and disintegrin and metalloprotease 17 (ADAM17). ADAM17 can generate TNF-α, induction IL-6Rα to the form soluble form (sIL-6Rα), and then mediated by gp130-mediated to forms sIL-6Rα-IL-6 complexes, ultimately activate signal transducer and activator of transcription 3 (STAT3). Both NF-κB and STAT3 can activate IL 6 amplifier (IL-6 Amp), which in turn causes various pro-inflammatory cytokines and chemokines (**Figure 1**).

Cigarette smoke is known to cause abnormal inflammatory activation of the bronchial epithelium and promote damage to macrophage function, increasing the risk of infection (Hiemstra, 2013; Aghapour et al., 2018). More and more studies suggest cigarette smoke activates the NLRP3 inflammasome in the lung epithelium, increasing the expression of NLRP3, pro-IL-1β, and caspase-1, enhancing the activity of Caspase-1 and the release of inflammasome related cytokines IL 1β and IL 18 in **Figure 1** (Li et al., 2016; Yi et al., 2018). Buscetta et al. (2020) found that cigarette smoke extract could increase the activity of caspase-1 through an NLRP3-independent and TLR4-TRIF-caspase-8-dependent pathway, leading to a decrease in basic glycolytic flux and response to lipopolysaccharide, which may eventually lead to macrophage dysfunction and increase the risk of infection in smokers. Simultaneously, in the context of subchronic electronic cigarette exposure, the body increases the release of pro-inflammatory factors and ACE2 receptors by regulating nAChRα7, leading to an inflammatory response and dysregulated repair (Wang Q. et al., 2020). The De Cunto team (De Cunto et al., 2016) showed that, under chronic smoking conditions, the expression of some pro-inflammatory cytokines was upregulated (e.g., Il-6, TNF-α, KC).

Paradoxically, nicotine was indicated to be an agonist of the cholinergic anti-inflammatory pathway. It has anti-inflammatory

properties and can regulate the body's immune response. It can also inhibit the release of pro-inflammatory cytokines (e.g., TNF, IL-1, IL-6) without inhibiting the release of anti-inflammatory cytokines (e.g., IL-10) (Wang H. et al., 2003). Because of long-term chronic respiratory infection, the level of antibodies against streptococcus pneumoniae in smokers' bodies will increase, making it possible to have certain anti-inflammatory capabilities (Sankilampi et al., 1997; Nuorti et al., 2000). The most effective evidence supporting the therapeutic anti-inflammatory potential of nicotine is the epidemiological study of smokers with ulcerative colitis; 90% of patients with ulcerative colitis are non-smokers, and the symptoms of colitis in smokers who smoke intermittently can be improved when smoking (Jenkins et al., 2005). Nicotine has been used in clinical trials to treat ulcerative colitis; however, its non-specific effects and collateral toxicity limit its clinical use (Ghia et al., 2006; de Jonge and Ulloa, 2007). At present, there is no certain evidence that nicotine increases anti-COVID-19 antibody levels in humans, and the mechanism of its action remains unclear.

These contradictions may be related to the concentration of nicotine. Stable nicotine dose therapy has good anti-inflammatory effects. However, smoking can cause a continuous increase in plasma nicotine concentration, resulting in addiction and adverse consequences. The WHO also stated that there is not enough information to confirm any connection between tobacco or nicotine in the prevention or treatment of COVID-19. Therefore, smoking is by no means a protective measure against COVID-19. Doctors and public health professionals should collect accurate smoking data, and further independent studies are necessary to analyze the impact of smoking on the incidence rate, progression, and COVID-19 mortality.

TOBACCO USE AND COVID-19 VIRUS TRANSMISSION

The COVID-19 virus can infect individuals of any age. The virus has a wide range of transmission methods. It may spread through contact, droplets, air, pollutants, faces, etc.; however, its main mode of transmission is respiratory droplets. In addition to contact transmission, the source can be respiratory secretions, droplets, aerosols and contaminated surfaces (Hui et al., 2020). People with or without symptoms can transmit the virus (Nair et al., 2017). Therefore, strict cross-infection control measures are difficult to maintain due to smoking behavior (Wang D. et al., 2020). Smokers' hands and cigarette filters are contaminated by SARS-CoV-2 in many ways. Smokers gather together in closed environments where they chat, drink, and pass cigarettes to other smokers, which increases the risk of COVID-19 infection (**Figure 2**). In the meantime, cigarette smoke causes users to cough or sneeze. This process produces large amounts of aerosols containing SARS-CoV-2, which can remain in the air for 3 h in the form of droplets or micro-aerosols, and they can survive as aerosols and on the surface (plastic paper and steel) for several hours to several days (Sherman, 1991; Benjamin, 2011). Even if strict social distance policies are maintained, contaminated humans may still be infected. Also, smokeless tobacco products may contribute to the COVID-19 epidemic and increase susceptibility (Singh and Chaturvedi, 2020). Users put smokeless tobacco products in their mouths, chew repeatedly, and finally spit them out along with saliva, which contains a variety of pathogens, including SARS-CoV-2. Smokeless tobacco users may exhibit behavior like spitting, and they often exhibit respiratory symptoms, cancer, and other diseases at high incidence rates, which are closely related to the COVID-19 epidemic (Siddiqi et al., 2015). Therefore, smokers' social culture may promote the spread of the virus.

COVID-19 PANDEMIC AND SMOKING CESSATION

Do the home isolation policies implemented during the COVID-19 pandemic increase smokers' motivation to quit? Heerfordt and Heerfordt (2020) investigated the global search trend of smoking cessation information during the COVID-19 pandemic (January–April 2020) from the perspective of Google's hot search trend and found that the search volume of smoking cessation information on Google did not increase significantly (Tieks et al., 2019). In contrast, because of the prolonged home isolation and regional blockade during the pandemic, residents experienced a series of symptoms, including panic, anxiety, sleep disturbance, etc. (Banerjee, 2020). This isolation may also have had a certain impact on nicotine addicts (Pfefferbaum and North, 2020), driving them to use substances, especially tobacco or alcohol, to relieve stress and negative emotions (García-Álvarez et al., 2020). One report showed that smokers' daily smoking rates increased by 49.9% during the epidemic, which could explain why the tobacco industry was unaffected during such a severe epidemic (Tobacco Reporter, 2020). A survey of smoking behavior and psychological dynamics in Italy in April 2020 (including 1,825 participants) showed that staying at home caused most exclusive smokers to consider quitting; however, most e-cigarette users did not consider stopping e-cigarettes use, and cigarettes and e-liquid purchases increased; one-third of former smokers considered relapse (Caponnetto et al., 2020). Although the governments around the world was not very active regarding efforts to encourage smoking cessation during the epidemic, the government can still encourage smokers to quit and maintain their health.

To date, many governments and health institutions have not included smokers in the high-risk population for COVID-19 and seldom issue warnings regarding the effects of smoking on COVID-19's spread. With the studies on the relationship between smoking and COVID-19, this situation may change.

SUMMARY

This review clearly indicate the complexity of the relationship between smoking and COVID-19. Smoking can increased mucosal permeability, reducing airway purification function and the screening of harmful microorganisms, increasing the risk of viral of respiratory infections and aggravating the severity

of respiratory diseases among smokers. There is also growing evidence to support the WHO's conclusion that smokers are at a higher risk of severe COVID-19 symptoms. Meanwhile, ongoing smoking can aggravate the condition of COVID-19 patients and increase the risk of death. Smokers' social behaviors, such as gathering to smoke, can increase of COVID-19 transmission. Although the mechanism of nicotine's effect on ACE2 expression needs further study, upregulation of ACE2 expression can increase the susceptibility to and risk of COVID-19. Also, nicotine can increase pneumococcal and streptococcal antibody levels to an extent. However, there is insufficient evidence to indicate that there is any link between nicotine in tobacco and the prevention or treatment of COVID-19. Smoking is not a measure to prevent COVID-19. Therefore, governments and health care providers should identify the importance of tobacco control, especially during the COVID-19 epidemic. Public health messages should increase publicity about tobacco hazards, the benefits of quitting, and provide strict, safe, and healthy tobacco management in public and the workplace to encourage smokers to quit (**Figure 2**).

AUTHOR CONTRIBUTIONS

JX and RZ wrote this review. WW and YZ supervised the entire work and critically revised the manuscript. OC read and amended the final manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Physiology

REVIEW
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Cigarette Smoke Exposure, Pediatric Lung Disease, and COVID-19

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The detrimental effects of tobacco exposure on children's health are well known. Nonetheless, the prevalence of secondhand or direct cigarette smoke exposure (CSE) in the pediatric population has not significantly decreased over time. On the contrary, the rapid incline in use of e-cigarettes among adolescents has evoked public health concerns since increasing cases of vaping-induced acute lung injury have highlighted the potential harm of these new "smoking" devices. Two pediatric populations are especially vulnerable to the detrimental effects of cigarette smoke. The first group is former premature infants whose risk is elevated both due to their prematurity as well as other risk factors such as oxygen and mechanical ventilation to which they are disproportionately exposed. The second group is children and adolescents with chronic respiratory diseases, in particular asthma and other wheezing disorders. Coronavirus disease 2019 (COVID-19) is a spectrum of diseases caused by infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that has spread worldwide over the last year. Here, respiratory symptoms ranging from mild to acute respiratory distress syndrome (ARDS) are at the forefront of COVID-19 cases among adults, and cigarette smoking is associated with worse outcomes in this population, and cigarette smoking is associated with worse outcomes in this population. Interestingly, SARS-CoV-2 infection affects children differently in regard to infection susceptibility, disease manifestations, and complications. Although children carry and transmit the virus, the likelihood of symptomatic infection is low, and the rates of hospitalization and death are even lower when compared to the adult population. However, multisystem inflammatory syndrome is recognized as a serious consequence of SARS-CoV-2 infection in the pediatric population. In addition, recent data demonstrate specific clinical patterns in children infected with SARS-CoV-2 who develop multisystem inflammatory syndrome vs. severe COVID-19. In this review, we highlight the pulmonary effects of CSE in vulnerable pediatric populations in the context of the ongoing SARS-CoV-2 pandemic.

Keywords: cigarette smoke exposure, E-cigarette, vaping, lung, coronavirus disease 2019, pediatric, infection, inflammation

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INTRODUCTION

The detrimental effects of cigarette smoke exposure (CSE) on pediatric respiratory function have been well demonstrated (Jaakkola and Jaakkola, 1997; Weiss et al., 1999; Walker et al., 2003; Jackson et al., 2014; Liptzin et al., 2015). Although the number of cigarette smokers continues to decrease, new smoking devices (e-cigarettes) have spread among adolescents[1] carrying with them the perception that they are "safer" than traditional tobacco products. This is especially concerning, since it is now well demonstrated that use of e-cigarettes is associated with subsequent use of traditional cigarettes, and vaping itself can cause significant lung injury, particularly among teenagers.[2]

Cigarette smoke exposure in children not only leads to wheezing, recurrent pulmonary and ear infections, and asthma, but it may also worsen pre-existing lung conditions (Weiss et al., 1999; Walker et al., 2003; Jackson et al., 2014). Here, former premature babies who suffer from chronic lung diseases following oxygen therapy and/or respiratory support are at increased risk. Similarly, children with asthma or chronic wheezing disorders often experience recurrent exacerbations and poor disease control when exposed to cigarette smoke.

The ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has dramatically challenged healthcare systems and daily life worldwide and its ultimate impact on children affected by chronic lung conditions remains to be seen. At the beginning of the outbreak, few cases and less severe manifestations with low mortality rates were described in children (Chang et al., 2020), suggesting that adults, especially the elderly, were predominantly affected. Initially, children were largely considered a significant source of spread but not a population that would be critically impacted by infection. However, as the pandemic has unfolded, a shift toward the younger population has been reported in the United States (Boehmer et al., 2020) and Europe[3], and children's unique infection susceptibility, disease manifestations, and health consequences are increasingly appreciated.[4] In addition, societal restrictions imposed by the health crisis are challenging children's daily life, education, and socialization opportunities, thereby impacting the management of their chronic medical conditions. This is particularly true in lower-income settings. Currently, the long-term consequences of coronavirus disease 2019 (COVID-19) for children's health remain difficult to foresee.

With this review, we attempt to highlight the detrimental effects of CSE in the pediatric population with particular focus on vulnerable children, including premature babies and children with pre-existing lung conditions, and to discuss the potential interplay of SARS-CoV-2 infection in these settings.

[1]National Institute of Health (2018)
[2]Center for Disease Prevention and Control (2020)
[3]European Center for Disease Prevention and Control (2020)
[4]AAP (2020a)

HOW BIG IS THE PROBLEM?

Cigarette Smoke Exposure in Children and Adolescents

Cigarette smoke derives from burning tobacco products (e.g., cigarettes, cigars, and pipes) or smoke that has been exhaled by a person smoking. Secondhand smoke (SHS) exposure represents the typical source of CSE in children. In the US, more than 128 million nonsmokers are exposed to SHS at any age (Best et al., 2009). Children, however, have a higher risk of SHS exposure compared to the nonsmoking adult population because of their frequent exposure from household members (Gergen et al., 1998; Pirkle et al., 2006). The World Health Organization estimates that around 700 million children – almost half of the world's pediatric population – have significant exposure to SHS.

SHS exposure in children has a strong impact on their lifelong health and places a significant burden on the health care system. In the United States in 2010, exposure to SHS in children caused 101,570 visits to the emergency department with a total of 62.9 million dollars spent (Yao et al., 2019). Analysis from the Global Burden of Disease Study 2017 estimated that 63,822 pediatric (<10 years of age) deaths worldwide were ascribable to SHS exposure that year (GBD 2017 Risk Factor Collaborators, 2018).

Detrimental effects on respiratory function are common in children exposed to SHS. A systematic review and meta-analysis of 60 studies concluded that passive smoke exposure increases the risk of pediatric lower respiratory tract infections, with the risk becoming even greater in cases where both parents smoke (OR single smoking parent 1.22–95% Cl 1.10–1.35 – vs. OR both smoking parents 1.54–95% Cl 1.40–1.69; Jones et al., 2011). Not only does SHS exposure facilitate the onset of respiratory diseases and infections, but it may also exacerbate symptoms of pre-existing diseases such as asthma.

Airway diseases such as wheezing and asthma are the most common chronic respiratory disorders in the pediatric population and may have significant lifelong impact on children's health, even spanning into adulthood (von Mutius, 2001; Tai et al., 2014; Um-Bergstrom et al., 2019). According to the Center for Disease Control and Prevention (CDC)[5], 5.5 million children under age 18 in the United States are currently affected by asthma, and 7.1 million suffer from respiratory allergies. CSE – both direct and secondhand – enhances the risk of developing asthma in infancy, childhood, and adolescence (Goksor et al., 2007; Burke et al., 2012; Thacher et al., 2014, 2018). A systematic review by Burke et al. (2012) showed that SHS exposure in children and young adults increases the incidence of asthma by 20%. Moreover, SHS exposure is associated with more severe asthma symptoms (Akinbami et al., 2013; Hollenbach et al., 2017), increased risk of asthma exacerbations (Wang et al., 2015; Neophytou et al., 2018), and hospitalizations (Das, 2003; Wang et al., 2015).

Within the last decade, the introduction of e-cigarettes and vaping has evoked significant public health concerns,

[5]CDC (2020a)

specifically in regard to youth. In fact, the number of adolescents who vape has constantly and significantly increased during the past few years (Centers for Disease Control and Prevention, 2013; Carroll Chapman and Wu, 2014; Miech et al., 2019). In 2019, over 5 million high schoolers (27.5%) in the United States reported using e-cigs in the past 30 days, and, alarmingly, teenagers with asthma are even more likely than their non-asthmatic peers to try e-cigarettes (Choi and Bernat, 2016; Fedele et al., 2016; Larsen et al., 2016; Kim et al., 2017; Reid et al., 2018; Turner et al., 2018; Martinasek et al., 2019). Moreover, initial use of e-cigarettes during adolescence is strongly associated with subsequent traditional cigarette smoking, contributing to the increase of smoking in the adult population. Since March of 2019, outbreaks of e-cigarette and vaping product use-associated lung injury (EVALI) have been reported throughout the United States, in particular among adolescents[2] (Layden et al., 2020). EVALI is a clinical syndrome that comprises constitutional, respiratory, and gastrointestinal symptoms associated with inflammatory response and pulmonary infiltrates in patients who have recently used (within 90 days) e-cigarette products, and that is not ascribable to any other cause. EVALI is most associated with cannabinoid vaping and vitamin E acetate containing products[2]; however, the mechanisms responsible for vaping-induced lung injury are still under investigation. COVID-19 and EVALI have similar presenting symptoms. Therefore, a high level of suspicion for EVALI is still recommended during the COVID-19 pandemic since the possible overlap of clinical manifestations between COVID-19 and EVALI may lead to late EVALI diagnosis and treatment. This is highlighted by some recently reported clinical case series (Darmawan et al., 2020; Hassoun et al., 2020; Anderson et al., 2021).

COVID-19 in Children

The American Academy of Pediatrics together with The Children's Hospitals Association provides a "Weekly State Data Report" to trace COVID-19 cases in children in the United States.[6] As of December 31, 2020, children represent 12.4% of all the United States COVID-19 cases (2,128,587/17,137,295). Of significance, pediatric COVID-19 cases have constantly increased over the last few months (17% increases in child cases over the last 2 weeks 12/17-12/31/2020). Hospitalizations and deaths are fortunately uncommon in United States children: between 0.2 and 3.4% of all pediatric COVID-19 cases resulted in hospitalization and 0.00 and 0.08% of all pediatric COVID-19 cases resulted in death. Similar trends are reported in Europe.[7]

Children of all ages are susceptible to SARS-CoV-2 infection, although most infected children are asymptomatic (Bellino et al., 2020; Davies et al., 2020; Dong et al., 2020; Parri et al., 2020). When symptomatic, children present with different clinical manifestations, laboratory, and radiological findings compared to adult patients (Verma and Amin, 2020; Xia et al., 2020). The incubation period is like adults, 2–14 days with an average of 6 days.[8] Pediatric patients typically have a milder disease course overall. Significantly, children from low-income settings represent a high proportion of pediatric hospitalized patients; however, this seems not to be consistently related to worse outcome (Fernandes et al., 2021).

Presenting symptoms are mostly non-specific and self-limiting, which makes it difficult to distinguish COVID-19 from other common viral illnesses, possibly driving a lower testing rate in this population. Children may or may not have fever, and usually present either with cough or gastrointestinal symptoms in addition to more general symptoms such as rhinorrhea, sore throat, headache, and myalgia (Verma and Amin, 2020; Yoon et al., 2020; Fernandes et al., 2021). Of note, 40–50% of COVID-19 pediatric cases have a documented coinfection with other respiratory pathogens (Wu et al., 2020). Inflammatory markers are commonly not elevated in mild cases (Verma and Amin, 2020). Chest CT findings in pediatric COVID-19 cases with respiratory involvement showed unilateral or bilateral subpleural ground-glass opacities and consolidations with surrounding halo sign (Xia et al., 2020).

Since April of 2020, cases of children becoming severely ill after SARS-CoV-2 infection with features resembling Kawasaki disease have been increasingly reported[9] (Riphagen et al., 2020; Verdoni et al., 2020). This severe clinical manifestation has been named multisystem inflammatory syndrome in children (MIS-C) associated with COVID-19. Initial CDC criteria[10] for MIS-C included "(1) persistent fever ≥38.0°C; (2) laboratory evidence of inflammation; (3) evidence of clinically severe disease requiring hospitalization; (4) multisystem (≥2) organ involvement; (5) positive for current or recent SARS-CoV-2 infection; and (6) no alternative plausible diagnoses." It has become clear that MIS-C is a separate clinical and possibly pathological, entity from severe pediatric SARS-CoV-2 (Jiang et al., 2020). Interestingly, MIS-C cases lagged the COVID-19 case curve by 1 month and only one third of children diagnosed with MIS-C had RT-PCR positivity for SARS-CoV-2 depicting an active infection, while most of them were antibody positive (Jiang et al., 2020). A recent case series of 1,116 hospitalized pediatric patients from United States surveillance data has provided insights into the clinical characteristics and outcomes of children and adolescents with MIS-C compared with severe COVID-19 (Feldstein et al., 2021). Patients with MIS-C were more likely to be age 6–12 years, non-Hispanic black race, presenting severe cardiovascular or mucocutaneous involvement, and more severe inflammation. On the other hand, preliminary reports suggested that severe pediatric COVID-19 cases were more frequent in younger infants (<1 year of age; Gotzinger et al., 2020; Liu et al., 2020; Yang et al., 2020a), however, recent reports did not confirm this trend. COVID-19 severity in children seems to be associated with age ≥ 10 years, hypoxemia, obesity, and one or more underlying medical conditions (Bellino et al., 2020; Dong et al., 2020; Shekerdemian et al., 2020; Feldstein et al., 2021; Fernandes et al., 2021; Ouldali et al., 2021).

[6]AAP (2020b)
[7]European Center for Disease Prevention and Control (2021)
[8]CDC (2020b)
[9]NYC Health (2020)
[10]CDC (2020c)

PATHOPHYSIOLOGIC INSIGHTS OF CIGARETTE SMOKE- AND SARS-CoV-2-INDUCED LUNG INFLAMMATION

As we learn more about the effects of SARS-CoV-2 in children, the question arises whether and how cigarette smoke and SARS-CoV-2 represent a new intertwined threat for children with underlying lung disease. There is concern about the possible "second hit" effect of COVID-19 on developing lungs that are already chronically impacted by environmental factors and/or prematurity and its consequences. Understanding the pathophysiologic mechanisms of CSE and SARS-CoV-2 induced inflammation and lung injury demonstrates potential areas of overlap or synergism that may lead to long-term pulmonary consequences in vulnerable pediatric populations. In this section, we review some of those mechanisms and their impact on pulmonary inflammation and injury, as summarized in **Figure 1**.

Cigarette Smoke and Vaping

Tobacco smoke contains more than 7,000 chemicals, including hundreds that are toxic and about 70 that can cause cancer. Tobacco burning releases a complex mixture of chemicals, both in gaseous and particulate form, that have cytotoxic and mutagenic properties, along with antigenic capacities that contribute to chronic inflammation (Lee et al., 2012). Oxidative imbalance and stress caused by CSE are responsible for direct genetic or epigenetic effects that lead to altered barrier function of the epithelial layer, epithelial to mesenchymal transition (EMT), and immune dysfunction.

Due to their anatomic location, the epithelial layers of the oral cavity and airways act as the first barrier to cigarette smoke's effects and are the cornerstone of cigarette smoke-induced inflammatory response. In regard to epithelial barrier function, cigarette smoke impairs mucociliary clearance by reducing ciliary beating and enhancing mucus production. Moreover, cigarette smoke derivatives disrupt epithelial cellular junctions, allowing for a deeper penetration of toxins and infectious agents (Aghapour et al., 2018).

Epithelial to mesenchymal transition is associated with redox imbalance and oxidative stress (Milara et al., 2013; Gohy et al., 2015) and represents an important consequence of cigarette smoke effects. EMT of airway epithelium promotes airway wall thickening and remodeling, thereby leading to airway obstruction (Eurlings et al., 2014). Alveolar EMT, rather, impacts re-epithelization (Eurlings et al., 2014; Shen et al., 2014) and contributes to the development of emphysema (Aghapour et al., 2018). EMT leads to increased fibroblast migratory capacity, invasiveness, resistance to apoptosis, and greatly increased production of extracellular matrix (ECM) components (Kalluri and Neilson, 2003).

Another important consequence of cigarette smoking is immune dysfunction. Epithelial and innate immune cells are highly responsive to cigarette derivates. Oxidative stress triggers the activation of transcription factors involved in inflammatory

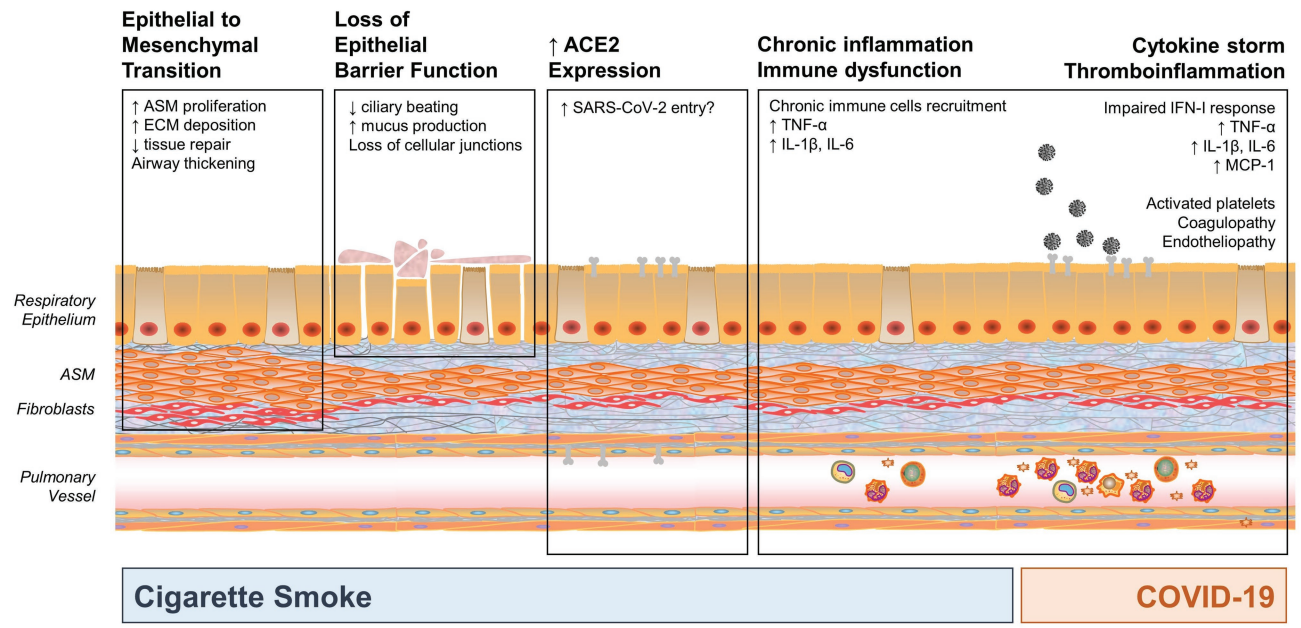

FIGURE 1 | Mechanisms by which cigarette smoke exposure (CSE) and coronavirus disease 2019 (COVID-19) may impact the developing and pediatric airway, highlighting potential areas of synergy. CSE results in three main impacts: epithelial to mesenchymal transformation, loss of epithelial barrier function (which may increase susceptibility to infection), and chronic inflammation and immune dysfunction. In addition, smoking increases angiotensin-converting enzyme 2 (ACE2) expression, which is a key entry point for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), thus potentially increasing susceptibility to infection. COVID-19 also results in significant inflammation and cytokine response. Synergy between CSE and SARS-CoV-2 may occur in activation of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, and other mediators that may result in cytokine storm, pronounced inflammatory state, and immune dysfunction. (ASM, airway smooth muscle; ECM, extracellular matrix).

responses, such as NF-kB and AP-1. Under the activation of these transcription factors, CSE promotes a huge production of proinflammatory mediators (e.g., IL-1β, IL-6, TNF-α, and granulocyte/monocyte CSF) and chemokines that are responsible for sustained immune cell recruitment and activation (Lee et al., 2012). This hyperinflammatory environment contributes to enhanced reactivity to inhaled antigens, tissue damage, and remodeling. Of note, despite being hyperactivated, innate and adaptive immune responses are highly dysfunctional in this setting (Lee et al., 2012). AP-1 activation has been related to corticosteroid resistant inflammation (Walters et al., 2005). Constitutively activated inflammation pathways are associated with reduced response to acute infectious challenges. Similarly, immune responses to viral antigens are significantly reduced, as cigarette smoke downregulates Toll-like receptor (TLR) 3-mediated responses to double-stranded RNA (Todt et al., 2013). Cigarette smoke-induced oxidative stress impairs the phagocytic activity of alveolar macrophages leading to accumulation of cellular debris and the initiation of necrotic processes. In regard to T-cells adaptive immune responses, cigarette smoke suppresses T helper (Th) 1 activation and enhances Th2 and Th17 inflammation that have been associated with eosinophilic inflammation, epithelial dysfunction, and virus-induced exacerbations in COPD patients (Papi et al., 2006).

Furthermore, CSE directly influences airway smooth muscle (ASM) function, enhancing proliferation, ECM deposition, and mitochondrial dysfunction (Aravamudan et al., 2014, 2017; Vogel et al., 2014).

The pathophysiology associated with EVALI is only beginning to emerge, but among the clinical manifestations are inflammation, wet cough, phlegm, and mucociliary dysfunction (Hedman et al., 2018; Chung et al., 2019). Data suggest that e-cigarette users produce significantly more sputum than smokers and show increased markers of inflammation in their airways. As airway inflammation and mucus hypersecretion are central features underlying asthma pathology, asthmatics might be at increased risk for e-cigarette-induced health effects (Kim et al., 2017).

SARS-CoV-2 Infection

Severe acute respiratory syndrome coronavirus 2 infection starts with the viral spike protein (S-protein) binding to angiotensin-converting enzyme 2 (ACE2) receptors on the cell surface (Walls et al., 2020). ACE2 is largely expressed in epithelial cells of the respiratory tract, vascular endothelial cells, and alveolar monocytes and macrophages (Lu et al., 2020). For SARS-CoV-2 to enter cells, the S-protein must be cleaved at two different sites by host cell proteases Furin and transmembrane serine protease 2 (TMPRSS2). This process promotes cell membrane fusion and internalization of the virus. Throughout the whole process of virus contact, internalization, and replication, infected host cells are provided with a series of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and allow the antiviral response to start. SARS-CoV-2's lipids, proteins, genetic materials, together with intracellular calcium homeostasis alterations, are recognized by PRR as TLRs 3, 4, 7, 8, and 9, RIG-I, and MDA-5. Viral recognition leads to activation of various inflammatory pathways as NLRP3 inflammasome that induces proinflammatory cytokine release, caspase activation, and cell death; MAPK pathway leads to IL-1β/2/6/10/18, and TNF*α*; secretion of interferon (IFN)-α/β (Kumar et al., 2021). INFs are key players in anti-viral host responses through inhibition of virus replication and immune activation. Interestingly, SARS-CoV-2 can inhibit an early type I IFN response in infected cells (Arunachalam et al., 2020; Miorin et al., 2020). This suppressed IFN-I production allows higher viral replication and tissue damage, further enhancing lung inflammation (Brodin, 2021). Bronchial epithelial cells, type I and II alveolar epithelial cells, and capillary endothelial cells are the site of primary infection leading to immune cell recruitment into the alveolar space and cytokine release. Many cases of severe COVID-19 are characterized by fever, elevated acute phase response markers, coagulopathy, and hemophagocytosis, suggesting a role for lung-derived cytokine storm in the pathogenesis of acute respiratory distress syndrome (ARDS), immunothrombosis, and multi-organ dysfunction (Fajgenbaum and June, 2020; Mcgonagle et al., 2021). Recent data demonstrate the persistence of anti-spike IgG neutralizing antibodies up to several months after the initial infection in >90% of infected individuals (Gudbjartsson et al., 2020; Wajnberg et al., 2020).

The significant variability in the clinical course of SARS-CoV-2 infections – ranging from asymptomatic infection, cold-like symptoms, to severe ARDS and multi-organ failure – has prompted the scientific community to identify determinants of disease severity. Male sex, age, and pre-existing conditions, such as hypertension, diabetes, and obesity are poor prognostic factors in COVID-19 (Sanyaolu et al., 2020). Impaired type I IFN responses, $T_H1 > T_H2$ cell response, high neutrophil-to-lymphocyte ratio, pre-existing inflammatory states associated with aging, and chronic diseases have been described as associated with worse outcomes (Del Valle et al., 2020; Brodin, 2021).

Focusing on children, lower ACE2 gene expression has been reported in the nasal epithelium of younger children (4–9 years old) which may in part explain the lower susceptibility to SARS-CoV-2 infection in children (Molloy and Bearer, 2020). On the other hand, children may rely on a more robust innate immune response (Consiglio et al., 2020; Gruber et al., 2020; Pierce et al., 2020) that allow them to limit viral replication and experience milder infection.[11] The higher number of naïve T-cells in children (Kumar et al., 2018), as well as a more recent memory for other coronaviruses infections (Mateus et al., 2020) have also been implicated in the difference in infections. There are also reports describing a different antibody response in the pediatric population regardless of disease severity (Weisberg et al., 2021). Independently from MIS-C development, SARS-CoV-2-infected children produce predominantly IgG anti-S antibody, with lower titers of anti-S IgM and IgA compared to adults. Moreover, children show significantly lower anti-N IgG that may be consistent with a lower viral load (Weisberg et al., 2021). The other aspect that has puzzled the scientific community is MIS-C pathophysiology. The temporal relationship between COVID-19 and MIS-C cases suggests that it is not

[11]Nature News (2020)

directly related to viral infection but rather to the development of adaptive immunity. The massive cardiovascular involvement suggests an underlying immune-mediated disease, and proposed mechanisms involve the production of autoantibodies as a result of viral mimicry of the host, T-cells recognition of viral antigens on infected cells, and immune complexes (Jiang et al., 2020).

COVID-19 and Smoking

Smoking has definitely been associated with COVID-19 progression and worse outcomes in adults (Gulsen et al., 2020; Patanavanich and Glantz, 2020; Vardavas and Nikitara, 2020; Zhao et al., 2020). COPD patients, who typically have a significant history of smoking, are at increased risk for severe COVID-19 (Zhao et al., 2020). ACE2 gene expression in airway epithelium is upregulated by tobacco smoking (Cai et al., 2020; Leung et al., 2020), possibly through the α7 subtype of the nicotine acetylcholine receptor (Russo et al., 2020). Because ACE2 receptors are an important entry point for COVID-19, this may represent an increased risk for viral entry and infection in smokers. Significantly, a recent *in vitro* study demonstrated that acute CSE induced increased infection severity in air-liquid interface cultures derived from human airway basal stem cells by impairing IFN responses and altering tissue repair (Purkayastha et al., 2020).

Cigarette smoking is associated with underlying chronic inflammation, oxidative stress, and epithelial and immune dysfunction that may also contribute to COVID-19 progression (Gulsen et al., 2020; Shastri et al., 2021). Even if not extensively tested, the same increased risk may apply to SHS and nicotine vapers.

PEDIATRIC LUNG DISEASES, CIGARETTE SMOKE EXPOSURE, AND COVID-19: A POTENTIAL INTERPLAY?

As described above, the overarching impact of cigarette smoke, e-cigarettes, and COVID-19 is a robust inflammatory pulmonary response. While cigarette smoke-induced inflammation is meant to serve as a natural/protective response, it may make children more vulnerable to illness, since the inflammatory mediators associated with CSE may result in exacerbation of inflammatory and remodeling pathways typical of chronic pediatric lung disease. This vulnerability in combination with an immature immune system – depending on the age of the child – serves as an additional insult to the patients' respiratory and general health. While the specific contribution of COVID-19 to pediatric lung disease remains to be fully elucidated, other respiratory infections have been shown to exacerbate pediatric lung disease by increasing activation of inflammatory pathways. These multiple "hits" of CSE and pulmonary infections may certainly exacerbate preexisting chronic pediatric pulmonary disease or lead to the development of pulmonary disease in at-risk children.

Wheezing Disorders, Cigarette Smoke Exposure, and COVID-19

Children with wheezing disorders represent a pediatric population at particular risk from additional "hits" such as CSE and respiratory infections. Asthma and other wheezing disorders present a heterogeneous spectrum characterized by chronic airway inflammation, airway hyperresponsiveness, and airway remodeling (Homer and Elias, 2005; Siddiqui and Martin, 2008; Bush and Menzies-Gow, 2009; Britt et al., 2013; Bonato et al., 2019). Clinically, asthma presents with respiratory symptoms, such as wheezing, shortness of breath, chest tightness, and cough, which cause variable expiratory flow limitations. These symptoms and their intensity can vary over time and may be induced by superimposed respiratory infections or environmental exposures (Deshpande and Morgan, 2016; Testa et al., 2020). Atopy, allergic diseases such as allergic rhinitis, and maternal asthma are all risk factors increasing the likelihood of airway hyperreactivity and asthma (Martinez et al., 1995; Savenije et al., 2011). In fact, even in utero smoke exposure (maternal smoking) has been shown to increase the likelihood of developing asthma in vulnerable children (Liptzin et al., 2015). SHS exposure increases the risk of developing asthma and exacerbation of asthma symptoms (Jaakkola and Jaakkola, 1997; Weiss et al., 1999; Walker et al., 2003; Jackson et al., 2014). Indeed, chronic SHS exposure is known to exacerbate asthma by contributing and/or further enhancing airway hyperresponsiveness and structural changes (Aravamudan et al., 2017). Here, it seems that structural changes due to increased ASM proliferation and increased airway contractility due to enhanced intracellular calcium response play a major role (Hartman et al., 2012; Vogel et al., 2014).

In regard to COVID-19 and pediatric wheezing disorders, a cross-sectional study (Shekerdemian et al., 2020) conducted in North-America's pediatric intensive care units (PICU) in May 2020 found that, among children with COVID-19 infections that required PICU admission, 80% had comorbidities such as developmental delay and/or genetic anomalies, but only 4% were reported as suffering from chronic lung disease. Overall PICU mortality was reported as <5%. Interestingly, asthma is rarely reported as comorbidity in pediatric COVID-19 cases although asthma is the most common chronic respiratory disease in children. This lack of overlap between COVID-19 and asthma is particularly interesting, as respiratory infections are typically a significant source of asthma exacerbation and morbidity. A systematic review of the current literature (Castro-Rodriguez and Forno, 2020) revealed no current data on the impact of COVID-19 in children with asthma. However, given the potentially significant implications of COVID-19 in children with asthma, the CDC emphasized the positive impact mask wearing and social distancing can make in this vulnerable patient population. Right now, health communities are encouraged to study affected populations in more detail to answer questions about the impact of COVID-19 on children with asthma, asthma severity, and the potential effects of asthma medications in treatment of COVID-19 infections (Castro-Rodriguez and Forno, 2020).

Prematurity, Cigarette Smoke Exposure, and COVID-19

Infants born prematurely are a second group at particularly high risk of pulmonary morbidity and mortality due to the

consequences of interrupting normal prenatal pulmonary development and maturation (Pramana et al., 2011; Vrijlandt et al., 2013; Been et al., 2014). The extent and form of pulmonary compromise varies greatly depending on the level of prematurity and any additional perinatal risk factors to which the infant is exposed (Britt et al., 2013). Extremely preterm infants [<28 weeks gestational age (GA)] have the highest rate of pulmonary insult because they are born prior to the saccular stage of lung development, before even primitive alveoli have started to form. These infants typically present with bronchopulmonary dysplasia (BPD), characterized by alveolar simplification, dysmorphogenesis of the alveolar capillaries, ASM proliferation, and abnormal ECM deposition (Jobe, 1999). Late preterm infants (33–36 weeks GA) are much less likely to develop BPD but are at increased risk of developing reactive airway diseases such as wheezing and asthma (Martin et al., 2013; Mcevoy et al., 2013).

Due to the pulmonary compromise that attends preterm birth, these infants commonly require respiratory support in the form of supplemental oxygen, mechanical ventilation, or continuous positive airway pressure (CPAP) to prevent hypoxia and maintain adequate alveolar recruitment and ventilation. Unfortunately, these necessary therapies may result in unintentional exacerbation of the pulmonary insults of prematurity.

In light of the numerous pulmonary insults to which preterm infants are commonly exposed in the perinatal period, they are particularly susceptible to the addition of environmental insults, such as cigarette smoke and infection. CSE is a well-documented risk factor for development of respiratory disease in the pediatric population (Carlsen and Carlsen, 2008; Vanker et al., 2017; Grant et al., 2020). From the perspective of prematurity, maternal tobacco use is clearly associated with increased risk for premature birth as well as placenta previa, placental abruption, intrauterine growth restriction, and premature rupture of membranes (Andres and Day, 2000). Preterm infants with BPD who are exposed to second-hand smoke have been found to require supplemental oxygen for longer periods of time and to be more likely to require steroid treatment as neonates (Collaco et al., 2014; Martinez et al., 2015).

The impact of COVID-19 on preterm infants is an emerging area of investigation. SARS-CoV-2 infection appears to increase risk of preterm birth and is associated with increased risk of admission to the neonatal intensive care unit (NICU; Allotey et al., 2020; Golden and Simmons, 2020; Mimouni et al., 2020; Pettirosso et al., 2020; Woodworth et al., 2020; Yang et al., 2020b). Intriguingly, one study found that mothers hospitalized with COVID-19 earlier in pregnancy (23–33 weeks GA) were less likely to delivery early than those who were infected later in pregnancy (34–36 weeks GA; Gulersen et al., 2020). In regard to prenatal vertical transmission, there have been case reports of neonates with respiratory symptoms who tested positive for SARS-CoV-2 within 24 h of birth, raising concern for possible vertical transmission (Rivera-Hernandez et al., 2020; Sisman et al., 2020). However, in the vast majority of cases, infants born to mothers with COVID-19 do not test positive or develop symptoms of infection, indicating that if vertical transmission does occur, it is very rare (Pettirosso et al., 2020; Woodworth et al., 2020). Indeed, one study found that postnatal transmission between SARS-CoV-2 positive mothers and infants was very low, even when breastfeeding and allowing the infants to room with their mother after birth. In a series of 120 cases, 83% of the infants roomed with their mother after birth with the majority breastfeeding. All neonates were negative for SARS-CoV-2 after birth. About 96% of these infants were retested for SARS-CoV-2 at 5–7 days of life and none tested positive (Salvatore et al., 2020). In neonates, COVID-19 most often presents with fever and mild respiratory symptoms, though it may present with gastrointestinal symptoms and abdominal distension in a subset of neonates (Golden and Simmons, 2020; Karabay et al., 2020; Marin Gabriel et al., 2020; Ng et al., 2020; Smith et al., 2020). However, there have been case reports of COVID-19 presenting with severe ARDS in the neonatal population (Frauenfelder et al., 2020; Kalyanaraman et al., 2020; Trieu et al., 2020; Wardell et al., 2020).

There is no data at this time evaluating the overlapping exposures of CSE and SARS-CoV-2 in premature infants. Both secondhand cigarette smoke and respiratory infections have been previously shown to act as additional perinatal "hits" to the vulnerable preterm lung that increase the risk of development of chronic lung disease later in life. It is therefore possible that infants infected with SARS-CoV-2 early in life may sustain lasting consequences from this pulmonary insult.

SUMMARY AND CONCLUSIONS

Overall, pediatric lung diseases remain a significant source of morbidity and mortality in children. Although advances have been made in the treatment of wheezing disorders and prematurity-related lung diseases, the health care burden remains high. The pathogenesis of such chronic lung conditions is complex and multifactorial. It is now clear that environmental insults play a role increasing susceptibility to pediatric asthma and wheezing disorders and worsen pre-existing lung diseases.

Among the environmental factors that affect the developing lung, cigarette smoke is certainly one of the most prevalent despite being preventable. While the number of adult smokers and the consequent risk of SHS exposure have decreased over time, the introduction of e-cigarettes and other vaping devices has brought up new health concerns such as EVALI and increased susceptibility to subsequent traditional smoking among adolescents. The long-term effects of CSE are ascribable to chronic inflammation and immune disfunction that lead to tissue remodeling and fibrosis, infectious susceptibility, and eventually lung function decline.

Severe acute respiratory syndrome coronavirus 2 represents a new addition to the vast number of infectious diseases that may affect children, and the current "pandemic dimension" makes COVID-19 a very prevalent threat. The clinical spectrum of COVID-19 in children is wide, ranging from asymptomatic to severe respiratory compromise and multisystem inflammatory syndrome. Determinants of COVID-19 clinical syndromes

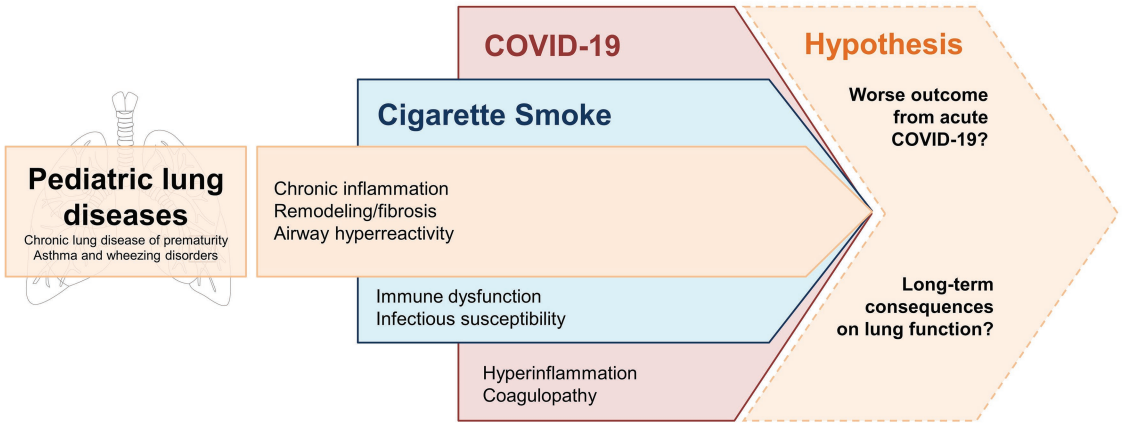

FIGURE 2 | Potential "multiple hits" effect of CSE and COVID-19 on developing lungs affected by common pediatric lung diseases – i.e., chronic lung disease of prematurity, asthma, and wheezing disorders. Underlying processes of inflammation and tissue remodeling are enhanced by chronic CSE. SARS-CoV-2 infection triggers an acute inflammatory response that may compromise the respiratory function of these vulnerable patients. Longitudinal clinical data are needed to confirm whether these factors have an additive effect that leads to more severe COVID-19 manifestations and/or long-term consequences in terms of lung function decline.

among the pediatric population are still under investigation and new contributing factors continue to emerge as we move through the pandemic. The varied manifestations suggest a role for genetic or acquired predisposition to more severe inflammatory responses. The long-term impact of SARS-CoV-2 infection on respiratory function of children with or without underlying lung diseases remains to be seen. Epidemiological data are key to identifying possible contributing factors and to guide research to better understand COVID-19 pathogenesis.

In this review, we wanted to highlight the possible detrimental interplay between the chronic inflammatory environment induced by cigarette smoke and the hyperinflammatory stimulus of COVID-19. As outlined in **Figure 2**, this combination has the potential to exacerbate pulmonary injury in developing lungs already affected by underlying conditions, such as chronic lung disease of prematurity, asthma, and wheezing disorders. While evidence regarding the intersection of cigarette smoke and COVID-19 pulmonary effects remain limited at this time, we agree with recommendations coming from major health associations to strongly encourage cigarette smoking cessation during the ongoing SARS-CoV-2 pandemic[12] (Ahluwalia et al., 2020). Children and adolescents with pre-existing lung conditions represent an extremely vulnerable population, in particular during this unprecedented time of reduced access to healthcare resources, stay-at-home mandates that may increase the risk of SHS exposure from household members, and social isolation that may encourage addictive behaviors such as vaping in young people.

[12]WHO (2020) and CDC (2020d)

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript equally and were involved throughout the entire process.

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frontiers in Physiology

BRIEF RESEARCH REPORT
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Vaping Exacerbates Coronavirus-Related Pulmonary Infection in a Murine Model

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Though the current preponderance of evidence indicates the toxicity associated with the smoking of tobacco products through conventional means, less is known about the role of "vaping" in respiratory disease. "Vaping" is described as the use of electronic cigarettes (E-Cigarettes or E-Cigs), which has only more recently been available to the public (~10 years) but has quickly emerged as a popular means of tobacco consumption worldwide. The World Health Organization (WHO) declared the SARS-CoV-2 outbreak as a global pandemic in March 2020. SARS-CoV-2 can easily be transmitted between people in close proximity through direct contact or respiratory droplets to develop coronavirus infectious disease 2019 (COVID-19). Symptoms of COVID-19 range from a mild flu-like illness with high fever to severe respiratory distress syndrome and death. The risk factors for increased disease severity remain unclear. Herein, we utilize a murine-tropic coronavirus (beta coronavirus) MHV-A59 along with a mouse model and measures of pathology (lung weight/dry ratios and histopathology) and inflammation (ELISAs and cytokine array panels) to examine whether vaping may exacerbate the pulmonary disease severity of coronavirus disease. While vaping alone did result in some noted pathology, mice exposed with intranasal vaped e-liquid suffered more severe mortality due to pulmonary inflammation than controls when exposed to coronavirus infection. Our data suggest a role for vaping in increased coronavirus pulmonary disease in a mouse model. Furthermore, our data indicate that disease exacerbation may involve calcium (Ca^{2+}) dysregulation, identifying a potential therapeutic intervention.

Keywords: coronavirus, inflammation, E-cigarette, spirometry, cytokines

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INTRODUCTION

From late December 2019 to current, the novel coronavirus SARS-CoV-2 has emerged as a highly communicable respiratory virus. While many patients infected with SARS-CoV-2 do not exhibit severe or life-threatening symptoms, approximately 5% go on to develop the potentially lethal disease known as COVID-19 (Bray et al., 2020). COVID-19 has a significant impact on the

pulmonary system that includes pneumonia-like bilateral infiltrates, which are clearly visible by X-ray (Omer et al., 2020). Many patients go on to require oxygen and often need to be mechanically ventilated. In addition to COVID-19 lung disease, there are additional extrapulmonary effects, which include cardiomyopathy and potentially neurological and renal effects. To date, there are no effective therapies for COVID-19, and the mortality rate remains high, both nationally and worldwide. While disease burden appears to be more significant among older minority populations, the disease is significant worldwide regardless of age, gender, or racial associations (Bray et al., 2020). It is likely that unknown risk factors may contribute to disease severity and must, therefore, be further investigated (Tsatsakis et al., 2020).

Electronic cigarettes (E-Cigs) are a relatively new and novel method of tobacco consumption. E-Cigs differ from conventional cigarettes in that they contain no combustible tobacco but rather utilize a battery-operated coil to heat and aerosolize the nicotine (or marijuana or CBD if present) in a liquid vehicle (e-liquid) to the lungs (Besaratinia and Tommasi, 2014). In a relatively short time, E-Cig sales and usage have penetrated most countries worldwide, with high levels of usage in Asian, European, and American markets (Hammond et al., 2019; Huang et al., 2019; Mallock et al., 2020). This relatively new and fast-growing subset of nicotine users, described as "vapers" rather than smokers, utilize products that are very efficient at delivering nicotine so that plasma nicotine levels comparable to those observed with conventional tobacco smoking have now been recorded (Etter and Bullen, 2011). However, since E-Cigs have only recently been available to the population at large (∼10 years), relatively little is known about their physicochemical properties and as to whether long-term E-Cig use will result in respiratory diseases similar to cigarette smoke, none at all, or something entirely different (Davis et al., 2017; Urman et al., 2018). Initially generally regarded as safe (GRAS) by the FDA, recently accumulated data indicate the adverse effects of E-Cig intake and have demonstrated the need for better assessment and regulation of e-liquids as necessary for population safety (Orzabal et al., 2019). Furthermore, there is concern that E-Cig consumption may emerge as an additional variable contributing to increased severity of COVID-19-related pulmonary disease across the worldwide population (Tsatsakis et al., 2020).

Published work has demonstrated murine hepatitis virus (MHV) as a causative agent of SARS-like pneumonia after intranasal exposure in mice (Yang et al., 2014). As is the case with both SARS-CoV1 and CoV2, MHV is a class II beta-coronavirus that also uses a spike protein to enter cells. However, whereas SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) and is then cleaved by ACE2 and TMPRSS2 during the entry process (which may also involve cleavage by the intracellular convertase furin), MHV spike protein binds to carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (Hingley et al., 1998; De Albuquerque et al., 2006; Shang et al., 2020). Although MHV-A59 receptor binding differs from SARS-CoV-2, MHV-A59 spike protein is also cleaved by furin and trypsin-like serine proteases. When administered to mice intranasally (IN) at sub-lethal doses, MHV-A59 causes progressive pneumonia that is characterized by alveolar damage, severe weight loss, and inflammation, including significant increases in IL-1β, IL-6, and TNFα (Yang et al., 2014). A similar lung disease has been observed with the MHV-1 strain (De Albuquerque et al., 2006). Together, these data indicate that MHVs are excellent models of SARS/MERS-like disease. We have recently developed a novel method to expose mouse models and cell lines to an intranasal (IN) vaped e-liquid condensate, which we will herein refer to as "vape" or a "vaped" e-liquid, and used this reagent to evaluate *in vivo* pulmonary function and *in vitro* toxicity. Using this model, we questioned how "vaped" mice would respond to a coronavirus pulmonary challenge.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all reagents and materials were purchased from either Thermo Fisher Scientific (Waltham, MA, United States) or Sigma-Aldrich (St. Louis, MO, United States) at the highest level of purity possible. A 50-mM 2-APB stock was first made up in DMSO, which was then diluted with sterile PBS to 500 μM, as has been previously described for the intravenous use of 2-APB in a mouse model (Morihara et al., 2017).

Purchase of E-Liquid Products and Vape Distillate Generation

The e-liquids ("Mint" JUUL pods) were purchased locally from retailers in Durham, NC, United States, between July 1, 2020 and October 15, 2020. These products were inventoried and stored at room temperature until used. Manufacturer's label information stated that ingredients include only vegetable glycerin (VG), propylene glycol (PG), nicotine, flavoring, and benzoic acid, with each pod containing 0.7 ml of the flavored fluid at 3% nicotine.

The e-liquid was vaped using a previously described (Panitz et al., 2015) apparatus to produce an e-liquid vapor distillate. Briefly, e-liquid vapors were produced using a JUUL E-Cig device (battery powered with a prefilled pod) connected to a silicon tubing and to the mouthpiece of the JUUL E-Cig on one end. The other end was placed in the lower part of a 50-ml conical tube, in which the distillate was condensed and collected, suspended above liquid nitrogen inside a thermal container. The JUUL device was utilized for periods of up to 5 s with at least 10 s between activations to simulate "puffs." To reduce the chance of "dry puffing" the e-liquid pods, only three-fourths of a pod fluid was vaped, which occurred over an ∼3-h duration per pod. The vaped e-liquid condensates were then stored at −20°C until they were used.

The vaped e-liquid was analyzed for nicotine content using a previously published (Pagano et al., 2015) GC/MS protocol (Mass Spectrometry Facility, Louisiana State University, Baton Rouge, LA, United States) and determined to contain a nicotine concentration of ∼2 mg/ml, which is in contrast to the unvaped e-liquid, 35 mg/ml (Jackler and Ramamurthi, 2019). Because each JUUL pod (0.7 ml) can produce ∼200 "puffs"[1], a "puff"

[1] juul.com

should contain ∼125 μg of nicotine. However, for our studies, we utilized 10-μl (20 μg nicotine) doses of our vaped e-liquid (∼2 mg/ml) in an attempt to minimize any potential morbidity after assessing the effects of several volumes (5, 10, and 20 μl). During gross examination, the latter two doses did not exhibit a noticeable difference (data not shown). Therefore, and again being mindful of minimizing potential morbidity, we chose to use the 10-μl dose.

Cells and Virus

A549 (CCL-185) and CALU-3 (HTB-55) cells were also obtained from ATCC and maintained in DMEM (Dulbecco's modified Eagle medium) and MEM (minimum essential medium) alpha (Gibco, Thermo Fisher Scientific), respectively, supplemented with 10% fetal bovine serum (GE Healthcare-HyClone, VWR International) and 100 U/ml of penicillin and 100 μg/ml of streptomycin (BioWhittaker-Lonza from VWR International), 1% L-glutamine (GE Healthcare-HyClone), 1% non-essential amino acids (GE Healthcare-HyClone), and 1% pyruvate (Gibco). Both cell lines were routinely cultured per their instructions and maintained at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2.

Coronavirus strain MHV-A59 and delayed brain tumor (DBT) cells were kind gifts from the laboratory of Ralph Baric (The University of North Carolina at Chapel Hill, Chapel Hill, NC, United States). DBT cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 0.05 μg/ml of gentamicin and 0.25 μg/ml of kanamycin. DBT cells express a relatively uniform and abundant amount of MHVR, the receptor for MHV-A59 docking and entry into cells. Thus, the virus was both generated and quantified [by standard plaque assay to determine plaque-forming units (PFUs), which are also known as infectious units (IUs) (Langlet et al., 2007)] using DBT cells for all experiments.

Cell Treatments and Viability Assay

A549 (2.0×10^4) or CALU-3 (3.0×10^4) cells were plated in 96-well black-walled tissue culture dishes (Costar catalog #3603 Millipore-Sigma; St. Louis, MO, United States) and grown overnight. E-liquids were serially diluted into the appropriate complete medium to produce the desired final concentrations and administered to the cells for 24 h.

Cell viability was determined using a resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide)-based assay (Acros Organics; Fair Lawn, NJ, United States). The resazurin stock solution (1 mg/ml) was prepared in diH_2O and added to the 96-well assay plates for a final concentration of 0.1 mg/ml. After the treatments, 10 μl of the dye was added to 100 μl of complete culture medium in each well. After 3 h of incubation in 5% CO_2 (37°C), fluorescence was measured using a PHERAstar Microplate Reader (BMG Labtech; Durham, NC, United States) and the appropriate filter set (ex: 540 nm, em: 590 nm). The relative fluorescence of the mock-treated cells was then arbitrarily converted to 100% for cell viability.

Mice and Treatments

All mice were obtained from The Jackson Laboratory (Bar Harbor, ME, United States). Young adult mice (6- to 8-week-old male and female C57-BL/6J) were used for all experiments (Finlay and Darlington, 1995). After being received, the mice were allowed to acclimate and recover from shipping stress for 1 week in the NCCU Animal Resource Complex, which is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal care and use were conducted in accordance with the guide for the care and use of the laboratory animals (National Institutes of Health), and mice were maintained at 25°C and 15% relative humidity with alternating 12-h light/dark periods.

Once acclimated, mice were provided anesthesia (isoflurane via a SomnoSuite system), and the e-liquid distillate, vehicle control [50:50 (vol/vol) PG/VG] or saline (10 μl) was delivered dropwise intranasally (IN) using a micropipette, as has been previously described (Miyashita et al., 2018; Gotts et al., 2019), once daily for 3 days to each animal in the appropriate treatment group. After these initial treatments, MHV-A59 infection proceeded IN within a 24-h time period, with the mice anesthetized via an i.p. injection of ketamine (100 mg/kg) and xylazine (50 mg/kg) prior to infection.

Previous studies have demonstrated peak effects of IN infections with 1.5×10^4–1.5×10^6 PFU MHV-A59/mouse at days 5–6 post-infection (p.i.). For our studies, the mice were inoculated with MHV-A59 or vehicle (naïve), and body weight was monitored daily per IACUC protocols for 2–8 days p.i. We chose a broad time period to capture potential differences in infection outcomes between treatments (**Figure 2A**). At time points of experimental completion, mice were humanely euthanized using an overdose of sodium pentobarbital, as per our accepted animal protocol.

Cytokine Analysis

At experimental endpoints, bronchoalveolar lavage (BAL) was performed, and supernatants were isolated for cytokine analysis. Inflammatory cytokine proteins were evaluated using ELISA (OptEIA, BD Pharmingen) or Millipore Milliplex reagents and a Luminex 200 system (Millipore Sigma, Burlington, MA, United States).

Spirometry

Spirometry analysis was conducted using a SomnoSuite low-flow anesthesia system (Kent Scientific Corporation, Torrington, CT, United States). Briefly, mice were sedated with ketamine/xylazine and then attached to a nose cone to monitor average peak CO_2 wave forms for ∼2–3 min.

Histopathology

At time points of experimental completion, mice were injected with an overdose of sodium pentobarbital, and then lungs were inflated with 1 ml of 10% neutral-buffered formalin, then removed and suspended in 10% formalin for 12 h. Lungs were washed once in PBS and then immersed in 70% ethanol. Tissues were then embedded in paraffin, and three 5-μm sections 200 μm

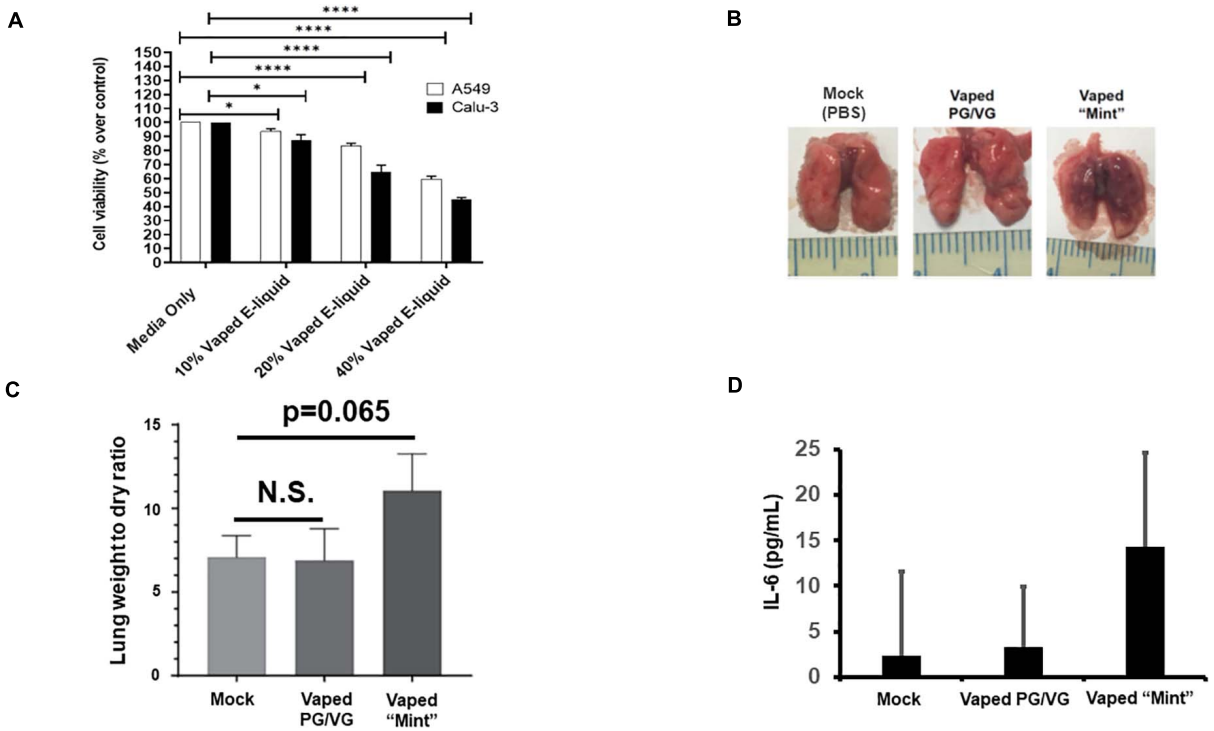

FIGURE 1 | Acute *in vitro* and *in vivo* models of vaping indicate cytotoxicity and pro-inflammatory responses. **(A)** A549 (2.0×10^4) and CALU-3 (3.0×10^4) cells were plated overnight in 96-well plates. Vaped e-liquids were added at the indicated concentrations (vol/vol) in complete medium. The plates were then incubated for 24 h, stained for viability, and read using a PHERAstar plate reader. Media-only mock control treatments were also performed. n = 18–24 wells per pretreatment. **(B)** Mice [four mice (two males and two females)/ group] received either PBS (mock), vaped PG/VG vehicle, or vaped "Mint" e-liquid (10 μl, containing ∼20 μg of nicotine) once daily intranasal (IN) for 3 days and were then sacrificed on the 4th day. Representative macroscopic images of dissected lungs are displayed. **(C)** Lung wet/dry weight ratios of mice receiving either PBS (mock), vaped PG/VG vehicle, or vaped e-liquid. n = 4 mice (two males and two females) per treatment group. **(D)** The indicated whole lung supernatants were evaluated for IL-6 abundance via ELISA. ANOVA was performed to compare among different groups and compared with the mock or media only treatment using Dunnett's *post hoc* test. Symbols and bars represent the mean ± SEM compared with the media only treatment (*$P < 0.05$, ****$P < 0.001$). N.S., not significant.

apart per lung were stained with hematoxylin/eosin (H&E) for examination by the NCCU histopathology core (directed by Dr. X. Chen). Sections were evaluated blindly for gross pathology, and disease score was evaluated as a measure of average pixel number density (pixelation) from multiple images per group using ImageJ software (Harris et al., 2018).

Statistics

Power analysis was performed using $\alpha = 0.05$ and power at 0.7. Statistics for analysis were performed using GraphPad Prism (La Jolla, CA, United States) and Microsoft Excel analysis. Appropriate statistical tests (Student's t-test, ANOVA) were determined after discussion with NCCU biostatistics faculty.

RESULTS

In vitro and *in vivo* Vaping Models Display Acute Signs of Toxicity and Inflammation

Our previous studies have evaluated the differences in toxicity of resting/"unvaped" e-liquids upon human pulmonary cells (Zhang et al., 2020). Though our data demonstrated clear increased inflammatory responses and toxicity associated with the e-liquids, our more recent work attempts to evaluate e-liquids after the "vaping" process, that is, heating the e-liquid to aerosolize it into an inhalable vapor. We have also recently developed a method to "vape" e-liquids and also to approximate an appropriate "puff" rate during the process, leading to the production of an e-liquid distillate or "vaped" e-liquid.

For our preliminary studies, we exposed A549 and CALU-3 human pulmonary epithelial cells to various concentrations of the vaped e-liquid, with toxicity assessed after 24 h (**Figure 1A**). Our results clearly delineate an increasing cytotoxicity with increasing concentrations (vol/vol) of the vaped e-liquid. We next evaluated the toxicity of our vaped e-liquid using our mouse model. To evaluate *in vivo* pathology, we delivered this same vaped e-liquid to mice [10 μl, containing ∼20 μg of nicotine, which is an equivalent amount of nicotine compared with the 10% cell treatments (**Figure 1A**), intranasal] intranasal (IN) for 3 days to evaluate acute pathology associated with vaping. On day 4, the mice were euthanized, and lungs were removed for analysis. Excised lungs from animals exposed to the vaped vehicle (PG/VG) visually appeared similar to the mock control, with

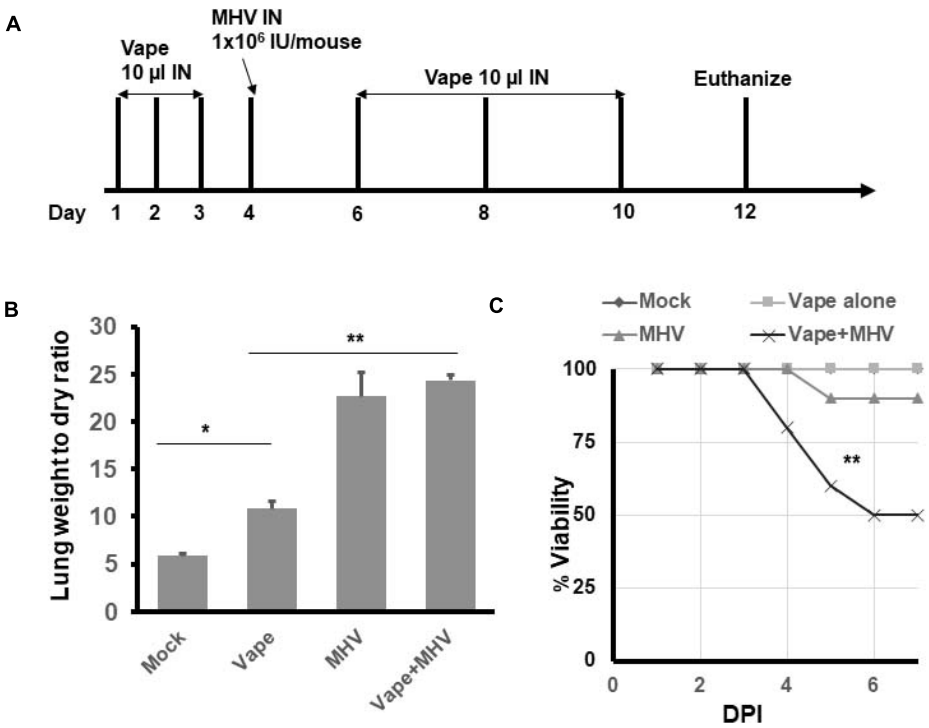

FIGURE 2 | Vaping contributes to increased pulmonary pathology in mice infected with MHV-A59. **(A)** Study design. **(B)** Lung wet/dry ratios of mice receiving either PBS (mock), vaped e-liquid, infection with MHV or vaped and infected with MHV (compared with mock). All lungs were harvested on day 12. $n = 4$ mice (two males and two females) per treatment group. **(C)** Viability study of vaped mice or mice infected with either MHV or vaped + infected with MHV (compared with mock). $n = 4$ mice (two males and two females) per group. The mock and vape-alone groups overlay each other so that the mock is not visible. ANOVA was performed to compare among different groups and compared with the mock control using Dunnett's *post hoc* test. Symbols and bars represent the mean ± SEM compared with the mock control (*$P < 0.05$, **$P < 0.01$). N.S., not significant.

no visible signs of inflammation or necrosis. However, upon examination, the lungs from the vaped e-liquid-exposed mice appeared both inflamed and darkened (**Figure 1B**). Lung weights were then measured to evaluate the "wet/dry" ratio (Parker and Townsley, 2004; Xu et al., 2006), which is a clinical measure of acute lung injury (**Figure 1C**). From the whole lung soluble lysate, we also observed an increase in the pro-inflammatory cytokine IL-6 in the mice exposed to the vaped e-liquid, while the vehicle-treated mice were more similar to the mock-treated animals (**Figure 1D**).

Modeling a SARS-Like Infection in Vaping Primed Lungs

To evaluate the effects of vaping on coronavirus pathogenesis, the murine coronavirus MHV-A59 was selected due to its published ability to induce SARS-like pneumonia in IN-exposed mice (Yang et al., 2014). Using our acute vape exposure model, we exposed animals to 1×10^6 infectious units (IU) of MHV-A59 IN (a published sub-lethal dose) and monitored the mice for 8 days post-infection before euthanizing the animals (**Figure 2A**). We observed MHV-infected lung weights to be approximately five- and 2.5-fold higher than the mock and vape-alone treatment, respectively, indicating signs of clinical pneumonia associated with successful viral infection. While vaping appeared to exacerbate MHV-dependent pneumonia, this difference was not significant (**Figure 2B**). Viability of the mice was observed over time post-exposure to MHV (**Figure 2C**). While the vape-alone group suffered no mortality, the sub-lethal dose of MHV inoculum was confirmed by the limited mortality observed in the MHV-infected animals (MHV alone). However, mortality was significantly increased in animals exposed to the vaped e-liquid and MHV infection.

These results suggest that MHV infection leads to pulmonary pneumonia within our model and that the contribution of vaped e-liquid exposure to MHV-dependent pulmonary pathogenesis is a decreased survival rate for the co-exposed animals (**Figure 2C**).

A Role for Ca^{2+} Flux in MHV-A59-Infected and Vape-Primed Lungs?

Our previous work (Ghosh et al., 2020; Zhang et al., 2020) as well as the work of others (Rowell et al., 2020) have implicated the potential role of increased intracellular Ca^{2+} as the mechanism of e-liquid-induced cytotoxicity *in vitro* (**Figure 1A**). In particular, it has been questioned if e-liquids may activate endoplasmic reticulum (ER)-resident Ca^{2+} release as the mechanism of toxicity (Ghosh et al., 2020). To further examine this premise, we utilized our model and the well-described inositol triphosphate

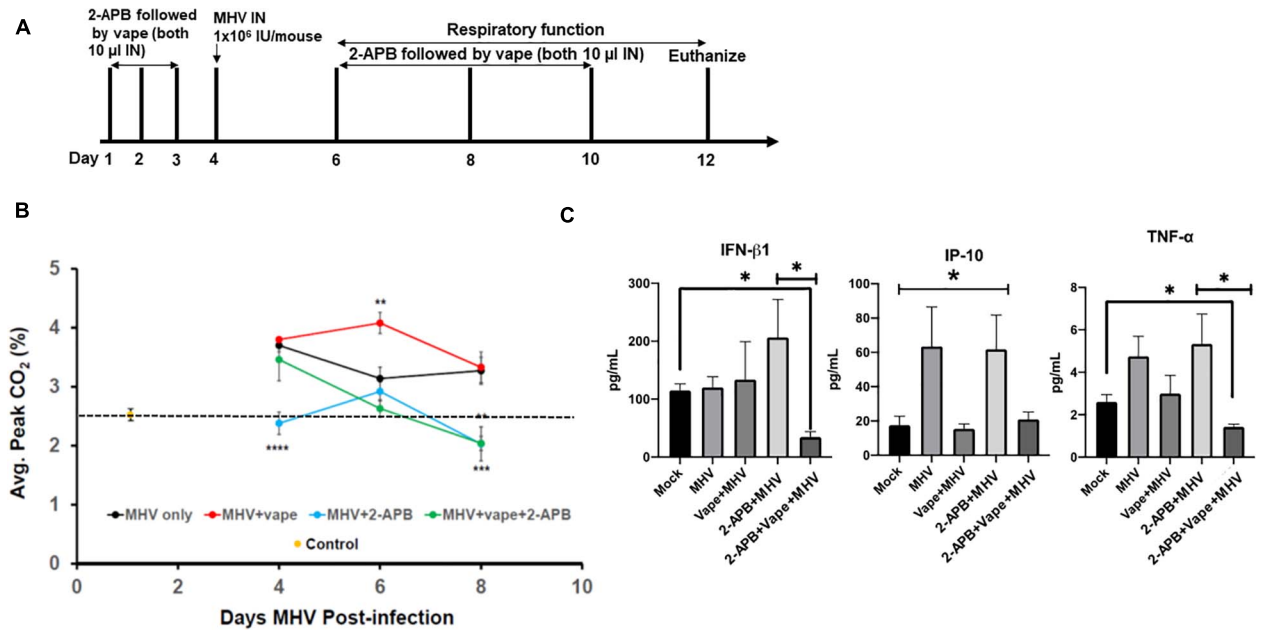

FIGURE 3 | 2-APB treatment reverses some of the effects of MHV infection in mouse lungs. **(A)** Study design. **(B)** Average peak CO_2 levels detected from the treatment groups 4, 6, and 8 days PI. The data were acquired using a SomnoSuite low-flow anesthesia system equipped with a nose cone. Mice were sedated with ketamine and monitored for ∼2–3 min to acquire peak CO_2 wave forms. *N* = 6 mice (three males and three females) per group. The dotted line is a reference point for the control. **(C)** Inflammatory cytokine analysis of BAL fluid from mice infected with MHV, vape + MHV, 2-APB + MHV, and 2-APB + vape + MHV (compared with mock control). ANOVA was performed for multigroup comparisons (Tukey's multiple comparison tests). The statistically significant IP-10 comparisons (*$P < 0.05$) are made between the mock and MHV groups, and the mock and 2-APB + MHV groups only. Symbols and bars represent the mean ± SEM compared with the mock control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$). N.S., not significant.

(IP_3) receptor antagonist 2-APB, i.e., the receptor that regulates an ER-resident calcium channel that releases calcium into the cytosol when activated (Maruyama et al., 1997).

Our acute vaping model followed by MHV infection on day 4 was performed in the presence and absence of 2-APB IN (10 µl of a 500-µM stock, which is 44 ng/g body weight) on each day the animals received the vape treatment, that is, days 1–3 prior to infection and days 2, 4, and 6 post-MHV infection (**Figure 3A**). However, in each case of dosing, the 2-APB was provided 30 min before the IN vaped e-liquid was dosed, that is, a prophylactic treatment. Our data indicate that the animals receiving the 2-APB treatment displayed improved respiratory function, as measured via spirometry (peak CO_2 output, SomnoSuite analysis), compared with either the MHV- or MHV + vape-treated groups, with the MHV + vape-treated group displaying the worst overall respiratory function (as seen on D6 of the time course, **Figure 3B**). Interestingly, 2-APB treatment also ameliorated respiratory function within MHV-alone groups, indicating an important role for Ca^{2+} signaling in viral pathogenesis in general. In addition, and in contrast to the treatment groups illustrated within **Figure 2C**, there was no mortality observed within the MHV + 2-APB- or MHV + vape + 2-APB-treated groups, i.e., 100% viability for these two groups was observed across the entirety of the treatment regime (data not shown).

Inflammatory cytokines were then assessed from bronchoalveolar lavage (BAL) fluid. MHV infection led to increases in pro-inflammatory cytokines, while the effect of the 2-APB treatment proved confounding and difficult to interpret (**Figure 3C**). For example, the 2-APB treatment did appear to increase the INF-β1 level when provided together with MHV infection though not significantly – a result we cannot currently explain. Our cytokine profiles from our preliminary analysis do appear to demonstrate the activation of TNF-α in the MHV-infected lungs, which is diminished to near mock-treated animal levels in the presence of the 2-APB treatment but only with the dual MHV + vape treatment. Again, the 2-APB + MHV treatment group has activated cytokine levels. In contrast, the IP-10 level is not increased in the MHV + vape group. Therefore, the 2-APB + vape + MHV treatment does not appear to alter the abundance relative to the mock control.

Importantly, the H&E lung histology of the MHV + vape + 2-APB-treated group was more similar to the control animals than to the MHV- or MHV + vape-treated groups (**Figures 4A,B**). Constriction of air space and consolidation of alveoli (demonstrative of viral pneumonia) were both demonstrated in MHV and vape + MHV lungs, while inflammation was diminished in the presence of 2-APB treatment.

In sum, these data suggest that vaping may significantly exacerbate the severity of pulmonary coronavirus infection, leading to increased pulmonary infiltrate of inflammatory cells (**Figures 4A,B**). Importantly, this disease burden may be mediated by Ca^{2+} signaling, such that a calcium antagonist may alleviate pathology.

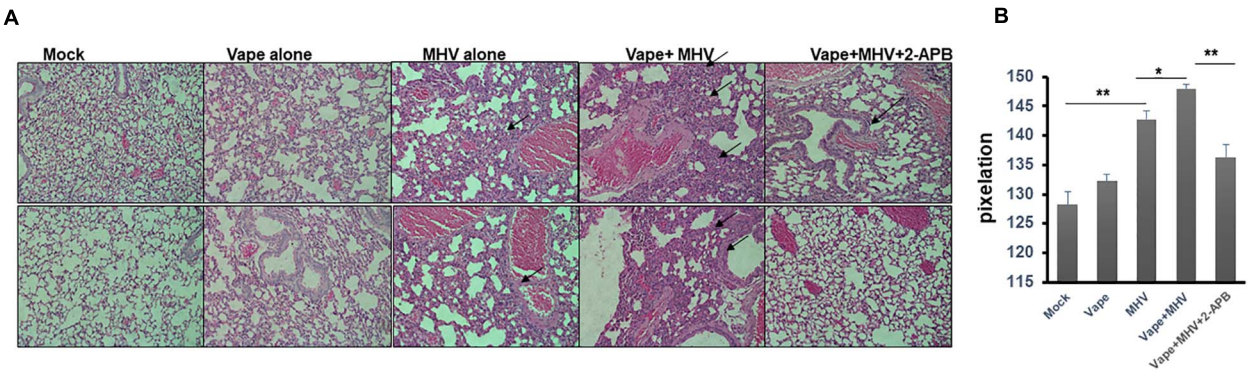

FIGURE 4 | Histopathology indicates that 2-APB treatment reverses some of the effects of MHV infection in mouse lungs. **(A)** Histopathology of lung tissues. H&E staining of sections of lung tissue isolated from mock-, vape alone-, MHV alone-, vape + MHV-, and vape + MHV + 2-APB-treated mice. Alveolar wall thickening and the infiltration of inflammatory cells into the interstitial spaces were particularly observable in the lungs from the MHV-alone and vape + MHV mice (indicated by arrows). However, these features were much less pronounced in the vape + MHV + 2-APB animals. 200× magnification. **(B)** Pixelation was quantified as a measure of inflammatory foci with five images per lung. Student's *t*-test was performed between groups. Symbols and bars represent the mean ± SEM compared with the mock control (*$P < 0.05$, **$P < 0.01$). N.S., not significant.

DISCUSSION

In the latter part of 2020, the COVID-19 pandemic brought to light a glaring lack of knowledge of the causative factors that may contribute to the severity of acute viral pneumonia due to SARS-CoV-2. SARS-CoV-2 has rapidly spread across the globe and affected a diverse populace, leading to a high variability in disease prognosis. Health professionals and infectious disease experts are still unclear as to which risk factors may contribute to disease severity. Tobacco smoking is known as a major risk factor for the development of disease and disability. Despite package health warnings, advertising bans, and increased taxes on tobacco products, an estimated 20% (1.3 billion people) of the world's population (36.5 million people in the United States) still smoke tobacco (Rom et al., 2013; Jamal et al., 2016). The role of vaping in pulmonary disease initiation and progression is still relatively unknown.

Herein, we have developed a mouse model to evaluate vaping/E-Cig exposure as a risk factor for coronavirus-dependent pulmonary disease. While exposing mice to vaped e-liquid IN, we observed increasing, though not significant, levels of acute inflammation (**Figure 1**), demonstrating pathology. Using this model, we went on to evaluate the effects of vaping upon coronavirus-dependent pulmonary pathogenesis using a mouse-tropic coronavirus strain, MHV-A59 (**Figure 2**). As we hypothesized, our preliminary studies indicate that vaped e-liquid increases the mortality and the pathology of MHV-induced pulmonary infection. However, vaping appears to dysregulate cytokine activation in our studies (**Figure 3C**), suggesting a complex and complicated role for vaping-related Ca^{2+} mobilization in inflammation and perhaps ultimately in respiratory disease development. This observation is pertinent and topical as E-Cig use (particularly among minors and young adults) rates are increasing (King et al., 2018), potentially indicating this population is at a greater risk for hospitalization due to coronavirus infection.

However, we must also acknowledge the limitations of our study. We employed *in vitro* immortalized pulmonary epithelial cells for our preliminary studies. As such, we have not yet performed similar studies with primary cells, which would provide more biological significance as the data obtained from immortalized cell lines do not always accurately replicate the data obtained when using primary cells (Kaur and Dufour, 2012). Such studies, which will also include using air–liquid interface (human airway epithelial cells) culture systems, will be incorporated into further works to evaluate the effects of vaping and MHV upon ciliated epithelial cell function as well as better model potential drug effects. Next, as indicated in **Figure 1**, our data indicate increasing trends for both lung weight/dry ratio and IL-6 level due to IN exposure to the vaped e-liquid. Even so, the data are not statistically significant, likely owing to the small sample size or "n" utilized within the context of this small preliminary study. Thus, statistical differences might be reached if using a larger sample size. Next, we have employed an intranasal route of exposure using a vaped e-liquid distillate, which does not exactly recapitulate the vaping experience. Our use of a condensed vaped e-liquid distillate has been termed an "intermediate approach" but is not a direct exposure route. In addition, the use of the e-liquid distillate does overcome some of the shortcomings of direct exposure. For example, a weakness of direct exposure routes is that E-Cig topographies, also known simply as smoking behavior and including such characteristics as puff duration, are poorly understood and will change as new E-Cig devices emerge (DeVito and Krishnan-Sarin, 2018). This fact is in contrast to traditional combustible cigarette puff topographies, which are well studied and defined. Second, doses received during direct exposures can be variable and include such issues as dermal and oral absorption, i.e., mice licking deposited vape aerosols off of their fur when whole body exposure is performed, among others (Oyabu et al., 2016; Noel et al., 2018).

However, there is clearly value to direct exposure routes such as whole body and nose-only exposure routes, and we are working to develop these models, in particular, the nose-only exposure model, and to compare/contrast these direct exposure data with our own (Miyashita et al., 2018; Gotts et al., 2019).

Our previous work also focused on the role of Ca^{2+} signaling in E-Cig-related cytotoxicity *in vitro* (Zhang et al., 2020). Therefore, we evaluated whether an antagonist for calcium signaling (2-APB) could alter the prognosis of the animals within our treatment groups. Indeed, our results do suggest that pathology is diminished in the 2-APB-treated mice [both the spirometry and gross pulmonary histology (**Figures 3**, **4**)], suggesting potential novel therapeutic interventions that may currently exist and that can be improved and repurposed. For example, based upon the understood mechanism of 2-APB, which is to perturb the ER stress pathway by inhibiting ER-resident Ca^{2+} release into the cytosol (Maruyama et al., 1997), 2-APB could potentially prevent viral replication (Tanaka et al., 2013; Jiang et al., 2020). Again, the potential pro-inflammatory role of 2-APB will require a more comprehensive study design that includes more subjects and variable times, doses, and types of exposures to confirm our results in future studies. However, it is possible that the normal lung response to MHV is to increase IP-10 and TNF-α levels, although these data were not significant in our study. Therefore, with the dual exposure, i.e., adding the vape treatment, this antiviral response is prevented. This lack of a response may be more apparent in the animals who died, which is an avenue of study for our future directions.

In sum, our model suggests that vaping exacerbates coronavirus-dependent pulmonary disease in mice. However, the exact mechanism of disease in MHV-infected and E-Cig condensate-treated mice remains to be established, which will benefit from our future studies that will include larger cohorts and a more robust and rigorous experimental design. Therefore, this model has a potential use for testing promising therapeutic interventions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the North Carolina Central University IACUC.

AUTHOR CONTRIBUTIONS

VS and RO were responsible for the study concept and design, acquiring the data, data analysis, manuscript writing, manuscript editing, and study supervision. De'JP, RZ, and MJ were responsible for acquiring the data, data analysis, and manuscript editing. All authors contributed to the article and approved the submitted version.

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Persistently Increased Systemic ACE2 Activity Is Associated With an Increased Inflammatory Response in Smokers With COVID-19

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Background: Tobacco smoking is known to be involved in the pathogenesis of several cardiopulmonary diseases. Additionally, smokers are highly susceptible to infectious agents due to weakened immunity. However, the progression of lung injury based on SARS-CoV-2-mediated COVID-19 pathogenesis amongst smokers and those with pre-existing pulmonary diseases is not known. We determined the systemic levels and activity of COVID-19 associated proteins, cytokine/chemokines, and lipid mediators (lipidomics) amongst COVID-19 patients with and without a history of smoking to understand the underlying susceptible factor in the pathogenesis of COVID-19.

Methods: We obtained serum from healthy (CoV−), COVID-19 positive (CoV+), and COVID-19 recovered (CoV Rec) subjects with and without a history of smoking. We conducted a Luminex multiplex assay (cytokine levels), LC/MS (eicosanoids or oxylipin panel), and ACE2 enzymatic activity assays on the serum samples to determine the systemic changes in COVID-19 patients.

Results: On comparing the levels of serum ACE2 amongst COVID-19 (positive and recovered) patients and healthy controls, we found a pronounced increase in serum ACE2 levels in patients with COVID-19 infection. Furthermore, ACE2 enzyme activity was significantly increased amongst COVID-19 patients with a smoking history. Also, we analyzed the levels of Angiotensin 1–7 (Ang1–7) peptide, the product of enzymatic action of ACE2, in the serum samples. We found significantly high levels of Ang1–7 in the serum of both CoV+ and CoV Rec patients. Our data further demonstrated a smoking-induced increase in serum furin and inflammatory cytokine [IFN_{γ}($p = 0.0836$), Eotaxin ($p < 0.05$), MCP-1 ($p < 0.05$), and IL-9 ($p = 0.0991$)] levels in COVID-19 patients as compared to non-smoking controls. Overall, our results show that smoking adversely affects the levels of systemic inflammatory markers and COVID-19 associated proteins, thus suggesting that COVID-19 infection may have severe outcomes amongst smokers.

Keywords: COVID-19, inflammation, ACE2, furin, smokers

Abbreviations: COVID-19, Coronavirus Disease-2019; ACE2, Angiotensin converting Enzyme 2; GM-CSF, Granulocyte-macrophage Colony stimulating Factor; HIV, Human Immunodeficiency Virus; COPD, Chronic obstructive pulmonary disease; ARDS, Acute Respiratory Distress Syndrome.

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INTRODUCTION

The current pandemic of Coronavirus disease 2019 (COVID-19) has emerged as a significant public health threat worldwide. Viral pneumonia and acute respiratory failure are the most common clinical manifestations of severe COVID-19, featuring fever, cough, hypoxemia, dyspnea, and bilateral infiltrates on chest radiography (Brosnahan et al., 2020). In humans, SARS-CoV2 binds to the ACE2 (angiotensin-converting enzyme 2) receptor. ACE2 is abundant in the lung epithelium, specifically type II pneumocytes, goblet cells, nasal epithelial/ciliated cells and oral mucosa (Kaur et al., 2020; Sungnak et al., 2020; Ziegler et al., 2020). It converts angiotensin II (Ang II) to angiotensin 1–7 (Ang1–7), a metabolite known to exert vasodilatory effects and oppose the actions of the ACE2 homolog, ACE, within the cell. However, since the onset of this pandemic, ACE2 is being widely studied in the context of COVID-19 (Zamorano Cuervo and Grandvaux, 2020). In this regard, the specific nature of changes in ACE2 levels and activity induced by SARS-CoV-2 infection remains elusive.

With the continual spreading of the virus and reports of novel mutant strains causing further alarm and panic, questions regarding COVID-19 risk factors have become even more urgent. While old age, heart disease, hypertension, and diabetes are the universally accepted risk factors for COVID-19, many other factors are subject to debate. One such risk factor is smoking. While initial reports on COVID-19 risk factors have indicated little to no risk amongst smokers, recent data suggest otherwise (Grundy et al., 2020; Kaur et al., 2020). A meta-analysis of 15 studies with a total of 2473 confirmed COVID-19 patients reported that COPD patients (63% vs. 33.4 in people without COPD) and current smokers (22%) are at a higher risk of severity and mortality due to COVID-19 compared to disease-free and non-smoking individuals, respectively (95% confidence interval) (Alqahtani et al., 2020; Leung and Sin, 2020). Similar findings were reported by Patanavanich and Glantz (2020), whose research suggested that smoking nearly doubles the rate of COVID-19 progression amongst patients. Despite these findings, the exact pathogenesis of COVID-19 and the clinical features resulting in severe outcomes amongst smokers is largely unexplored.

Considering this lack of research and the growing call of concern for further understanding about the COVID-19 risk-factors, we investigated whether a variation in systemic markers for inflammation and COVID-19 infection existed between smokers and non-smokers. We also studied the gender-based differences in the levels and activity of COVID-19 related proteins (ACE2 and Furin) to understand disease pathogenesis amongst both sexes. Evidence from previous literature has suggested upregulated levels of ACE-2 in the lungs of smokers (Cai et al., 2020; Leung et al., 2020). However, there is no evidence correlating this increased expression to COVID-19 disease development and associated susceptibility. Our study investigates the relationship between COVID-19 associated proteins and shows a marked increase in the lung-to-serum inflammatory spillover amongst COVID-19 positive patients with a smoking history compared to controls. Lipid profiling further shows slight increases in the levels of prostaglandins ($F2_{\alpha}$), 15-hydroxyeicosatetraenoic acid (15-HETEs) and 5(6)-epoxyeicosatrienoic acid (5(6)-EET) in serum collected from COVID-19 positive patients as compared to COVID-19 recovered individuals.

MATERIALS AND METHODS

Ethics and Approvals

All the procedures performed in this study comply with the protocols approved by the Institutional Biosafety committee at the University of Rochester Medical Center, Rochester, NY, with an approval number Rahman/102054/09-167/07-186. The patient samples and information used in this study were procured from a commercial provider- BioIVT (Westbury, NY, United States). All the laboratory procedures were performed per the regulations specified by the BSL2+ level of containment for Clinical and Research Safety.

Human Blood Serum Collection

Sera from healthy (CoV−), COVID-19 positive (CoV+), and COVID-19-Recovered (CoV Rec) subjects were obtained from BioIVT (Westbury, NY, USA). The samples were collected between April-July, 2020. Disease status was confirmed by BioIVT using RT-PCR and/or antibody (Diazyme serological assay) test for COVID-19. The patients grouped as "COVID-19 recovered" were tested "positive" and were determined convalescent 30 days post symptoms per the CDC guidance. The patient population was categorized based on smoking status; both current and previous tobacco smokers were considered "Smokers" for subsequent analyses. The patient population that has "never smoked" is termed as non-smokers. The characteristics of the study subjects used for the experiments are presented in **Table 1**.

Assessment of Pro-inflammatory Mediators in Blood Sera Using Luminex Multiplex Assay

The levels of pro-inflammatory cytokines/chemokines like MCP-1, IL-8, IFN_{γ}, TNF-α, and IL-7 in the sera were measured by Luminex multiplex assay using Bio-Plex Pro^{TM} Human cytokine 27-plex assay (Cat#M500KCAFOY, Bio-Rad Labs, Hercules, CA)

TABLE 1 | Characteristics of study subjects.

Characteristics	CoV−	CoV+	CoV Rec	*p*-value[a]
N	18	16	21	
Age (years), mean (SD)	36.055 (8.39)	39.75 (14.68)	45.5 (13.81)	0.4836
Male Sex, n (%)	9 (50)	8 (50)	10 (47.62)	0.0667
Smoker, n (%)	9 (50)	3 (18.75)	6 (28.57)	0.9333
Caucasian**	9 (50)	13 (81.75)	20 (90.23)	>0.9999

[a]*Kruskal-Wallis test.*
***p < 0.05 per Chi-square test.*

as per the manufacturer's directions. Blood sera were diluted fourfold. The levels of 27 pro-inflammatory mediators were measured using the Luminex FlexMap3D system (Luminex, Austin, TX) and plotted in pg/mL.

Assessment of Furin Levels Using ELISA

To determine the level of Furin in sera collected from CoV−, CoV+ and CoV Rec subjects, we employed a Human Furin ELISA kit (Cat # ab113322, Abcam, Cambridge, MA) as per the manufacturer's protocol. Colorimetric detection was performed at 450 nm using Cytation 5 microplate reader (BioTek Instruments, Inc. Winooski, VT). Furin levels were expressed as pg/mL.

Assessment of ACE2 Levels Using ELISA

We employed a commercially available Human ACE2 ELISA kit (Cat#ELH-ACE2, Ray-Biotech, Peachtree Corners, GA) to determine the ACE2 levels in the patient's serum samples, per manufacturer's directions. Colorimetric detection was performed at 450 nm using Cytation 5 microplate reader (BioTek Instruments, Inc., Winooski, VT) and serum ACE2 levels were measured in ng/mL.

Assessment of ACE2 Activity

We utilized the ACE2 Activity Assay kit (Cat #: K897 BioVision, Milpitas, CA, United States) to determine the ACE2 activity in the human serum samples. The assay was performed as per the manufacturer's instructions. In brief, serum samples were diluted by adding equal volumes of ACE2 Lysis Buffer and ACE2 Assay Buffer. In addition to the appropriate standards and controls (positive, negative, and background), the diluted serum samples were then added to a 96-well plate. After that, we added ACE2 substrate to both sample and control wells. Subsequently, fluorescence was measured at an excitation maximum of 320 nm and an emission maximum of 420 nm using Cytation 5 microplate reader (BioTek Instruments, Inc., Winooski, VT). Total protein content per sample was determined using the Bradford protein assay kit (Thermo Fisher Scientific, Waltham, MA). Sample ACE2 activity for each sample was calculated using the following formula:

$$\text{Sample ACE2 Activity} = B * D/(\Delta T * P)$$

where, B = Released MCA (cleaved product of ACE2 substrate) in Sample based on standard curve slope (pmol), ΔT = Reaction time (in min), P = Sample used (in mg), and D = Sample Dilution factor.

Assessment of Angiotensin II Levels Using ELISA

To determine the levels of Angiotensin II in sera collected from COVID-19 negative, COVID-19 positive, and COVID-19 recovered subjects, we utilized the Human Angiotensin II Competitive ELISA kit (Cat #: RAB0010, Sigma-Aldrich, St. Louis, MO) as per the manufacturer's protocol. The serum samples were diluted 20-fold for this experiment. Colorimetric detection was performed at 450 nm using Cytation 5 microplate reader (BioTek Instruments, Inc. Winooski, VT). Angiotensin II levels were expressed as pg/mL.

Assessment of Angiotensin I-7 Levels Using ELISA

We utilized the Human Angiotensin 1–7 ELISA kit (Cat #: NBP2-69078, NOVUS Biologicals, Littleton, CO) to determine the serum Ang1–7 levels as per the manufacturer's protocols. The serum was diluted 5-fold for this study. Colorimetric detection was performed at 450nm using the Cytation 5 microplate reader (BioTek Instruments, Inc. Winooski, VT). Angiotensin 1–7 levels were expressed as pg/mL.

Determination of Serum Eicosanoid/Oxylipins Levels Through Lipidomic Analysis

Serum eicosanoid/oxylipin profiling was outsourced to and performed by Cayman Chemical (Ann Arbor, MI). Lipid profiling was done using ultraperformance liquid chromatography in tandem with mass spectroscopy (UPLC-MS/MS). Lipidomes were prepared using serum from six different age-, sex-, and smoking status-matched patients from each group.

Nomenclature: The abbreviations used for various classes of lipids include the following:

6-keto $PGF1_{\alpha}$: 6-keto Prostaglandin $F_{1\alpha}$, TXB2: Thomboxane B_2, $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$, PGE2: Prostaglandin E_2, 12-HHTrE: 12-Hyrdoxyheptadecatrenoic acid, LTB4: Leukotriene B_4, LXA4: Lipoxin A_4, HETE: Hydroxyeicosatetraenoic acid, EET: epoxyeicosatrienoic acid, HODE: Hydroxyoctadecadienoic acid and HDHA: Hydroxy Docosahexaenoic Acid.

Lipid extraction: Lipid extraction from the serum samples was performed by protein precipitation followed by solid-phase extraction (SPE). Protein precipitation was performed by the addition of H_2O: Acetonitrile solution to each sample. After that, SPE was performed using Strata-X cartridges (33 μm, 200 mg/10 mL; Phenomenex, PA). The extracted lipids were finally dissolved in water/acetonitrile 60:40 (v:v) solution. To prepare the calibration curves, a mixture of the 20 calibration standards was prepared in methanol.

UPLC-MS/MS: Equal volumes of calibration standards and samples was added to Kinetex (2.6 μm C18 100 Å 100×2.1 mm, Phenomenex OOD-4462-AN) column, and Reverse phase liquid chromatography (LC) using Sciex ExionLC Integrated System was used for lipid separation. The lipid quantification in the samples was performed using Sciex 6,500+. The total amount of eicosanoids present in each sample was determined using MultiQuant software (Sciex). The lipid abundance ratios were calculated in terms of log base twofold change and plotted as a heat map using GraphPad Prism 8.0.

Statistical Analyses

All statistical calculations were performed using GraphPad Prism 8.0. Data was expressed as mean ± SE. Comparisons between two data groups were made using unpaired *t*-test or Mann Whitney's test, while One-way ANOVA was used for multiple comparisons.

Differences were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ when compared with respective controls.

RESULTS

Serum ACE2 Levels Are Increased by COVID-19 Infection

In humans, ACE2 binds to the SARS-CoV2 spike protein (Lan et al., 2020). In light of this, we first investigated changes in the levels of serum ACE2 amongst COVID-19 (positive and recovered) patients and healthy controls using an ELISA-based assay. Our results indicate a significant increase in the serum ACE2 levels on COVID-19 infection as shown in **Figure 1A**.

ACE-2 Activity Varies as a Function of Smoking History Among COVID-19 Patients

Next, we investigated the serum ACE2 activity in the patient sera from COVID-19 positive and recovered patients and compared it to healthy subjects. We found a significant increase in the serum ACE2 activity in both COVID-19 positive and COVID-19 recovered patients. We found a higher ACE2 activity in serum from COVID-19 recovered patients compared to serum from COVID-19 positive patients (**Figures 1Bi**). These results show that the ACE2 activity remains persistently high amongst COVID-19 patients.

To determine the smoking-related changes in the ACE2 function amongst smokers and non-smokers, we next compared the ACE2 activities amongst COVID-19 (positive and recovered) patients with and without a smoking history. We found a significant increase in the serum ACE2 activity from COVID-19 patients with a smoking history as compared to non-smokers (**Figure 1Bii**). ACE2 activity was more pronounced among male patients than females (**Figure 1Biii**). Our results show that smoking status plays a crucial role in governing the COVID-19 related enzyme activity in human subjects, suggesting a role in individuals' disease pathogenesis.

Differential Levels of Angiotensin II and Angiotensin 1–7 Based on COVID-19 Status

Having observed a differential ACE2 activity between smokers and non-smoker who have had a history of SARS-CoV-2 infection, we were next interested in comparing the Angiotensin II (Ang II) and Angiotensin 1–7 (Ang1–7) levels in our experimental groups. ACE2 catalyzes the conversion of Ang II to Ang1–7. Interestingly, our data indicated increased levels of Ang II and Ang1–7 in the serological samples from CoV+ and CoV Rec patients compared to healthy subjects (**Figures 2A**, **3A**). It is pertinent to mention however, that increase in the Ang1–7 levels was comparatively higher (∼6-fold) (**Figure 3A**) than that observed for Ang II (∼4-fold) (**Figure 2A**) in our experiments.

We further compared the Ang II and Ang1–7 levels based on the smoking history of our study participants. Here, we observed a noticeable increase in the ACE2 substrate (Ang II)

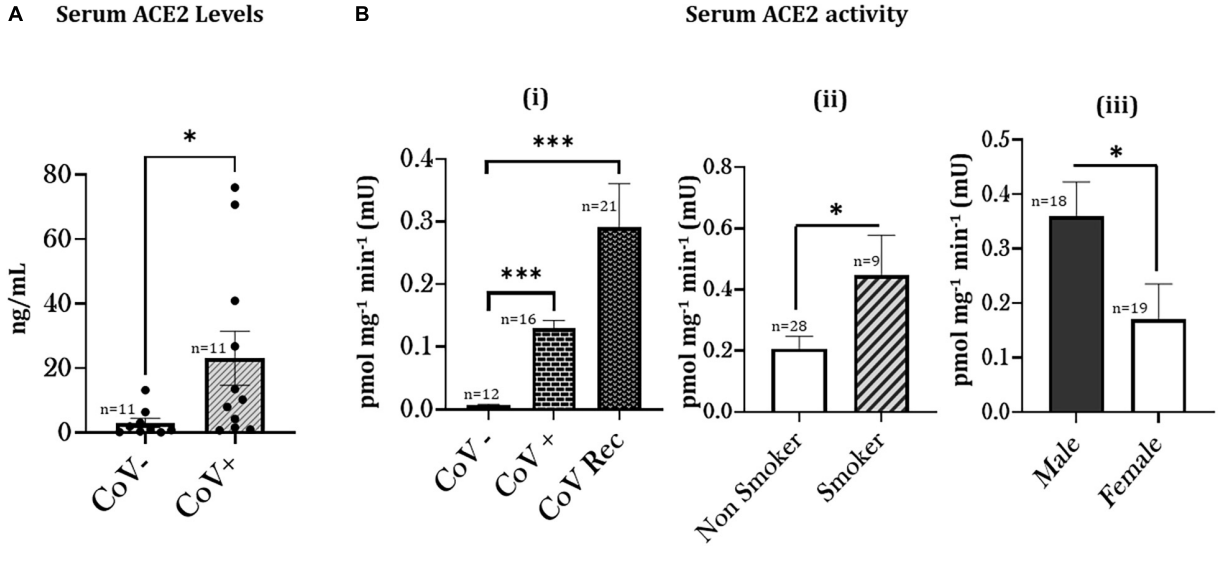

FIGURE 1 | Increased ACE2 levels and activity amongst COVID-19 patients with a smoking history. **(A)** Blood serum samples from COVID-19 negative (CoV–) and COVID-19 (positive and recovered) patients were obtained and the ACE2 level was quantitatively measured. $^{*}p < 0.05$ as per Mann Whitney's test for pairwise comparisons. CoV+: COVID19 positive (current and recovered) patients. **(B)** Patient Sera from healthy (CoV–), COVID-19 positive (CoV+), and COVID-19 recovered (CoV Rec) subjects were obtained, and ACE2 Activity was quantitatively measured. The obtained results were plotted based on serum ACE2 activity in (i) healthy subjects vs. COVID-19 positive and COVID-19 recovered patients. Data are shown as mean ± SEM. ($^{***}p < 0.001$, as per one-way ANOVA for multiple comparisons), (ii) COVID-19 (positive and recovered) patients with or without a smoking history, and (iii) COVID-19 patients based on their gender. Data are shown as mean ± SEM. $^{*}p < 0.05$, *** $p < 0.001$ as per unpaired *t*-test.

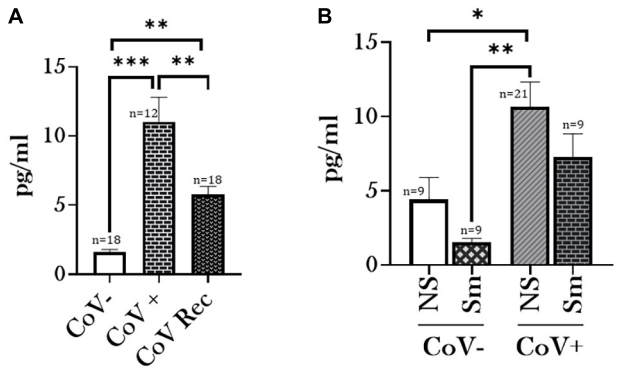

FIGURE 2 | Angiotensin II levels increase amongst patients with a history of COVID-19. Blood serum samples from healthy (CoV–), COVID-19 positive (CoV+), and COVID-19 recovered (CoV Rec)subjects were obtained and Angiotensin II (AngII) levels were quantitatively measured. The obtained results were plotted based on serum levels in **(A)** CoV–, CoV+, and CoV Rec subjects and **(B)** CoV- and COVID-19 (positive and recovered) patients with and without a smoking history. Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as per One-Way ANOVA for multiple comparisons. NS, Non-Smokers and Sm: Smokers.

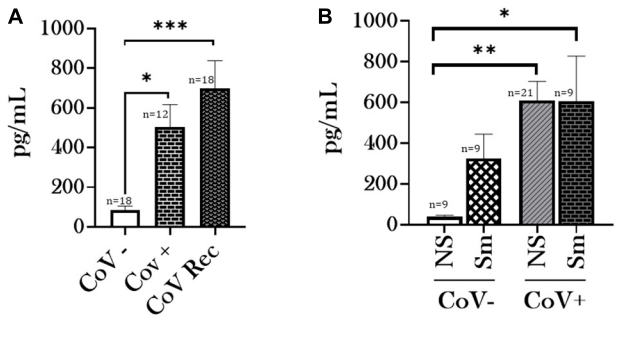

FIGURE 3 | Serum Angiotensin 1–7 levels increase amongst patients with a history of COVID-19. Blood serum samples from healthy (CoV–), COVID-19 positive (CoV+), and COVID-19 recovered (CoV Rec) subjects were obtained, and Angiotensin 1–7 (Ang1–7) levels were quantitatively measured. The obtained results were plotted based on serum levels in **(A)** CoV-, CoV+, and CoV Rec subjects and **(B)** CoV- and COVID-19 (positive and recovered) patients with and without a smoking history. Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as per One-Way ANOVA for multiple comparisons. NS, Non-Smokers and Sm: Smokers.

and product (Ang1–7) levels amongst non-smokers on COVID-19 infection as compared to healthy controls (**Figures 2B**, **3B**). Such an increase was not observed between COVID-19 positive and healthy individuals with a smoking history. Of note, we did not observe any gender-based disparity in the AngII or Ang1–7 levels amongst the CoV−, CoV+, and CoV Rec subjects in our study (data not shown).

Smoking Alters Furin Levels in Serological Samples

Another key to understanding COVID-19 virulence as a function of susceptibility to viral entry is analyzing changes in Furin levels. Unlike other Coronaviruses, SARS-CoV-2 has a lower dependence on target host-cell proteases and depends more on pro-protein convertase Furin for its viral entry (Johnson et al., 2021). Based on this, we measured Furin-levels in sera from CoV−, CoV+, and CoV Rec groups using ELISA. We observed a noticeable (though not significant) decrease in the serum Furin levels in COVID-19 recovered patient groups as compared to healthy controls. Though not significant, the serum Furin levels in CoV+ patients was also lower as compared to CoV− patients (**Figure 4A**). Likewise, the serum Furin level was lowered amongst smokers compared to non-smokers in patient sera collected from COVID-19 (positive and recovered) patients (**Figure 4B**). Though not shown here, the Furin levels were significantly higher amongst healthy smokers compared to healthy non-smokers (data not shown). Taken together, our results show that a SARS-CoV2 infection decreases the extracellular Furin levels irrespective of the smoking status.

Similarly, although not significant, Furin levels among females were elevated compared to males amongst COVID-19 (positive and recovered) patients (**Figure 4C**). However, due to the lack of enough gender-matched controls, we were unable to determine if such a gender-based variation is observed amongst healthy individuals.

Infection With SARS-CoV-2 Upregulates Pro-inflammatory Cytokine Levels in Smokers

It is well known that the severity of COVID-19 is associated with increased levels of pro-inflammatory mediators (Hojyo et al., 2020; Tang et al., 2020; Zhou et al., 2020). Given this, we analyzed the levels of 27 cytokines/chemokines in the serum samples from, CoV−, CoV+, and CoV Rec study populations using Luminex multiplex assay. Our results showed a significant increase in the levels of IL-8 and IL-1rα in COVID-19 positive patient sera compared to healthy controls (**Figure 5**). In contrast, the levels of IL-10 and PDGF-BB were significantly lowered amongst COVID-19 recovered patients as compared to healthy individuals. Parameters such as, IL-5, GM-CSF, IL-12(p70), and IL-15 were at non-detectable levels in the CoV+ and CoV Rec groups.

Intriguingly, when analyzing cytokine/chemokine levels in patient sera based on smoking status, a notable trend emerged. We noted a substantial increase in the production of pro-inflammatory markers like IFN_{γ} ($p = 0.0836$), MCP-1 ($p < 0.05$), and Eotaxin ($p < 0.05$) in the COVID-19 positive patient sera from those with a smoking history compared to the non-smoking controls. Furthermore, we found a moderate increase in the levels of IL-9 ($p = 0.0991$) amongst smokers infected with COVID-19 compared to COVID-19 positive non-smokers (**Figure 6**). It is important to mention here that the levels of inflammatory mediators in the serum did not show any gender-based variations in our study (data not shown).

Altered Serum Lipid Profile Amongst COVID-19 Positive Patients

It is known that infections can induce various alterations in lipid metabolism that can dampen inflammation or fight infection

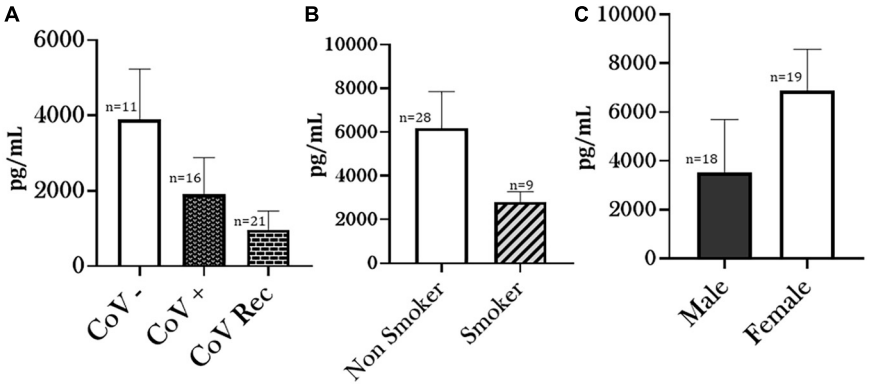

FIGURE 4 | Altered Furin levels amongst COVID-19 patients. Blood serum from healthy (CoV–), COVID-19 positive (CoV+), and COVID-19 recovered (CoV Rec) subjects were obtained, and the Furin levels were quantitatively measured. The obtained results were plotted based on serum Furin levels in **(A)** healthy subjects vs. COVID-19 positive or COVID-19 recovered patients, **(B)** COVID-19 (positive and recovered) patients with or without a smoking history, and **(C)** COVID-19 (positive and recovered) patients based on their gender. Data are shown as mean ± SEM.

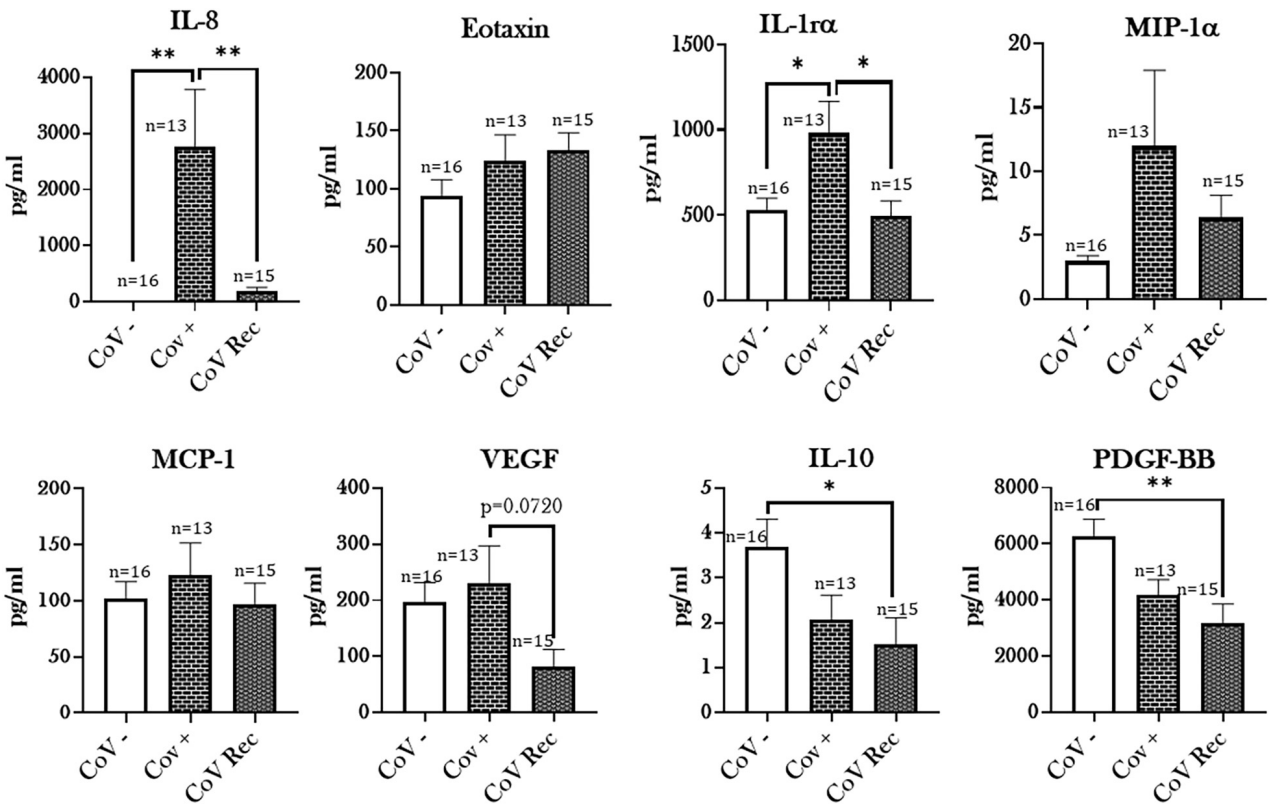

FIGURE 5 | Elevated cytokine levels in COVID-19 positive patients. Blood serum samples from healthy (CoV–), COVID-19 positive (CoV+), and COVID-19 recovered (CoV Rec) subjects were obtained, and the levels of cytokines/chemokines were assessed with the help of Luminex multiplex assay. The levels of detected cytokines were plotted. Data are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$; as per One-way ANOVA for multiple comparisons.

(Feingold and Grunfeld, 2000). Thus, we were next interested in studying the changes in the lipid profiles of patient sera from COVID-19 positive and COVID-19 recovered groups. **Figure 7A** depicts a generated heat map that shows alterations in the levels of the 17 most prevalent eicosanoids/oxylipins in sera from COVID-19 positive subjects compared to COVID-19 recovered individuals. Though none of the observed change was significant amongst CoV+ and CoV Rec groups, we found slight increase in the levels of $PGF2_{\alpha}$, 15-HETE, and 5(6)-EET in the COVID-19 positive patients as compared to the COVID-19 recovered patients (**Figure 7B**). The detailed account of the fold changes (Cov V+ vs. CoV Rec groups) in the levels of each of the studied

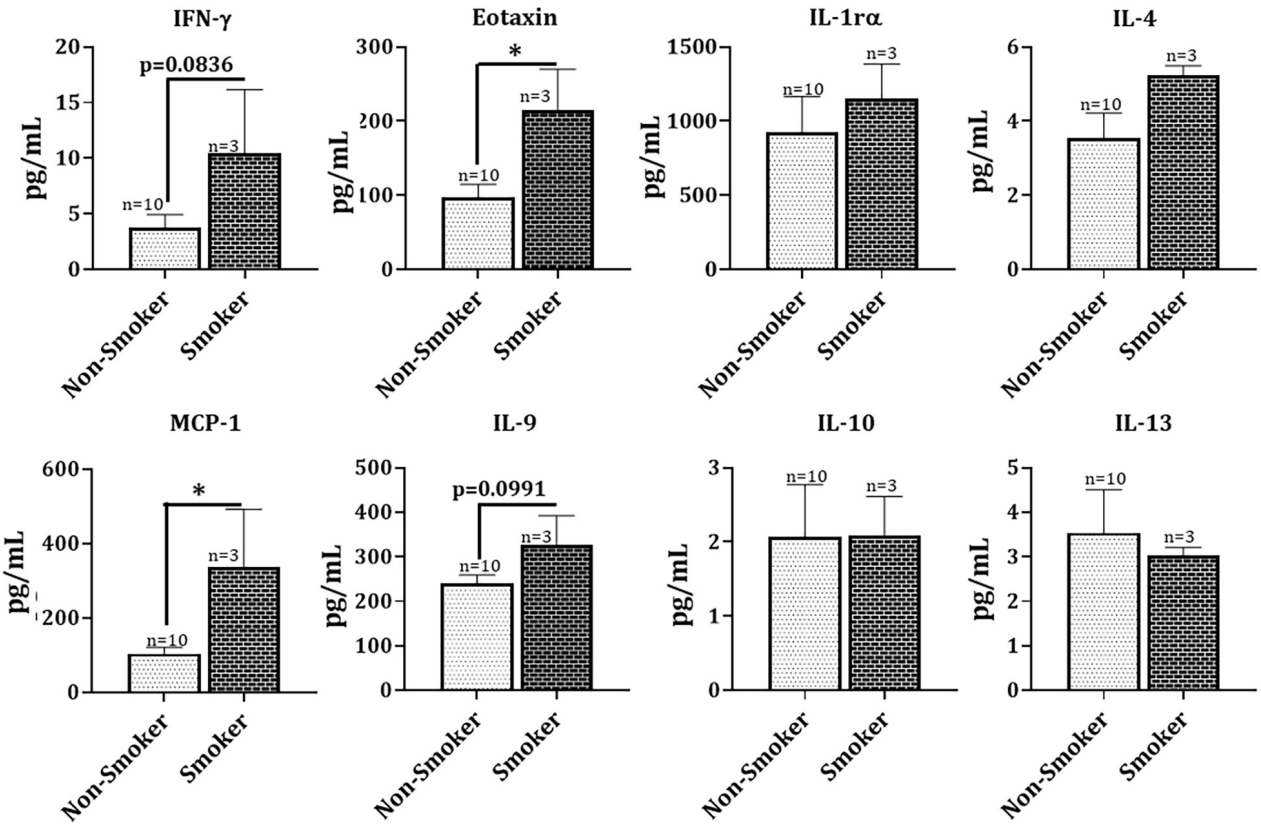

FIGURE 6 | Hyperinflammation in COVID-19 positive patients with a smoking history. Levels of cytokines/chemokines as assessed with the help of Luminex multiplex assay in blood serum samples from COVID-19 positive patients with and without a smoking history were collected. Data are shown as mean ± SEM. *$p < 0.05$, as per unpaired *t*-test.

lipids with *p*-values is provided in **Table 2**. It is pertinent to mention here that we did not find any statistically significant variations in the lipid profiles of COVID-19 positive and COVID-19 recovered patients based on age, sex, or smoking history.

DISCUSSION

The current pandemic of COVID-19 poses a serious threat to the global public health and health care. While the efforts of rolling out an effective vaccine are ongoing, there is panic and uncertainty about the new mutant strains of SARS-CoV2 (present as six variants worldwide so far), and long-term effects of COVID-19 infections (Baric, 2020; Mercatelli and Giorgi, 2020). It is important to identify the high-risk populations and the underlying differences in the disease pathogenesis to limit the viral spread efficiently. There have been contradictory pieces of evidence regarding the susceptibility of smokers toward COVID-19 infection. Still, recent work has demonstrated the association of smoking with adverse clinical outcomes in COVID-19 and emphasized the role of serum ACE2 levels in its pathogenesis (Saheb Sharif-Askari et al., 2020; Lowe et al., 2021). In this regard, we were interested in understanding the COVID-19 disease pathogenesis amongst smokers and investigated the systemic responses against SARS-CoV2 infection in COVID-19 positive, COVID-19 recovered, and COVID-19 negative subjects.

We first investigated the ACE2 levels in the serum from COVID-19 (positive and recovered) patients and healthy subjects. We found significantly high levels of ACE2 in the serum of COVID-19 positive individuals. ACE2 is well-known as the binding receptor for the SARS-CoV2 on the host's cell surface. Use of ACE inhibitors and ARB (Angiotensin II receptor blockers) in COVID-19 patients is being debated since the start of this pandemic (Behl et al., 2020; Kaur et al., 2020). On studying the ACE2 activity in serological samples from COVID-19 patients, we found a significant increase in the serum ACE2 activity in both COVID-19 positive and COVID-19 recovered subjects as compared to the controls. However, on comparing the ACE2 activities amongst COVID-19 patient (positive and recovered) cohorts, we found persistently increased ACE2 activity in COVID-19 recovered patients as compared to COVID-19 positive individuals. In this regard, Patel et al. (2021) have shown persistently elevated plasma ACE2 activity amongst patients with SARS-CoV2 infection. They further showed a direct correlation between disease severity and plasma ACE2 activity in their study participants. They speculate that this may be due to increased ectodomain shedding of ACE2 and could be responsible for the symptoms of prolonged ill-health amongst

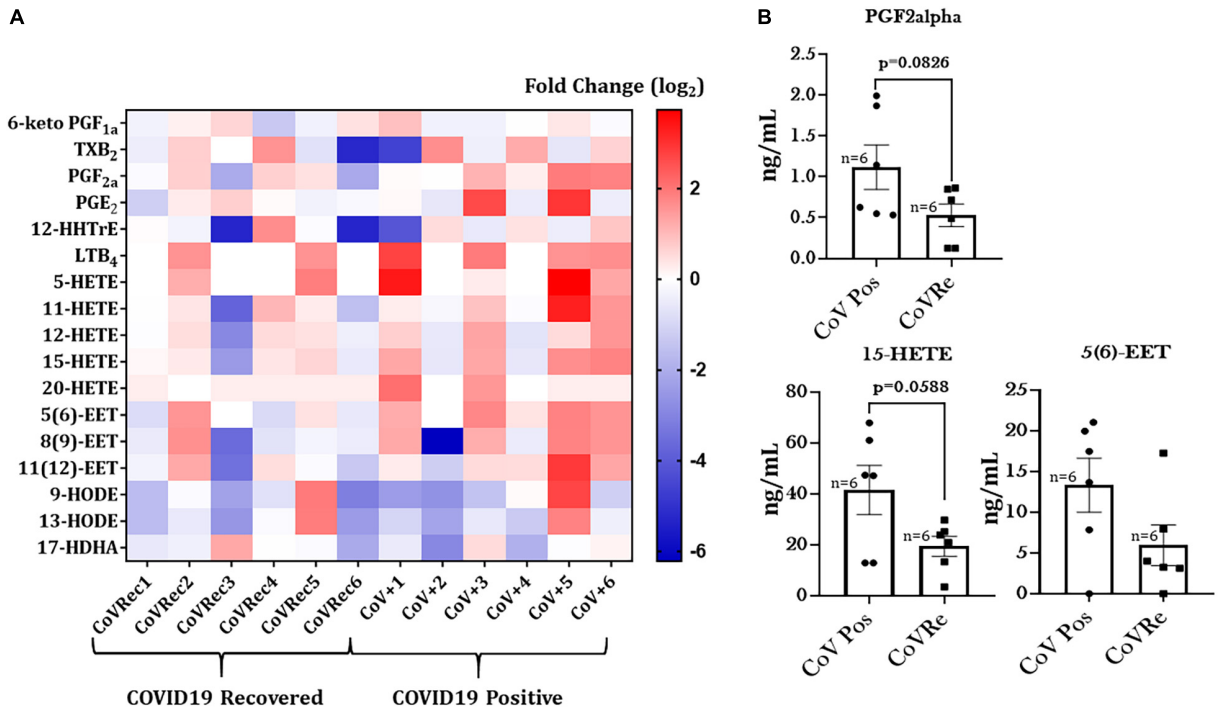

FIGURE 7 | Heat map of eicosanoid levels in COVID-19 positive and COVID-19 recovered patient serum. **(A)** The fold changes in the eicosanoid levels in COVID-19 recovered patient serum as compared to COVID-19 positive serum. Each horizontal row represents the analyte in a specific lipid class, and each vertical column represents the individual sample being tested. Lipid abundance ratios are colored according to the fold changes and the color key indicates the magnitude of log2 fold change. **(B)** The actual changes in the levels (ng/mL) of oxylipins showing slight variations amongst COVID-19 positive and COVID-19 recovered patients.

patients with COVID-19 (Patel et al., 2021). Our observations corroborate with these findings and point toward probable disruption of ACE2/Ang1–7/MasR, the receptor for Ang1–7 axis. Evidences from literature show that ACE2 has a protective role in the pathogenesis of ARDS (Li et al., 2016; Liu et al., 2020). In contrast, previous evidences have correlated increased serum ACE2 activity with increased cardiovascular risk and obstructive coronary artery disease (Úri et al., 2016; Ramchand et al., 2018; Ramchand and Burrell, 2020). In light of these evidences, further investigation in this area is imperative.

We also found a significantly higher ACE2 activity for COVID-19 (current and recovered) patients with a smoking history. As noted previously, increased plasma ACE2 activity has been shown amongst severe patients with a SARS-CoV2 infection (Patel et al., 2021). It could be speculated that smokers might have severe outcomes due to COVID-19. Despite this speculation, a future study with a larger sample size and age- and sex-matched controls is important to establish such a correlation. As reported previously (Sama et al., 2020); our data also indicate gender-based upregulations in ACE2 activity amongst males. These findings could correlate to the increased morbidity and mortality amongst male patients.

Since we show increased ACE2 activity in COVID-19 positive patients, we further analyzed the levels of Ang II (ACE2 substrate) and Ang1–7 (ACE2 product), in the serological samples from patients and normal subjects. Angiotensins are key physiological peptides involved in regulating several biological processes predominantly involved in the regulation of vascular tone and aldosterone secretion. Ang II levels play a pivotal role in adverse myocardial remodeling, while increased levels of Ang1–7 indicate counter-balancing the vaso-constrictive effects of Ang II (Patel et al., 2016). Our results demonstrated elevated levels of Ang II and Ang1–7 in serological samples from COVID-19 positive patients compared to healthy controls. However, we observed a ∼6-fold increase in the Ang1–7 levels in COVID-19 positive patient sera instead of a ∼4-fold increase for Ang II levels. This indicates the counter-regulatory effects of Ang1–7 in COVID-19 positive individuals, however, further investigation is required to confirm this.

In contrast, increased levels of Ang II might indicate non-ACE2 mediated production of Ang II with the help of protease, chymase, Cathepsin G, or CAGE. It is known that increased serum Ang II levels lead to increased vasoconstrictions, inflammation, fibrosis, and eventually heart failure, a common complication amongst patients with COVID-19 (Long et al., 2020; Samidurai and Das, 2020), even after the infection is long over (i.e., long Covid-19). Elevated serum Ang II levels play a crucial role in the progression of hypertension and heart failure. The levels of Ang II have been found to be elevated in optimally treated (using ACE blockers or ARB) patients of heart failure, thus adding to the adverse health effects (Jorde et al., 2000; Petrie et al., 2001; Li et al., 2004; Patel et al., 2016). In addition, Ang II action leads to activation of ADAM-17, resulting in increased ACE2 shedding (Patel et al., 2014). Ectodomain shedding refers

TABLE 2 | Differentially altered lipid analytes in COVID-19 positive patient sera with respective fold changes with COVID-19 recovered subjects.

Analyte	Fold change	*p*-value
6-keto $PGF1_{\alpha}$	1.110515	0.656547
TXB2	1.416495	0.545928
$PGF2_{\alpha}$	2.109126	0.082623
PGE2	2.857159	0.193622
12-HHTrE	0.999787	0.999655
LTB4	2.770774	0.174837
5-HETE	4.558867	0.177288
11-HETE	2.911128	0.209095
12-HETE	1.631523	0.177848
15-HETE	2.139606	0.058767
20-HETE	1.586839	0.428033
5(6)-EET	2.247237	0.104477
8(9)-EET	1.969702	0.192472
11(12)-EET	2.398283	0.228698
9-HODE	1.462013	0.705169
13-HODE	1.014798	0.984878
17-HDHA	0.766199	0.537526

to the release of a cell-membrane receptor from the cell surface to the extracellular space, notably in plasma/serum. Recent studies have shown that SARS-CoV2 induces ACE2 ectodomain shedding through the increased activation of disintegrin and metalloprotease, ADAM-17 (Palau et al., 2020). Although, the biological and clinical significance of this shedding has not been determined; elevation in plasma ACE2 activity in heart failure has been linked to worsened prognosis (Epelman et al., 2008; Putko et al., 2014).

The binding of SARS-CoV2 to ACE2 is preceded by cleavage by Furin (Örd et al., 2020). Furin is abundant in the respiratory tract both intracellularly and in circulation as a free enzyme. It enhances the viral ACE2-affinity by exposing the viral binding site on the S1 domain and revealing the effusion site on the S2 domain, which makes it a crucial factor in SARS-CoV2 infection (Xia et al., 2020). Evidence suggests that furin cleavage plays a potent role in the virulence of dengue, HIV and avian flu (Fitzgerald, 2020; Walls et al., 2020). Our investigations found a marked, though non-significant, reduction in the serum Furin levels on COVID-19 infection in both COVID-19 positive and recovered patients as compared to the healthy controls. However, the serum Furin levels in COVID-19 positive patients was slightly higher than COVID-19 recovered patients. To date, there have been limited studies pertaining to circulating Furin levels. Still, there have been associations established between dysregulated serum furin levels and the occurrence of metabolic diseases like diabetes, hypertension, obesity and cardiovascular disease. While lower serum furin levels were indicative of abdominal obesity and hypertension, higher furin levels were associated with diabetes and myocardial infarction (Fernandez et al., 2018; He et al., 2019, 2020; Wang Y. K. et al., 2020). We speculate that lowered furin levels indicate the increased internalization of Furin for viral cleavage; however, further work is required to ascertain the role of intracellular and extracellular Furin in the development of COVID-19.

Additionally, we found that Furin levels were lowered amongst smokers with COVID-19, though again this change was not significant. It is pertinent to mention here that in healthy smokers Furin levels were significantly increased (data not shown) compared to non-smoking controls. Our results point toward decrease in the Furin levels in COVID-19 irrespective of the smoking status.

Coinciding with the existing literature (Hirano and Murakami, 2020; Huang et al., 2020; Mahmudpour et al., 2020), we also found increased levels of cytokines/chemokines in the patient serum from COVID-19 positive subjects. Additionally, we for the first time, show significant changes in the levels of these pro-inflammatory mediators in COVID-19 positive patients with a smoking history as compared to the non-smoking controls. Elevated levels of IFN-$_{\gamma}$ ($p = 0.0836$), MCP-1, and Eotaxin point toward increased inflammatory response amongst smokers. It may be possible that anti-inflammatory and pro-resolving mediators are decreased by smoking in COVID-19 patients. Of note, we also found some changes in the lipid profiles of COVID-19 positive and COVID-19 recovered patients. Viral infections cause changes in lipid metabolism and play a crucial role in regulating innate and adaptive immune responses (Heaton and Randall, 2011; Ketter and Randall, 2019). We did not find a significant change in the serum lipid profiles of COVID-19 positive and COVID-19 recovered samples, though slight variations in some of the lipids were noticed. Amongst the lipids that showed a slight increase in COVID-19 positive patients compared to COVID-19 recovered individuals were $PGF_{2\alpha}$, 15-HETE, and 5(6) EET. Of these, $PGF_{2\alpha}$ and15-HETE are bronchoconstrictors and cause lung injury (Fish et al., 1984; Martin et al., 1989; Zhu et al., 2003), whereas 5(6) EET has an anti-inflammatory role within the cells. It is difficult to speculate what could be the possible source or role of the observed changes in the serum lipid levels. However, in general, esterified eicosanoids are released by many cell types including immune cells (neutrophils, eosinophils, and macrophages), endothelial and epithelial cells (Hammond and O'Donnell, 2012; Mazaleuskaya et al., 2018).

Overall, our results provide evidence of systemic inflammatory spillover in COVID-19 positive patients (**Figure 8**), which is shown to be aggravated in patients who smoke. It is pertinent to mention here that pulmonary conditions like COPD and smoking-induced lung injury cause such spillovers into the systemic circulation (Tkacova, 2010; Liu et al., 2014). It will be interesting to study these inflammatory and lipid mediators along with anti-inflammatory mediators to understand the pathogenesis of SARS-CoV2 infection. However, we have not discussed this here, but the vaping population might be another group that may suffer from severe outcomes in the event of a SARS-CoV2 infection. Previous work by our group has shown gender-based variation in the ACE2 protein expression in lung tissues from C57Bl/6 mice exposed to e-cigarette (e-cig) aerosols (Wang Q. et al., 2020). E-cig use has been associated with loss of lipid homeostasis, eventually leading to pulmonary toxicity and lung injury (Madison et al., 2019; Chand et al., 2020).

Though we were able to show variations in systemic inflammatory and lipid mediators on SARS-CoV2 infection,

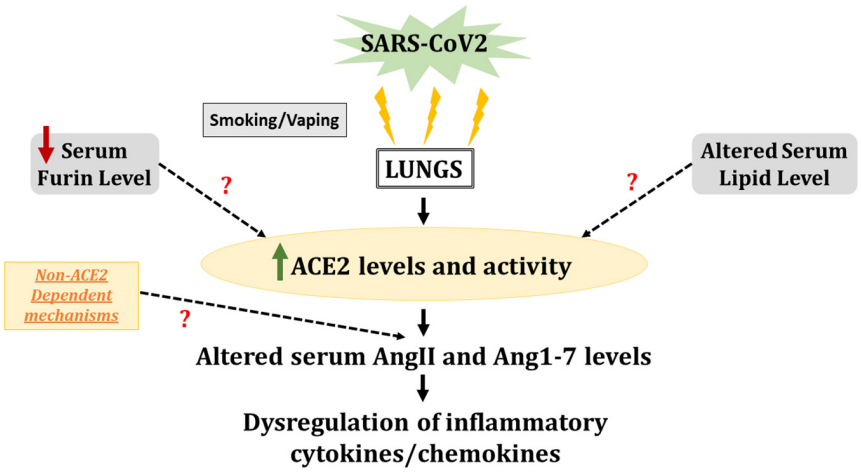

FIGURE 8 | SARS-CoV2 infection leads to increased ACE2 activity and serum cytokine levels in smokers. Serum samples from healthy individuals and COVID-19 positive patients with smoking history were compared for ACE2 activity and levels of inflammatory cytokines/chemokines. The results pointed toward increased ACE2 activity and altered AngII and Ang1–7 levels in the serum of COVID-19 patients as compared to normal individuals. The altered AngII and Ang1–7 levels could also be a result of non-ACE2 dependent mechanisms which is not studied here. Increased levels of pro-inflammatory cytokines/chemokines in COVID-19 positive patients with smoking history, indicates a heightened immune response on SARS-CoV2 infection in smokers. We also found evidence for lowered serum furin and altered lipid profiles amongst COVID-19 patients, which may or may not correlate with the ACE2 activity. These alterations can lead to heightened inflammatory response and lung remodeling with smoking/vaping history.

our study had some limitations. The sample cohort used for this study was relatively small. Since we obtained our samples from a commercial source, we did not have information about disease severity, the duration of hospitalization, and medications administered to COVID-19 positive and COVID-19 recovered individuals. Similarly, there is no data about the smoking habits and pack years for current smokers or years since quitting for the ex-smokers. We intend to include a larger and more heterogeneous cohort of patients in the future. Also, due to the limited sample number we had to pool the ex- and current smokers for this study which might have introduced some confounders. The persistently elevated ACE2 activity and elevated levels of AngII and Ang1–7 raise intrigue and warrant further investigation to understand better the role of ACE2 in COVID-19 development, progress, and remission. This will have ramifications on heightened inflammatory response and lung remodeling even in recovered patients (long Covid-19) with a smoking/vaping history. Such studies are crucial in understanding the mechanistic role of intact and circulating ACE2 in COVID-19 and deduce if recombinant ACE2 could develop as a therapy.

CONCLUSION

In conclusion, our data show that the systemic ACE2 activity and cytokine release are upregulated amongst COVID-19 patients with a smoking history. We also provide evidence for inflammatory systemic spillover due to SARS-CoV-2-induced COVID-19 infection, which could be crucial in identifying biomarkers in susceptible population and/or developing future therapies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human biospecimens were reviewed and approved by University of Rochester Institutional Biosafety Committee (IBC).

AUTHOR CONTRIBUTIONS

GK and SY designed and conducted the experiments. GK, SY, and IR wrote, edited, and revised the manuscript. TM analyzed the lipidomic data and edited the manuscript. IR conceptually designed the overall experiments and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.653045/full#supplementary-material

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Chronic E-Cigarette Aerosol Inhalation Alters the Immune State of the Lungs and Increases ACE2 Expression, Raising Concern for Altered Response and Susceptibility to SARS-CoV-2

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Conventional smoking is known to both increase susceptibility to infection and drive inflammation within the lungs. Recently, smokers have been found to be at higher risk of developing severe forms of coronavirus disease 2019 (COVID-19). E-cigarette aerosol inhalation (vaping) has been associated with several inflammatory lung disorders, including the recent e-cigarette or vaping product use-associated lung injury (EVALI) epidemic, and recent studies have suggested that vaping alters host susceptibility to pathogens such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To assess the impact of vaping on lung inflammatory pathways, including the angiotensin-converting enzyme 2 (ACE2) receptor known to be involved in SARS-CoV-2 infection, mice were exposed to e-cigarette aerosols for 60 min daily for 1–6 months and underwent gene expression analysis. Hierarchical clustering revealed extensive gene expression changes occurred in the lungs of both inbred C57BL/6 mice and outbred CD1 mice, with 2,933 gene expression changes in C57BL/6 mice, and 2,818 gene expression changes in CD1 mice (>abs 1.25-fold change). Particularly, large reductions in IgA and CD4 were identified, indicating impairment of host responses to pathogens *via* reductions in immunoglobulins and CD4 T cells. CD177, facmr, tlr9, fcgr1, and ccr2 were also reduced, consistent with diminished host defenses *via* decreased neutrophils and/or monocytes in the lungs. Gene set enrichment (GSE) plots demonstrated upregulation of gene expression related to cell activation specifically in neutrophils. As neutrophils are a potential driver of acute lung injury in COVID-19, increased neutrophil activation in the lungs suggests that vapers are at higher risk of developing more severe forms of COVID-19. The receptor through which SARS-CoV-2 infects host cells, ACE2, was found to have moderate upregulation in mice exposed to unflavored vape pens,

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and further upregulation (six-fold) with JUUL mint aerosol exposure. No changes were found in mice exposed to unflavored Mod device-generated aerosols. These findings suggest that specific vaping devices and components of e-liquids have an effect on ACE2 expression, thus potentially increasing susceptibility to SARS-CoV-2. In addition, exposure to e-cigarette aerosols both with and without nicotine led to alterations in eicosanoid lipid profiles within the BAL. These data demonstrate that chronic, daily inhalation of e-cigarette aerosols fundamentally alters the inflammatory and immune state of the lungs. Thus, e-cigarette vapers may be at higher risk of developing infections and inflammatory disorders of the lungs.

Keywords: e-cigarette, vaping, ACE2, COVID-19, immunomodulation, SARS-CoV-2, RNAseq, lipidomics

INTRODUCTION

Electronic cigarettes (e-cigarette) arose as a safer way to deliver nicotine than conventional cigarettes, with the ultimate goal of helping smokers quit tobacco (Bozier et al., 2020). E-cigarette use has increased significantly over the last 10 years (Fadus et al., 2019), despite of the lack of sufficient data about the effects of these devices and its content being delivered (Crotty Alexander L. et al., 2015; Crotty Alexander L. E. et al., 2015). This is due in part to the advertisement of these drug delivery devices as safer and containing less toxins than conventional cigarettes (Bozier et al., 2020). Unfortunately, e-cigarettes have not been found to be successful smoking cessation tools (Bullen et al., 2013; Glantz and Bareham, 2018), and in fact many users who tried using them for cessation end up as dual users of both conventional tobacco and e-cigarettes (Stratton et al., 2018). In addition, the use of e-cigarettes may contribute to relapse of smoking in ex-smokers (Gomajee et al., 2019; McMillen et al., 2019). More worrisome is the growing population of e-cigarette users whom are never smokers, including large numbers of adolescents and young adults (Berry et al., 2019; Fadus et al., 2019). Therefore, from a socioeconomical perspective to a self-awareness of the effect of these devices, it is crucial to understand the implications of their use in health.

Vaping of e-cigarettes has been associated with numerous inflammatory disorders of the lungs, including hypersensitivity pneumonitis, lipoid pneumonia, and eosinophilic pneumonia, demonstrating that the inhalation of chemicals within e-cigarette aerosols can alter the inflammatory state of the lung (Sommerfeld et al., 2018; Viswam et al., 2018; Arter et al., 2019). In 2019, an epidemic of lung injuries associated with vaping occurred in the United States (Chatham-Stephens et al., 2019). The primary clinical diagnosis in these patients was acute respiratory distress syndrome (ARDS) or acute lung injury (ALI). Almost 3,000 e-cigarette or vaping device-associated lung injury (EVALI) cases had been reported to the Centers for Disease Control and Prevention by late November 2019, with 68 deaths confirmed by February 2020 (Chatham-Stephens et al., 2019). Since then, data on EVALI has not been updated by the CDC, due to the coronavirus disease 2019 (COVID-19) pandemic.

Since there are numerous e-cigarette devices and a thousands of varieties of e-liquids (chemicals in liquid form that are aerosolized by e-devices), the specific chemicals responsible for the lung injuries have not yet been confirmed, but vitamin E acetate and tetrahydrocannabinol (THC) have been closely tied to this particular vaping induced lung disease (Blount et al., 2020; Crotty Alexander et al., 2020).

Outside of directly causing lung diseases, including EVALI, e-cigarette vaping may disrupt sleep (Boddu et al., 2019), induce fibrosis in the heart, liver, and kidneys (Crotty Alexander et al., 2018), alter neutrophil function and host defenses (Corriden et al., 2020), increase systemic inflammation (Crotty Alexander et al., 2018), and increase susceptibility to infections (Sussan et al., 2015; Madison et al., 2019; Corriden et al., 2020). Researchers have shown increased severity of influenza pneumonia in murine models of e-cigarette exposure (Madison et al., 2019), which raises the question of whether other viral lung infections might also be impacted by e-cigarette exposure. Because of a lack of medical coding for e-cigarette and vaping device use, and because healthcare professionals do not consistently ask patients about vaping and dabbing, using medical records to answer the critical question of how vaping impacts susceptibility to infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and development of COVID-19 will be difficult. Thus, we hypothesized that vaping may alter inflammatory responses in the lungs, which may relate to changes that are known to increase susceptibility to SARS-CoV-2 infection, which could explain why younger patients can develop COVID-19 even with no apparent pre-existing medical conditions or alternatively exacerbate the diseases if pre-existing conditions are present.

The aim of this study was to broadly assess the effects of e-cigarette aerosols on lung inflammatory responses and resting immune state *via* unbiased methodologies. We assessed overall gene expression changes in the lungs upon chronic inhalation exposure to e-cigarette aerosols in two disparate mouse backgrounds—the inbred C57BL/6 strain and outbred CD1 strain. We assessed changes in inflammatory factors and host defenses due to inhalation of aerosolized vehicle components alone, versus with the addition of nicotine. Because it is unknown whether vaping predisposes to higher severity of COVID-19, we specifically assessed for changes in inflammation in the lung, including activation of neutrophils, which are considered a primary driver of ARDS pathogenesis. It is also unknown whether vaping, like conventional cigarette smoking, increases susceptibility to infection with SARS-CoV-2, therefore we specifically assessed for angiotensin-converting enzyme 2

(ACE2) expression, which is crucial for the infectivity of SARS-CoV-2, finding that indeed e-cigarette use may increase ACE2 expression and that flavorant chemicals within e-liquids might drive ACE2 expression in particular.

MATERIALS AND METHODS

E-Cigarettes

Three types of vaping devices were used for these studies: vape pens, box Mods, and pod devices (JUUL). Kanger Mini-protank glassomizers with 1.5 Ohm coils, attached to Kanger eVOD variable voltage 1000 mAh batteries set at 4.8 Volts, were the vape pen devices used for these studies. The box Mod used was a Kanger base with direct electricity from the wall and glass and metal Kanger protanks. For vape pens and box Mods, chemical components of e-liquids were purchased from Sigma and mixed in the lab prior to filling the tanks. For vape pen exposures, propylene glycol (PG) was mixed 1:1 with glycerin (Gly) to create a 50:50 solution with nicotine at 24 mg/ml. Sixty-minute vape pen exposures corresponded with plasma cotinine (the primary metabolite of nicotine) levels of 269 ± 17 ng/ml. For box Mod exposures, e-liquids were mixed at 70:30 PG:Gly with and without 6 mg/ml nicotine, to produce both vehicle e-cigarette aerosol without nicotine and regular e-cigarette aerosol with nicotine (EV). Sixty-minute box Mod exposures with e-liquid containing nicotine corresponded with plasma cotinine levels of 253 ± 21 ng/ml. For the pod devices, we used the two most popular JUUL flavors in 2018–2019, mint and mango. JUUL batteries and pods were purchased directly from the manufacturer in bulk, and all lot numbers were recorded. E-liquids from JUUL pods have 30:70 PG:Gly, nicotine salts at 59 mg/ml, benzoic acid, and flavorant chemicals (Talih et al., 2019). Twenty-minute JUUL mint exposures corresponded with plasma cotinine levels of 194 ± 14 ng/ml, and JUUL mango with 216 ± 38 ng/ml.

E-Cigarette Aerosol Exposures in Mice

Inbred C57BL/6 and outbred CD1 mice were purchased from Envigo at 6–8 weeks of age. Two strains of mice with different genetic backgrounds, which have different susceptibilities to inflammatory and infectious challenges, were utilized to increase the likelihood of biological relevance to humans by identifying vaping induced changes which occur in both, and thus are not limited to one specific genotype or phenotype. Studies were first run in female mice (n = 6 per group) of both strains, with further studies conducted in male C57BL/6 mice. All animal studies were conducted with prior approval of both UCSD and VA Institutional Animal Care and Use Committees (IACUC). Mice were randomized prior to exposures. For vape pen and box Mod exposures, mice were placed into the Scireq inExpose whole-body exposure system for 60 min once daily for 3–6 months. JUUL exposures were done in 20-min blocks three times daily for 1 month. As previously described, e-cigarettes were activated and flow generated *via* application of negative pressure every 20 s, with puff duration of 4 s across all devices (Alasmari et al., 2017, 2019; Crotty Alexander et al., 2018; Corriden et al., 2020). Mice were recovered in prewarmed cages for 20 min after each exposure.

Bronchoalveolar Lavage and Lung Tissue Harvest

After the final e-cigarette aerosol exposure, mice were anesthetized and sedated with 100 mg/kg ketamine and 10 mg/kg xylazine and underwent tracheostomy. Bronchoalveolar lavage (BAL) was performed with 500 μl cold 1× PBS three times, with pooling of the recovered fluid. BAL was centrifuged at 1,800 μrpm at 4°C for 8 min and supernatant aliquoted and snap frozen prior to transfer to the lipidomics core at UCSD. Right lung lobes were harvested, placed into RLT buffer (Qiagen), snap frozen, and stored at -80°C prior to RNA extraction.

Lipidomics

Bronchoalveolar lavage samples were submitted to the LIPID MAPS Lipidomics Core at UCSD and underwent broad profiling and quantitative analysis of eicosanoids using liquid-chromatography mass spectrometry (LC-MS/MS) platforms. In brief, 157 eicosanoids and *N*-acylethanolamines were analyzed by UPLC-MS/MS and the steady-state levels fully quantitated by comparison with authentic standards. BAL (30–200 μL) was introduced, along with 1,000 μL of internal standard mix, and extraction was performed with SPE using strata-x polymeric reversed-phase columns (8B-S100-UBJ Phenomenex). Samples were injected into UPLC (Acquity UPLC System, Waters) followed by analysis by mass spectrometry (Sciex 6500 Qtrap). Data were normalized to volume (pmol/ml), and two-way ANOVA with Tukey multiple comparisons test was used to assess differences in lipids across the three exposure groups. Data represents the dynamic balance between synthesis and secretion, and catabolism and clearance, of eicosanoids in the airways of mice exposed to e-cigarette aerosols with and without nicotine.

RNAseq

Total RNA from whole lung was isolated (RNeasy mini kit, QIAGEN) and submitted to the UCSD IGM Genomics Core for processing, quality control checks, creation of libraries, and sequencing (HiSeq4000).

qPCR

Total RNA was isolated from whole lung (RNeasy mini kit, Qiagen), converted to cDNA (Applied Biosystems™ High Capacity cDNA Reverse Transcription Kit, Thermo Fisher), and underwent qPCR with Taqman Gene Expression Assays for mouse ACE2 (Mm01159006_m1) and mouse GAPDH (Mm99999915_g1) primers, oligonucleotides, FAM dye, with TaqMan® Fast Advanced Master Mix (Thermo Fisher), per manufacturer's RT-PCR instructions in a 384-well plate RT-PCR thermal cycler (Biorad). Data were analyzed by one-way ANOVA with Sidak's multiple comparisons test.

RNAseq Statistical Analysis

Sequencing data underwent DESeq2 normalization (Chen et al., 2018) followed by analysis at the La Jolla Institute of

Allergy and Immunology. An unbiased approach was used to identify e-cigarette aerosol inhalation-dependent gene expression changes by taking all lung samples from both strains of mice (C57BL/6 and CD1) and filtering for the largest gene expression changes upon e-cigarette exposure (genes showing a fold change of 1.25 and adjusted *p*-value of less than 0.1), followed by hierarchical clustering to create a heat map. A volcano plot was used to highlight the greatest changes. Bubble GUM (GSEA unlimited map) charts were drawn using the Benjamini–Yekutieli *p*-value correction method, using controls even if tests were dependent. The gene sets were filtered based on Benjamini–Yekutieli Padj (FDR) < 0.25 (allowing only for 25% false positives). For cell-specific heat maps, transcripts per kilobase million (TMP) were calculated for the genes and no clustering was performed to keep the cell-specific gene sets intact.

Calculation of Gene Fold Changes for qPCR Analysis

Gene expression fold change was calculated for ACE2 and ACE2 interactor genes in lung tissue from mice exposed to EV vs. air control mice. Fold change was calculated as the median of expression in the EV-exposed cohort divided by the median of expression in the normal cohort. A list of interactor genes was obtained from Pathways Commons[1], an aggregator of interactions drawn from major pathway databases.

Correlation of ACE2 Expression With Immune Infiltration and Immune-Associated Pathways

Estimates of abundance of immune cell populations were inferred using the program Cibersortx[2] (Chen et al., 2018), which deconvolutes bulk RNA sequencing data to derive the infiltration levels of 22 different immune cell types. These infiltration levels are correlated to ACE2 expression using the Spearman correlation test ($p < 0.05$). In addition, ACE2 expression was also correlated with the upregulation or downregulation of immune-associated pathways using gene set enrichment analysis (GSEA, $p < 0.05$). Pathways were obtained from the Pathways Interaction database (PID) and the Reactome database.

RESULTS

Exposure to E-Cigarette Aerosols Induces Profound Gene Expression Changes in Key Immune and Inflammatory Pathways in the Lungs of Two Disparate Mouse Genetic Backgrounds

Hierarchical clustering revealed extensive gene expression changes occurred in the lungs of both inbred C57BL/6 mice and outbred CD1 mice whom inhaled e-cigarette aerosols daily for 3–6 months, with 2,933 gene expression changes in C57BL/6 mice, and 2,818 gene expression changes in CD1 mice (>abs 1.25-fold change; **Figure 1A**). Examination of the largest changes by volcano plot revealed several genes of interest (**Figure 1B**). Expression of IgA was greatly reduced in CD1 mice, indicating that e-cigarette exposure may lead to altered IgA levels in the lungs. CD177, facmr, tlr9, fcgr1, and ccr2 were all reduced, suggesting diminished host defenses *via* decreased neutrophils and/or monocytes in the lungs upon e-cigarette aerosol exposure. In addition, assessing immune cell-specific gene expression changes in the lung tissue demonstrated alterations across lymphocytes, eosinophils, neutrophils, macrophages, and dendritic cells (**Figure 1C**). CD4 was also reduced in CD1 but not C57BL/6 mice, suggesting a strain-specific reduction of $CD4^+$ T cells from the lungs of CD1 mice upon e-cigarette aerosol exposure, again indicating immunomodulation that may increase the risk of infection and dysregulate the immune response to an infection. The most upregulated gene in both strains of mice was Krt83, an uncommon keratin gene expressed in epithelial cells. Krt8 expression has been found to be increased in some carcinomas (Chu and Weiss, 2002; Gires et al., 2006) and promotes tumor progression and metastases in gastric carcinoma in particular (Fang et al., 2017).

KEGG enrichment plots demonstrated profound downregulation of gene expression in key immune and inflammatory pathways in lungs of e-cigarette-exposed mice versus air controls (**Figures 1D–I**). Enrichment plots contain KEGG profiles of the running enrichment scores (ES) and positions of gene set members on the rank-ordered list in GSE. Cytokine receptor interaction signatures were downregulated in e-cigarette lungs versus air controls (ES -0.571, NES -2.32, *p* and *q*s = 0; **Figure 1D**). Notable cytokines represented in this pathway include IL-1, IL-2, IL-6, TNF, interferon gamma (IFNγ), and TGFβ. Chemokine signaling pathway signatures were downregulated in e-cigarette lungs (ES -0.585, NES -2.32, *p* and *q*s = 0; **Figure 1E**). Notable members of this pathway include β-arrestin, G-protein-coupled receptors, PI3K, JAK/STAT, ERK1/2, and IKK/IκB/NFκB). Genes associated with primary immunodeficiency were downregulated in e-cigarette lungs (ES -0.717, NES -2.16, *p* and *q*s = 0; **Figure 1F**). Genes in this pathway are specific for lymphoid lineages with members including CD3δ/ε, CD8α, CD45, IKKγ, CD40/CD40L, RAG1/2, IL1Rα, Igα, BTK, λ5, and RFX5/AP/ANK. Moreover, we also observed upregulation of gene expression related to cell activation specifically in neutrophils (**Figures 1G,H**) and dendritic cells (ES 0.654, NES 2.41, *p* and *q*s = 0; **Figure 1I**). With early (9 h; **Figure 1G**) neutrophil stimulation and (B) late (24 h; **Figure 1H**) neutrophil stimulation interaction signatures increased in e-cigarette-exposed mouse lungs versus air controls (ES 0.735 and 0.683, respectively; NES 2.75 and 2.58, respectively; *p* and *q*s = 0). Gene members of the neutrophil activation pathways include CXCR2, Stfa2l1, Csf3r, and Chi3l1, while early dendritic cell activation members include MAPK, NLRs, JAK/STAT, and TNF. These data suggest that e-cigarette aerosol exposure leads to immunomodulation of host defenses, suppressing and inducing key immune pathways (innate and adaptive) affecting the homeostatic state of the lungs.

[1] https://www.pathwaycommons.org/

[2] cibersortx.stanford.edu

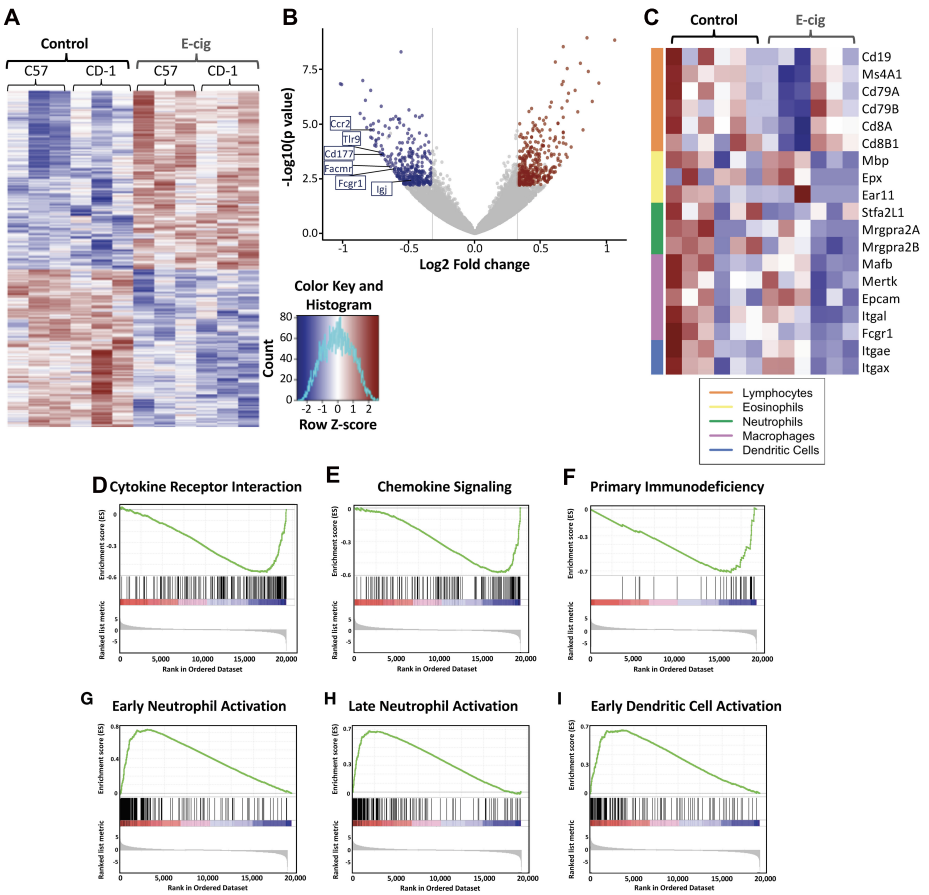

FIGURE 1 | Exposure to e-cigarette aerosol induces profound effects in overall inflammatory responses in two different mouse genetic backgrounds. Transcriptomic analysis of lung tissue performed by RNAseq from CD1 and C57BL/6 female mice exposed daily to e-cigarette aerosol generated by Vape pens with 50:50 PG:Gly and 24 mg/ml nicotine for 3 and 6 months, respectively, detected 2,818 (CD1) and 2,933 (C57BL/6) gene expression changes. **(A)** Heat map of differentially expressed (fold change of 1.25 and adjusted *p*-value less than 0.1) genes with hierarchical clustering revealed many genes with expression changes in both strains of mice after e-cigarette exposure as compared to Air controls. **(B)** Examination of gene expression changes above 1.5-fold (vertical lines) by volcano plot revealed several genes of interest. Igj was greatly reduced, indicating that e-cigs substantially impair lung IgA expression. CD177, Facmr, Tlr9, Fcgr1, and Ccr2 were reduced, indicating a further loss of host defenses. **(C)** Assessing immune cellspecific gene expression changes in the lung tissue demonstrated alterations across lymphocytes, eosinophils, neutrophils, macrophages and dendritic cells. Enrichment plots demonstrated profound downregulation of gene expression in broad immune signaling **(D,E)** pathways, important for **(D)** cytokine receptor interactions and **(E)** chemokine signaling. **(F)** Downregulation across genes of importance in primary immunodeficiency was seen, while patterns of gene upregulation were detected in innate immune cells: **(G)** Early neutrophil activation and **(H)** Late neutrophil activation, and cells that act as the intersection between innate immune and adaptive cells – antigen presenting cells: **(I)** Early dendritic cell activation. Data is representative of 6 mice per group, with total lung RNA from pairs of mice pooled, giving three data points per group. The enrichment score is shown as a green line **(D–I)**, which reflects the degree to which a gene set (the barcode where each black line is a gene in the gene set) is overrepresented at the top or bottom of a ranked list of genes (the heatmap axis – red/blue/white).

Inhalation of E-Cigarette Aerosols Diminished Eicosanoid Lipids Within the Airways

E-cigarette exposure led to decreased of two main eicosanoid lipids, including prostaglandin E2 (PGE2; $p < 0.05$) and 12-hydroxyeicosatetraenoic acid (12-HETE; $p < 0.001$), which suggests dysregulation of inflammatory pathways and host defenses (**Figure 2**). The non-nicotine chemicals (vehicles: PG and Gly) within the aerosols appeared to be the drivers behind these subtle changes, as vehicle mice had equal to greater changes as compared with EV with nicotine. Therefore, besides the variety of inflammatory proteins altered due to e-cigarette aerosol inhalation, there are also changes in important lipids involved in inflammation.

ACE2 Expression Is Upregulated by Exposure to E-Cigarette Aerosol

ACE2 was found to be upregulated by 33% in mice exposed to e-cigarette aerosols generated from vape pens (**Figure 3A**). The expression of genes that are associated with ACE2, including angiotensinogen (AGT) and SLC7 members, were also upregulated after exposure to e-cigarette aerosols (**Figures 3A,B**). Angiotensinogen is the precursor of angiotensin II, the substrate of ACE2, while SLC7 members have been documented to interact

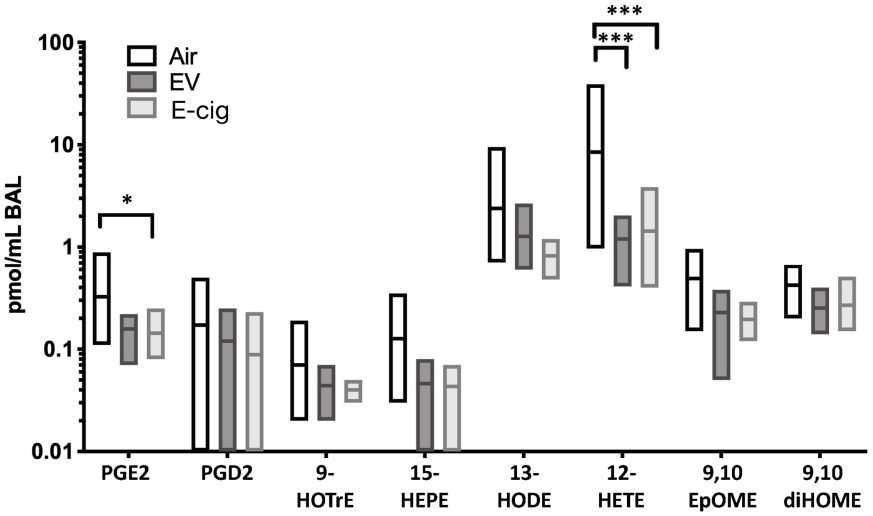

FIGURE 2 | Eicosanoid lipid profiles within BAL are modestly altered by both e-cigarette vehicle and nicotine-containing (E-cig) aerosol exposures. C57BL/6 female mice were exposed for 1 h daily to ecigarette aerosols generated with e-liquid containing 70:30 PG:Gly, with (E-cig) and without 6 mg/mL nicotine (Vehicle), generated from box Mods, or Air only, for 6 months. BAL underwent broad profiling and quantitative analysis of eicosanoids using liquid-chromatography mass spectrometry (LC-MS/MS). Mice exposed to Vehicle aerosols had decreased PGE2, which is produced in airway epithelium and is a bronchodilator. Both Vehicle (no nicotine) and E-cig (with nicotine) exposed mice had decreased 12-HETE in the BAL ($p < 0.001$), suggesting changes induced by non-nicotine chemicals within aerosols. Overall, eicosanoid levels trended lower in the BAL of both Vehicle and E-cig groups relative to Air controls, suggesting broad suppression of eicosanoid production or release. Data are representative of 6 mice per group. *$p < 0.05$, ***$p < 0.001$.

with ACE2 and are considered part of the ACE2 gene network (Dai et al., 2020; Soule et al., 2020; The Lancet Respiratory Medicine, 2020). SLC proteins can physically bind to ACE2 and may affect viral entry mechanisms (The Lancet Respiratory Medicine, 2020). Thus, ACE2, a currently relevant membrane-bound enzyme (and ACE2-related genes) in the context of SARS-CoV-2 infection is upregulated when mice are exposed to e-cigarette aerosols, and genes interacting with ACE2 are also upregulated by these inhalants.

ACE2 Expression Is Associated With Lung Immune Cell Levels and Immune-Associated Pathways

The abundance of immune cell types was assessed by deconvolving bulk RNA-sequencing data and correlated abundances to ACE2 expression. ACE2 upregulation is significantly correlated with a lower abundance of resting $CD4^+$ memory T cells but with a higher abundance of follicular helper T cells (Spearman, $p < 0.05$) (**Figure 3C**). In addition, we found that e-cigarette-induced upregulation of ACE2 is correlated with the downregulation of several immune-associated pathways, including interleukin receptor signaling, IFNγ signaling, and CD28 co-stimulation (**Figures 3D–G**). Interestingly, ACE2 upregulation in e-cigarette aerosol-exposed mice is associated with increased expression of genes associated with influenza infection and transcriptional regulation by runx3, a key regulator of tissue-resident memory $CD8^+$ T cell differentiation and homeostasis (**Figures 3D,E**). Thus, these data suggest that e-cigarette can impair the ability to fight infection in the lungs, particularly viral infections.

JUUL Mint Aerosols Induce ACE2 Expression

JUUL were the most popular devices on the market from 2017 until 2020, and their e-liquids are composed of different chemicals than vape pen and box Mod e-liquids, including nicotinic salts at elevated concentrations (69 mg/ml nicotine), benzoic acid, flavorants, and a flipped ratio of PG:Gly of 30:70. Mice exposed to JUUL mint aerosols daily for 3 months developed a 6.1-fold increase in ACE2 expression in their lungs ($p < 0.0001$; **Figure 4**). Inhalation of aerosols from JUUL mango did not alter ACE2 expression. Aerosols generated by box Mods, with and without nicotine (EV and vehicle, respectively) did not alter ACE2 expression. These data suggest that chemical flavorants in the JUUL mint e-liquid may be involved in the upregulation of ACE2.

DISCUSSION

The question of how e-cigarette use impacts health has come to the forefront since the 2019–2020 EVALI epidemic in the United States (Bozier et al., 2020). Now, with the COVID-19 pandemic and its related lung damage (ARDS and ALI), the potential associated risk of smoking and vaping at increasing the susceptibility to develop a life-threatening SARS-CoV-2 infection has brought up the interest of researchers in the field (Dai et al., 2020; McAlinden et al., 2020; Soule et al., 2020; The Lancet Respiratory Medicine, 2020).

Some research groups have looked at overall changes induced by e-cigarette aerosols by transcriptomics studies, most of them

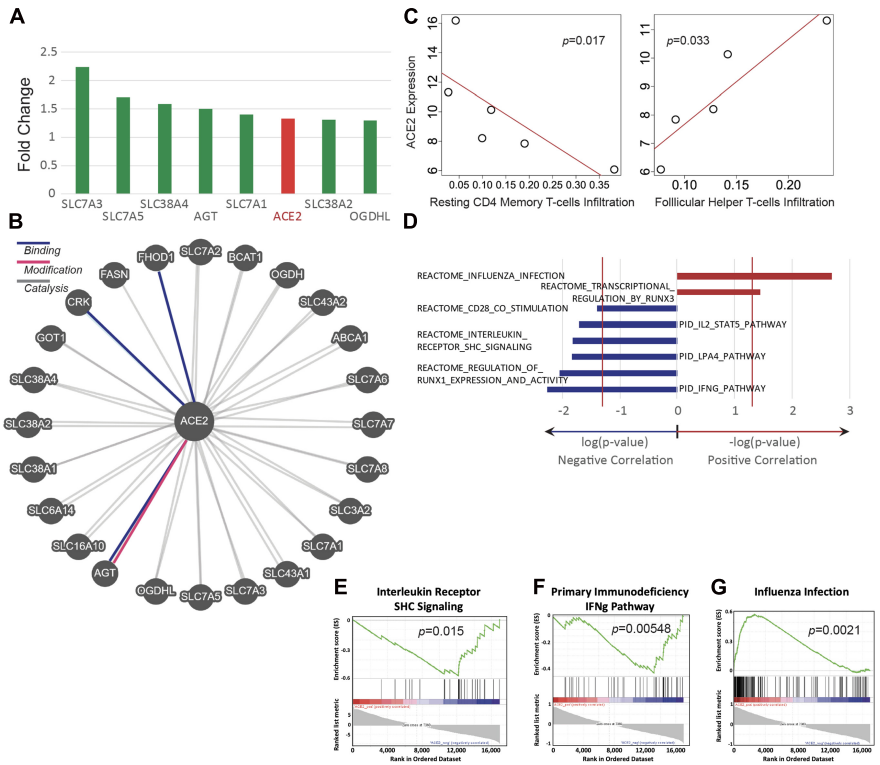

FIGURE 3 | ACE2 expression is upregulated in E-cig exposed mice and correlates with immune status. Transcriptomic data from the lungs of CD1 and C57BL/6 female mice, exposed to aerosols generated from Vape pens with 50:50 PG:Gly and 24 mg/ml nicotine for 3 and 6 months, respectively, was analyzed for ACE2 expression and assessed for correlation with immune pathways. **(A)** Changes in ACE2 and ACE2-associated gene expression in the lungs of E-cig exposed mice relative to Air control mice. **(B)** Schematic of genes associated with ACE2. **(C)** Scatter plots of ACE2 expression demonstrate correlations with immune cell infiltration levels (*y*-axis plots gene expression values in read count per million reads; *x*-axis plots the fraction of immune cells within the lung tissue that are of the specific cell type indicated). **(D)** Correlations between ACE2 expression and immunity-associated pathways. The vertical red lines represent significance cutoff at $p = 0.05$. Individual GSEA plots demonstrate correlations between ACE2 expression and **(E)** interleukin receptor signaling, **(F)** the IFNg pathway in primary immunodeficiency, and **(G)** immune pathways activated during infection with influenza. Data is representative of six mice per group, with pooling of RNA from pairs of mice prior to transcriptomics.

using *in vitro/ex vivo* human primary cell culture models, which include human nasal epithelial cells (Banerjee et al., 2017; Park et al., 2019), human airway/bronchial epithelia cells (Shin and Crotty Alexander, 2016; Crotty Alexander et al., 2018; Park et al., 2019; Herr et al., 2020), human buccal/oral epithelial (Iskandar et al., 2019; Tommasi et al., 2019), and even cell lines such as human bronchial epithelial BEAS-2B cells (Renegar et al., 2004; Anthérieu et al., 2017). However, to the best of our knowledge, only one study has utilized a rodent model, specifically Sprague-Dawley rat lungs (Phillips et al., 2017). Very few differences in gene expression were found, in contrary to the studies on human cells, although this study had significant conflicts of interest as it was funded solely by Philip Morris International (PMI), and all authors were employees of PMI (Phillips et al., 2017). Thus, our study may be the first to present an unbiased transcriptomic analysis of murine lung tissue as a model, which may provide significant insights as to the broad changes in gene expression of whole lung tissue occurring in response to daily e-cigarette aerosol exposure *in vivo* over time (model of chronic vaping).

In our work presented here, we found that chronic exposure (6 months) to e-cigarette aerosols generated by vape pens induced profound changes in gene regulation within the lungs of both C57BL/6 and CD1 mice. Remarkably, 2,933 gene expression changes occurred in C57BL/6 mice and 2,818 gene expression changes in CD1 mice (**Figure 1A**). A volcano plot revealed several genes of interest (**Figure 1B**). This included reduction in IgA in CD1 mice, which is important in lung mucosal immunity (Pilette et al., 2001; Gould et al., 2017). IgA has been found to protect mice against viral respiratory infections (Renegar et al., 2004; Muramatsu et al., 2014; Gould et al., 2017), and its deficiency correlates with airway inflammation and progression of chronic obstructive pulmonary disease (COPD) (Polosukhin et al., 2011). Moreover, other genes were significantly reduced in mice exposed to e-cigarette aerosol, such as CD177 (highly upregulated in patients with severe influenza infection) (Tang et al., 2019), fcmr (which can regulate early B cell activation and plasma cell development after influenza virus infection) (Nguyen et al., 1950), tlr9 (important for viral sensing and correlated with lower respiratory viral loads) (Fulton et al., 2010; Lee et al., 2013), fcgr1 (which contribute to the antiviral-specific antibody responses) (Teijaro et al., 2010; Job et al., 2019), and ccr2 (which promotes viral clearance by enhancing virus-specific T cell responses)

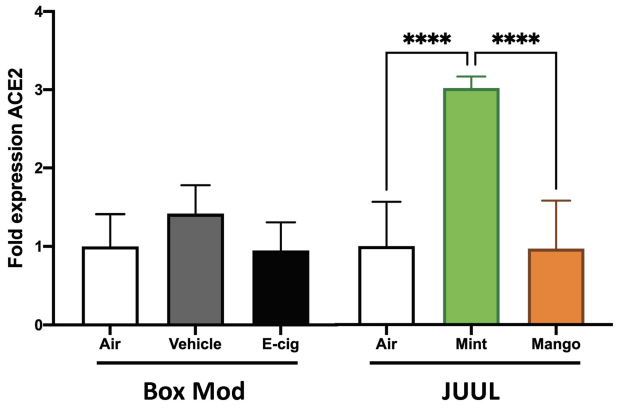

FIGURE 4 | JUUL Mint aerosol inhalation drives upregulation of ACE2 expression within the lungs. Relative gene expression by qPCR from lung tissues of mice exposed for 1 h daily to e-cig aerosols was analyzed. Male C57BL/6 mice were exposed to nicotine containing (Ecig) and no nicotine (Vehicle) e-cig aerosols generated by box Mods (70:30 PG:VG with 6 mg/mL nicotine) for 3 months, with no significant differences found relative to Air controls. Female C57BL/6 mice exposed for 20 min three times daily for 1 month to aerosols generated from JUUL (30:70 PG:Gly with 59 mg/mL nicotine) Mint flavored pods had three-fold greater ACE2 expression in the lung, while chronic JUUL Mango aerosol inhalation did not alter ACE2 expression. ****$p < 0.0001$. Data are representative of five mice per group.

(Pamer, 2009; Strunz et al., 2019). In addition, CD4 was also reduced in CD1 but not C57BL/6 mice, which are also important for antiviral responses in the lung (Fulton et al., 2010; Teijaro et al., 2010). Finally, the most upregulated gene in both strains of mice was Krt8, an uncommon keratin gene expressed in epithelial cells. Krt8 has been found to have increased expression in some carcinomas (Chu and Weiss, 2002; Gires et al., 2006) and to promote tumor progression and metastases in gastric carcinoma in particular (Fang et al., 2017), but it has also been involved in alveolar epithelial progenitors in lung regeneration and increased replication of respiratory syncytial virus (Shirato et al., 2012; Strunz et al., 2019).

The modulation of the inflammatory status of the lungs observed in the transcriptomic analysis was associated with impairment of key immune and inflammatory pathways defined by KEGG enrichment plots. On one hand, we found downregulation of pathways involved in cytokine receptor interactions (**Figure 1D**), chemokine signaling (**Figure 1E**), and primary immunodeficiency (**Figure 1F**). On the other hand, we found upregulation of pathways related to cell activation in neutrophils (**Figures 1G,H**) and dendritic cells (**Figure 1I**). Activation of neutrophils speeds their demise, by neutrophil extracellular trap formation (NETosis) and apoptosis, such that activation may not be paired with increased numbers of neutrophils if chemokines such as IL-8 are not released. In the particular case of increased activation of neutrophils, it has been shown that prolonged activation of these cells can lead to detrimental effects to the host and can even cause severe disease, including pneumonia and ARDS (Abraham, 2003; Galani and Andreakos, 2015), with ARDS being the main clinical feature of both EVALI (Alexander et al., 2020; Wang J. et al., 2020) and COVID-19 (Matthay et al., 2020; Xu et al., 2020). More recently, it has been shown that severity of COVID-19 is associated with increased activation of neutrophils (Leppkes et al., 2020; Radermecker et al., 2020; Wang J. et al., 2020; Zuo et al., 2020). Thus, this analysis suggests that e-cigarette exposure can affect crucial inflammatory pathways involved in host defense and immune-mediated tissue damage.

In addition, we assessed abundance in the BALF fluid of eicosanoid lipid inflammatory mediators and found decreases in prostaglandin E2 (PGE2) and 12-hydroxyeicosatetraenoic acid (12-HETE), with a trend in 15-hydroxyeicosapentaenoic acid (15-HEPE) (**Figure 2**). 12-HETE is the major product of 12/15-LOX (lipoxygenases) in rodents and is also generated by a dedicated 12-LOX (which has been reported to be upregulated in the lungs of hypoxic rats) (Preston et al., 2006; Sagliani et al., 2013). 12-HETE stimulates proliferation of pulmonary artery smooth muscle cells and have a possible role in the remodeling process in pulmonary hypertension (Legler et al., 2010; Sagliani et al., 2013). In the case of 15-HEPE, it has been shown to be able to dampen allergic rhinitis symptoms through increased production by eosinophils, leading to inhibition of mast cell degranulation (Sawane et al., 2019; Petrilli et al., 2020). In the context of PEG2, it is the most abundant eicosanoid and a very potent lipid mediator key in many biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity, and fertility (Legler et al., 2010; Grewal et al., 2013). PGE_2 can thus modulate various steps of inflammation in a context-dependent manner and coordinate the whole process in both proinflammatory and anti-inflammatory directions, contributing to the regulation of the cytokine expression profile, and can act as anti-inflammatory on innate immune cells like neutrophils, monocytes, and NK cells (Legler et al., 2010; Ricciotti and FitzGerald, 2011). Interesting enough, reductions in PGE2, 12-HETE, and 15-HEPE seem to be mediated by vehicle components (without nicotine) since vehicle-treated mice had equal to greater changes as compared with EV mice (with nicotine) (**Figure 2**). Therefore, besides the variety of inflammatory proteins altered due to e-cigarette aerosol inhalation, there are also changes in important lipid mediators involved in inflammation and pulmonary hypertension.

With all that being said, the current global COVID-19 pandemic has raised concern for those individuals with pre-existing conditions, including those with impaired immune response that could increased susceptibility to SARS-CoV-2 infection. This virus is novel and therefore scientists and clinicians have raced to try to understand factors influencing susceptibility to this infection. Early reports indicated that age was the primary risk factor, with mortality rates of >80% reported for patients >80 years of age in some cohorts (Hoffmann et al., 2020; Petrilli et al., 2020). However, more and more young individuals have developed life-threatening COVID-19, suggesting that other factors may influence susceptibility to life-threatening forms of this disease (Grewal et al., 2013; Lee et al., 2014). Such conditions might be inherited or induced by extrinsic factors. Among these extrinsic factors, the use of vaping devices may play a role and should be further studied.

Using deconvolving bulk RNA-sequencing data, we found increased expression of ACE2 in the lung tissue of vape pen-exposed mice (**Figure 3A**). ACE2 is a crucial protein for SARS-CoV-2 infectivity (Chen and Kolls, 2013; Hoffmann et al., 2020). In addition, we correlated the abundance of RNA-sequencing data to ACE2 expression and found a correlation of ACE2 with a lower abundance of resting $CD4^+$ memory T cells but with a higher abundance of follicular helper T cells (Spearman, $p < 0.05$) (**Figure 3C**). This indicates that there is impairment in adaptive immune responses that are crucial for many infectious diseases. Memory $CD4^+$ T cells provide much more immediate protection and are key for vaccine-mediated immunity (Mahmud et al., 2013; Gray et al., 2018). Follicular helper T cells can drive the differentiation of B cells into antibody-secreting cells and to induce the production of high-affinity class-switched antibody (Gray et al., 2018; Hutloff, 2018). Because we used whole lung tissue for RNA extraction, the source of the increased ACE2 gene expression is unknown. In addition, using GSEA, we found that e-cigarette-induced upregulation of ACE2 is correlated with the downregulation of several immune-associated pathways, including interleukin receptor signaling, IFNγ signaling [necessary for Th1 cells, which are essential for host defense against many pathogens, including viruses such as influenza viruses (Chen and Kolls, 2013; Smith et al., 2020)], IL-2/STAT5 pathway [involved in T regulatory cell differentiation and proliferation (Mahmud et al., 2013; Leung et al., 2020b)], and LPA4 pathways and CD28 co-stimulation [a key molecule for T cell activation (Esensten et al., 2016; Leung et al., 2020a)] (**Figures 3D–G**). This data seems to be linked with the lower abundance of resting $CD4^+$ memory T cells observed in **Figure 3C**. Notably, ACE2 upregulation in e-cigarette aerosol-exposed mice is associated with increased expression of genes associated with influenza infection (**Figures 3D,E**). Thus, these data suggest that vape pens can increase the expression of ACE2, which associated with impaired key immune pathways involved in the host ability to fight infection, particularly viral infection such as influenza, and potentially SARS-CoV-2.

Despite of the public concern about the potential role of smoking or vaping in COVID-19, so far just a few studies have shown that such relationship exists (Dai et al., 2020; Leung et al., 2020a,b; McAlinden et al., 2020; Russo et al., 2020; Smith et al., 2020). In the case of smoking, some studies have shown that cigarette smoke and COPD can increase the expression of ACE2 in the respiratory tract (Leung et al., 2020b; Smith et al., 2020) and nicotine has been attributed as a driver to this increased ACE2 expression in smokers (Pintarelli et al., 2019; Leung et al., 2020a; Russo et al., 2020; Wang Q. et al., 2020). In contrast, a recent study concluded that it is in fact tobacco and not nicotine that is driving increase in ACE2 expression (Lee et al., 2020), although another study suggested that α7 nicotinic acetylcholine receptor signaling is involved in inducing ACE2 expression (Wang Q. et al., 2020). In the context of e-cigarettes, it has been found that flavorless e-cigarette does not induce ACE2 expression (Lee et al., 2020). Similar to this, we found that chronic, daily inhalation of flavorless nicotine-containing Mod box e-cigarettes did not induce ACE2 expression by qPCR (although flavorless nicotine-containing vape pens increase 33% ACE2 expression based in RNA-sequencing expression analysis). However, daily inhalation of JUUL mint aerosol (but not JUUL mango) leads to increased expression of ACE2 in lung tissue, suggesting that specific flavorants used to create the mint flavor in JUUL pods are driving the increased expression of ACE2. This is of great concern since there are several thousands of chemicals used to provide flavor to e-cigarettes that might be increasing the risk of developing a severe COVID-19. Future studies dedicated to the effects of different flavorants might give insight into increased expression of ACE2 that may account for susceptibility to SARS-CoV-2 infection in relatively young and healthy individuals.

Limitations of this work include the absence of cellular or tissue-level pathology driven by the gene expression changes identified. Further studies are needed to assess for potential altered susceptibility to viral and bacterial pathogens caused by e-cigarette vaping. Although both female and male mice were exposed to e-cigarette aerosols, direct sex effects could not be distinguished due to differences between exposures. It is critical to drill down on potential temporal effects, effects specific to certain chemicals/flavorants, and sex effects of e-cigarette use. There is a need to identify protein, cellular, tissue level, and physiologic read-outs relevant to gene expression changes identified. By combining data across studies, the e-cigarette research community would be well placed to rapidly identify biologically relevant signals occurring from e-cigarette aerosol inhalation. While nicotine concentrations were different in the vape pen, box Mod, and JUUL exposures, the cotinine levels in the plasma of mice immediately after daily exposures were similar. This is consistent with what is known about these devices, that vape pens have poor delivery of nicotine to the bloodstream, while box Mods and pod devices have highly successful nicotine delivery.

Altogether, these data suggest that chronic inhalation of e-cigarette aerosols will lead to numerous gene expression changes within immune and structural cells of the lung, with an overall pattern of immunosuppression, which may diminish host defenses of both innate and adaptive responses in the lungs and lead to increased susceptibility to infections. Alteration of the immune state of the lung by aerosol inhalation is also very likely to impact healing (pulmonary fibrosis), inflammatory responses (hypersensitivity pneumonia, acute eosinophilic pneumonia, and acute interstitial pneumonia), and regulation of genetically damaged cells (lung cancer). Of particular note, we found that flavorants may induce ACE2 expression, increasing the risk to develop severe COVID-19. Flavorant effects escape from most e-cigarette studies due to the wide variety of chemicals used to provide flavor and to simplify the experimental approaches leaving most of the time only the three main components of e-juices, PG, vegetable glycerin, and nicotine.

In summary, beyond the specific impact of vaping on one pathway, these RNAseq data demonstrate a multitude of changes in gene expression which appear to be higher

than those seen with cigarette smoke inhalation. Immune (Pintarelli et al., 2019) and inflammatory pathways from both innate and adaptive responses were found to be impacted by chronic inhalation of e-cigarette aerosols, which again raise concern for altered susceptibility to lung infections and inflammatory diseases in human users of these vaping devices. Further studies are needed to define the roles of e-device composition, wattage, chemicals within e-cigarette aerosols (flavorants, vehicles, nicotine, and contaminants), and puff topography on gene expression changes in the lungs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by UCSD and VASDHS IACUC.

AUTHOR CONTRIBUTIONS

LC, SS, WO, and SC: conception and design of the experiments. AM, JM-S, JO, SN, IA, CB, SC, WL, WO, SS, and LC: acquisition, analysis, and interpretation of data. JM-S and LC: manuscript composition. All authors reviewed, contributed to, and approved the manuscript.

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