THE IMPACT OF ACTIVE AND PASSIVE SMOKING UPON HEALTH AND NEUROCOGNITIVE FUNCTION

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THE IMPACT OF ACTIVE AND PASSIVE SMOKING UPON HEALTH AND NEUROCOGNITIVE FUNCTION

Topic Editor:
Thomas Heffernan, Northumbria University, UK

A combusted cigarette emits thousands of different chemicals into the air, dozens of which are toxins.

Cover photo by Pixabay. Available at: https://pixabay.com/en/ashtray-smoking-smoke-cigarette-1502822/

Tobacco smoking is a major risk factor for a number of chronic diseases, including a variety of cancers, lung disease and damage to the cardiovascular system. The World Health Organization recently calculated that there were 6 million smoking-attributable deaths per year and that this number is due to rise to about eight million per year by the end of 2030. Recent work has demonstrated that habitual smoking in adults is not only associated with a range of health problems, but may also contribute to a number of neurocognitive deficits, including deficits in memory and attention. One area of growing concern is the health and neurocognitive consequences of exposure to second-hand smoke or "passive smoking" (where a non-smoker inhales another person's smoke, mainly in the form of side-stream smoke). In terms of tackling smoking-related problems, there has been a rise in the amount and range of smoking cessation and interventions techniques, including the emergence of e-cigarettes as one of the most popular forms of nicotine replacement therapies. The present book comprises a collection of manuscripts discussing (1) the impact of active and passive smoking upon health and neurocognitive function, (2) smoking cessation techniques and interventions used to tackle smoking-related problems, and (3) a critical consideration of

current issues surrounding the use of e-cigarettes as nicotine-replacement therapy. This collection of papers includes empirical, theoretical, and review papers. This Research Topic demonstrates the broad nature of research currently being undertaken in this field and should pave the way for future work.

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Table of Contents

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EDITORIAL
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Editorial: The Impact of Active and Passive Smoking upon Health and Neurocognitive Function

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Keywords: active smoking, passive smoke exposure, health, neurocognitive function, smoking cessation

The Editorial on the Research Topic

The Impact of Active and Passive Smoking upon Health and Neurocognitive Function

Tobacco smoking is a major risk factor for a number of chronic diseases, including a variety of cancers, lung disease, and damage to the cardiovascular system. The World Health Organization recently calculated that there were six million smoking-attributable deaths per year and that this number is due to rise to about eight million per year by the end of 2030. Recent work has demonstrated that habitual smoking in adults is associated with a range of health conditions, including cardiovascular disease, pulmonary dysfunction, and an increased risk of a variety of cancers. In terms of neurocognitive function, although some studies have found that acute smoking can enhance cognitive functions in the short term, actually chronic smoking is deleterious in the long term. Chronic smoking has been associated with reductions in working memory (the temporary storage and manipulation of information), executive function (planning tasks, focusing ones attention, and ignoring irrelevant distractions), and prospective memory (memory for everyday things, such as keeping an appointment, or taking an important medication on time). More recently, the focus on smoking-related health problems and neurocognitive deficits has expanded to include the study of "second-hand smoking" (also known as "passive smoking" – wherein a person who does not smoke him/herself inhales tobacco smoke either via side-stream smoke or via smoke being blown directly into his/her face). Research in this area has linked exposure to second-hand smoke in those who have never smoked to a range of health problems akin to smokers, including lung and cardiovascular disease, as well as deficits in neurocognitive function. In terms of neurocognitive function, exposure to second-hand smoke has been linked with an increased risk of mild cognitive impairments in older adults, reductions in working memory, as well as deficits in executive function. Interventions aimed at reducing cigarette consumption and improving the health of both smokers and those exposed to second-hand smoke continue to be developed. The aim of this Frontiers Research Topic is to bring together a collection of papers that look at what impact active and passive smoking has upon health and neurocognitive function; as well as to consider some of the wide variety of interventions aimed at reducing cigarette use and/or improving health.

Copeland examined pre-treatment characteristics among daily smokers (including smoking patterns, smoking outcome expectancies, and smoking-related health information) and how these related to success on a brief motivational enhancement intervention. Marshall et al. explored whether the combined (polydrug) effect of consuming excessive amounts of alcohol and smoking cigarettes exacerbated everyday memory problems when compared with the sum of their independent effects (excessive drinking alone, or smoking alone). Philibert et al. examined whether aryl hydrocarbon receptor repressor (AHRR) can be used to determine whether AHRR methylation status is a quantifiable biomarker for progress in smoking cessation that could have substantial impact on both smoking

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cessation treatment and research. Ling and Heffernan reviewed evidence in relation to the cognitive consequences of exposure to second-hand smoke in those who had no history of smoking. Payne et al. evaluated chronic obstructive pulmonary disease-related health factors in flight attendants exposed to second-hand cigarette smoke and assessed whether meditative movement was effective as a treatment in improving pulmonary function in these flight attendants. Jukosky et al. demonstrated how cigarette exposure alters the innate immune response and increases an individual's susceptibility to pathogen infection when compared with non-exposed individuals. Jovanovic and Jakovljevic discuss regulatory control of e-cigarette composition and raises concern regarding the quality control and health outcomes surrounding e-cigarettes. The commentary by Parrott discusses concerns about the paradoxical nature of using e-cigarettes; whether they may in fact be damaging to physical/psychological health of the users, as well as raising concerns about what impact e-cigarettes have upon those who are "passively vaping." Lasebikan and Ola assessed the efficacy of screening, brief intervention, and referral for treatment package to reduce tobacco smoking in two semi-rural community settings in South-West Nigeria.

Overall, the papers presented in this *Frontiers in Psychiatry* special topic demonstrates the broad nature of research currently being undertaken in relation to active and passive smoking and some of the current issues surrounding the use of e-cigarettes as nicotine-replacement therapy. The research cited here should pave the way for further work in this area. Areas for future research include the concern of what impact exposure to second-hand smoke might be having upon children's health, neurocognitive function, and educational achievement, an area of particular importance given the recent estimates from the World Health Organization that approximately 40% of children across the world are regularly exposed to second-hand smoke in the home. A further area that has received very little attention at all is whether exposure to "third-hand smoke" (the residue of nicotine and other chemicals left on indoor surfaces as a result of tobacco) smoking has a detrimental impact upon those who have never smoked, both in terms of health and neurocognitive function.

AUTHOR CONTRIBUTIONS

This is the sole work of the author and editor for this Special Topic.

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ORIGINAL RESEARCH
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In vivo Cigarette Smoke Exposure Decreases CCL20, SLPI, and BD-1 Secretion by Human Primary Nasal Epithelial Cells

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Smokers and individuals exposed to second-hand cigarette smoke have a higher risk of developing chronic sinus and bronchial infections. This suggests that cigarette smoke (CS) has adverse effects on immune defenses against pathogens. Epithelial cells are important in airway innate immunity and are the first line of defense against infection. Airway epithelial cells not only form a physical barrier but also respond to the presence of microbes by secreting antimicrobials, cytokines, and chemokines. These molecules can lyse infectious microorganisms and/or provide signals critical to the initiation of adaptive immune responses. We examined the effects of CS on antimicrobial secretions of primary human nasal epithelial cells (PHNECs). Compared to non-CS-exposed individuals, PHNEC from *in vivo* CS-exposed individuals secreted less chemokine ligand (C-C motif) 20 (CCL20), Beta-defensin 1 (BD-1), and SLPI apically, less BD-1 and SLPI basolaterally, and more CCL20 basolaterally. Cigarette smoke extract (CSE) exposure *in vitro* decreased the apical secretion of CCL20 and beta-defensin 1 by PHNEC from non-CS-exposed individuals. Exposing PHNEC from non-CS exposed to CSE also significantly decreased the levels of many mRNA transcripts that are involved in immune signaling. Our results show that *in vivo* or *in vitro* exposure to CS alters the secretion of key antimicrobial peptides from PHNEC, but that *in vivo* CS exposure is a much more important modifier of antimicrobial peptide secretion. Based on the gene expression data, it appears that CSE disrupts multiple immune signaling pathways in PHNEC. Our results provide mechanistic insight into how CS exposure alters the innate immune response and increases an individual's susceptibility to pathogen infection.

Keywords: cigarette smoke exposure, primary nasal epithelium, antimicrobial peptides, CCL20, beta defensing-1, SLPI, innate immune response, nasal epithelial cell culture

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INTRODUCTION

There is copious evidence that exposure to primary cigarette smoke (CL) and/or second-hand cigarette smoke (SHCS) is associated with increased frequency of infections or other symptoms of perturbations of the immune system. Exposure to primary CL or SHCS is a risk factor for recurrent otitis media, upper respiratory tract infection (1, 2), meningococcal

infection (3), bacteria pneumonia, oral candidiasis (4, 5), type 2 herpes simplex virus (6), pneumococcal disease, influenza (7), asthma (8–11), and rhinosinusitis (12). One recent review of the epidemiology of smoke exposure and infection concludes that "Cigarette smoking is a substantial risk factor for important bacterial and viral infections" (7).

Cigarette smoke exposure alters the physiology of the upper airway. Evaluation of adenoid tissue removed from children living in SHCS-contaminated environments demonstrates significant histopathological and ultrastructural differences in these upper airway immune tissues when compared to children not exposed to SHCS (1). In studies of the effect of CS-extract on adherence of respiratory pathogens to buccal epithelial cells, CS-extract increased bacterial binding to the cells (13). Upper airways of CS-exposed individuals harbor more potential pathogens than those of non-smokers (14). These findings are consistent with studies in mice. For example, when mice exposed to CS were compared to mice with sham exposure, CS-exposed mice were less able to clear *Pseudomonas aeruginosa*. Besides the delayed rate of clearance, mice exposed to CS experienced increased inflammation (15). In studies looking at changes in immune responses to bacterial challenge in mice exposed to nicotine, exposed mice exhibited significantly higher titers of influenza virus following infection (16). Subsequent studies of human monocytes exposed to bacterial toxins and then to cigarette smoke extract (CSE) demonstrated extremely aberrant immune responses relative to cells exposed to bacterial toxins but not exposed to CSE (17).

A major function of the mucosal epithelium is innate immune protection (18, 19). Beyond providing a physical barrier between the external environment and the body itself in the digestive, reproductive, and respiratory organs, the epithelium produces a complex multifaceted mucus layer that lines mucosal surfaces (20). To maintain balance with the body's normal flora and to suppress infections, epithelial cells monitor their environment and respond to microbial challenges by altering secretions into the luminal environment. Partners with the epithelial cells in the delicate balancing act with microbial flora are leukocytes that can also recognize a microbial threat and respond by altering secretions. In response to threatening microbes recognized by epithelial cells and/or by leukocytes, airway mucosal epithelium produce antimicrobial proteins, including defensins, lysozyme (LYZ), lactoferrin (LF), secretory leukocyte protease inhibitor (SLPI), and chemokine ligand (C–C motif) 20 (CCL20) (19, 21–23). LYZ is a cationic bacteriolytic protein produced by mucosal epithelial cells, another cationic protein is LF that impedes bacterial growth and replication by sequestering iron. LYZ and LF are both released by the nasal epithelium (24). SLPI, a low molecular weight protease inhibitor with an antimicrobial domain is also found in nasal secretions (25, 26). Small cationic peptides, including defensins and defensin-like CCL20, exert their effect through electrostatic interactions with bacterial membranes that puncture the microbial cell. Recently, CCL20 has also been found to exhibit antiviral activity as well (27). In addition to antimicrobial activity, many of these peptides, including human beta-defensin 2 (BD2) and CCL20, also serve as chemokines to recruit specialized immune cells carrying the CCR6 receptor to the site of an infection (24, 28, 29).

The airway mucosa produces antimicrobials both constitutively and in response to recognition of microbes. To recognize potential pathogens, the innate immune system relies on *pathogen associated molecular patterns* (PAMP) that are unique to microbes. One group of receptors that recognize PAMP is the toll-like receptors (TLR) that are expressed on epithelial cells as well as most specialized immune cells. PAMP ligands are repeated in a wide variety of pathogenic microbes, including bacteria, fungi, and viruses. TLR ligands include lipoteichoic acid (LTA), derived from Gram-positive bacteria (30). Common to TLR in mammals is the ability to induce effects through activation of the transcription factor NFκB, and through mitogen-activated protein kinases (MAPKs) independent of NFκB (31). TLR activation results in an induction of many molecules, including antimicrobials and cytokines necessary for innate and adaptive immune protection (16, 32, 33).

As noted above, it is clear that CS perturbs the response to pathogens. Although various experiments have associated CS exposure with specific immune system perturbations, the mechanisms involved are not yet fully understood. An understanding of those mechanisms will provide new prognostic, diagnostic, and therapeutic approaches to smoke-related morbidity and mortality. The experiments reported here tested the hypothesis that CS exposure *in vivo* or *in vitro* suppresses the release of antimicrobial peptides from primary human epithelial cells.

Our group has shown that *in vitro* CSE exposure reduces production of the antimicrobial peptide CCL20 in Beas-2b, immortalized human bronchial epithelial cells (34). However, Beas-2b, although not transformed, is a line that has been maintained in culture for long periods and is a genetic representation of a single individual. In order to examine the effect of CS exposure on antimicrobial peptide secretion in true primary cells, we established short-term cultures of primary human nasal epithelial cells (PHNECs) from smoke-naïve individuals and smokers, and assayed antimicrobial peptide secretion.

MATERIALS AND METHODS

Influence of *In vivo* Cigarette Smoke Exposure on the Antimicrobial Secretions of PHNEC

Beta-defensin 1 (BD1), CCL20, and SLPI secretion were assayed in cells obtained from CS-exposed individuals and individuals with no CS exposure. PHNECs were obtained from 13 individuals with no CS exposure and 7 individuals with significant primary CS or SHCS exposure. PHNECs were obtained and cultured as described in section below. As noted by other groups studying PHNEC, it was much more difficult to culture PHNEC from CS-exposed individuals compared to non-CS-exposed individuals (Carson, personal communication). Four experimental treatments were performed using both *in vivo* smoke-exposed PHNEC and cells from smoke-naïve individuals. These treatments were constitutive secretion where nothing was applied, CSE exposed, LTA stimulation (LTA stimulated) to simulate a bacterial infection, and CSE exposed with LTA stimulation. CSE-exposed primary human nasal cell cultures were treated apically with 300 μL 1× CSE in air–liquid interface (ALI) for 3 h, other treatments received ALI only. This CSE exposure

did not increase cell death significantly 24 h later (data not shown). CSE was then removed by suction and cells were rinsed twice with PBS. Then LTA-exposed cell cultures were stimulated apically with LTA from *Bacillus subtilis* (10 μg/mL) in ALI media by applying 300 uL in the apical compartment. PHNECs were incubated for 20 h, and after this period apical and basolateral supernatants were harvested. Cell supernatants were centrifuged at 17,000 × *g* for 10 min and the supernatants were removed and stored at −80°C. BD1, secretory leukocyte protease inhibitor (SLPI), and CCL20 were assayed by ELISA in apical and basolateral secretions either using commercially prepared assay kits or ELISAs developed from ELISA development kits (R&D systems, Mckinley, MN, USA, or Leinco Technologies, St. Louis, MO, USA).

A two-way analysis of variance (ANOVA) was used to examine the effect of treatment (constitutive, CSE exposed, LTA stimulated, and CSE exposed with LTA stimulation) or smoking status (*in vivo* smoke-exposed versus cells from smoke-naïve individuals) and any interactions between treatment and smoking status on PHNEC secretions. Data were natural log (ln) transformed for analysis to meet the underlying ANOVA assumption of a normal distribution. *Post hoc* analyses were carried out with Tukey's HSD tests. All statistics and data transformations were performed using JMP 11.

PHNEC Culture

Primary human nasal epithelial cells were obtained from smoke-exposed and non-smoke-exposed healthy human volunteers and differentiated *in vitro* in transwells as described by Carson et al. (35). The criteria for recruiting subjects were similar to those described previously (35, 36). We determined smoke exposure status via questionnaire and confirmed it through measurement of urine cotinine.

Primary human nasal epithelial cells were collected from healthy smoking and non-smoking adult volunteers by gently scraping the inferior surface of the turbinate five to eight times with a Rhinopro™ curette (Arlington Scientific, Arlington, TX, USA). The curette was inserted through a nasoscope, which was used to visualize the inferior turbinate. This protocol was approved by the Institutional Review Board of the Geisel School of Medicine at Dartmouth.

Primary human nasal epithelial cells from nasal scrapes were seeded on human collagen-coated wells of a 12-well plate and grown to 70% confluence in ALI media (Lonza Biologics). At 70% confluence, PHNECs were trypsinized and seeded into flasks and grown in media that was one part ALI and two parts bronchial epithelial growth medium (BEGM) (Lonza Biologics). PHNEC in flask were grown to 70% confluency and subsequently trypsinized and seeded into collagen-coated filter supports with a 0.4-μM pore size (Trans-CLR; Costar, Cambridge, MA, USA) and grown in ALI media. We promoted mucociliary differentiation of PHNEC after cells grew to confluency by supplementing the media with all-trans retinoic acid and the media was removed from the apical compartment to create ALI culture conditions. We observed mucociliary differentiation 14–21 days after ALI differentiation and the cultures of PHNEC were utilized experimentally at this stage.

Smoke Extract Generation

Cigarette smoke extract was generated by using vacuum suction to draw the smoke from a single research reference grade cigarette (Kentucky Cigarette Research and Development Center at the University of Kentucky, Lexington, KY, USA) through 100 mLs of ALI media in a controlled manner.

Time-Course Experiments

Primary human nasal epithelial cells were obtained from four non-smoke-exposed individuals, expanded, and differentiated as described previously. Constitutive, CSE, LTA, and LTA + CSE treatments were established as described previously. Three wells of cells per individual were used in each treatment. At time points of 1 and 6 h after LTA stimulation, we harvested cells apical and basolateral supernatants and RNA (for time-course gene expression). Apical and basolateral BD1 and CCL20 secretion were measured using ELISA. A repeated measures MANOVA was used to test for differences in CCL20 and BD-1 secretion between the four treatments previously described and time points (1 and 6 h post-LTA stimulation). Bonferroni-corrected *t*-tests were used *post hoc* to identify significant differences between specific treatments.

Expression of 511 immunology-related genes was quantified using Nanostring technology. We analyzed expression at the 1 and 6 h time point post-LTA stimulation and compared LTA and CSE + LTA treatments. RNA was extracted from individual transwells ($n = 12$) using a Qiagen Allprep kit. The total counts of mRNAs were assayed using nCounter GX Human Immunology gene expression code set that uses molecular barcodes attached to target-specific probes. Barcoded probes hybridize directly to their RNA targets in solution, and the probes are counted directly using microscopic imaging. Following the manufacturer's protocol, 100 ng mRNA was hybridized with the nCounter GX Human Immunology code set and loaded into the nCounter prep station followed by imaging and quantification using the nCounter Digital Analyzer. Quality control and data normalization were performed using the nSolver analysis software. Data were normalized to global means for internal positive controls, and subsequently normalized to the geometric means of a suite of housekeeping genes. Two reference genes (GUSB and HPRT1) were eliminated from the normalization panel because their average raw count data varied more than 1 SD between LTA and CSE + LTA treatments. The panel of genes used for normalization included genes with both low and high expression levels.

Specific samples from each individual were paired when experiments were designed and assigned to separate treatments. Gene expression in the LTA-stimulated treatment and the LTA + CSE treatments were compared by matched pair *t*-test separately at each time point. No corrections were made for multiple comparisons as each barcode is considered an independent assay.

RESULTS

PHNECs from CS-Exposed Individuals Were Difficult to Culture

To examine the difference in antimicrobial secretions between PHNEC from smokers and non-smokers and their response

to CSE exposure, cultures were established from each group. While PHNEC from non-smokers grew readily, PHNEC from smokers generally grew poorly and only a fraction of the donors provided cells that could be grown to the required level for the assays. Here, 86.6% of nasal scrapes from healthy donors ($n = 15$) were expanded in culture and differentiated into ciliated ALI cultures as described in our methods, while 33.3% of nasal scrapes from CS and SHS exposed individuals ($n = 12$) were able to grow and differentiate in the same manner. There was a significant difference between successful non-CS-exposed and CS-exposed PHNEC cultures using a z-test to compare two population proportions ($z = -2.83$, $p = 0.002$, **Figure 1**). These results reflect outcomes from a subset of nasal samples that were gathered after noticing this trend. This is similar to what has been reported by other researchers harvesting PHNEC from smoke-exposed individuals (Carson, personal communication).

In vivo Smoke Exposures Alters Antimicrobial Peptide Secretions by PHNEC

Chemokine Ligand (C–C Motif) 20

Primary human nasal epithelial cell from *in vivo* CS-exposed individuals secreted significantly less CCL20 apically and significantly more CCL20 basolaterally than PHNEC from non-CS-exposed individuals. **Figure 2A** illustrates this finding by pooling all treatment groups and compares PHNEC CCL20 secretion from non-CS and *in vivo* smoke-exposed individuals. Our measurements of CCL20 in the apical compartment showed that there was no significant effect of treatment (constitutive, CSE exposed, LTA stimulated, and CSE exposed with LTA stimulation) and no interaction between treatment and smoking status. However, there was a significant effect of smoking status on CCL20 secretion and this was highly significant in both the apical ($F_{1,235} = 24.07$, $p < 0.0001$) and basolateral ($F_{1,171} = 12.2$, $p < 0.001$) compartments. Regardless of the treatment group, *in vivo* smoke-exposed PHNEC secreted less CCL20 apically and more CCL20 basolaterally than non-smoke-exposed individuals (**Figure 2A**). There was a significant effect of treatment in the basolateral compartment ($F_{3,171} = 3.19$, $p = 0.025$). *Post hoc* analysis showed significantly increased CCL20 secretion in *in vivo* smoke-exposed PHNEC in the basolateral constitutive ($p = 0.017$) and basolateral CSE ($p = 0.01$) treatments (**Figure 2B**).

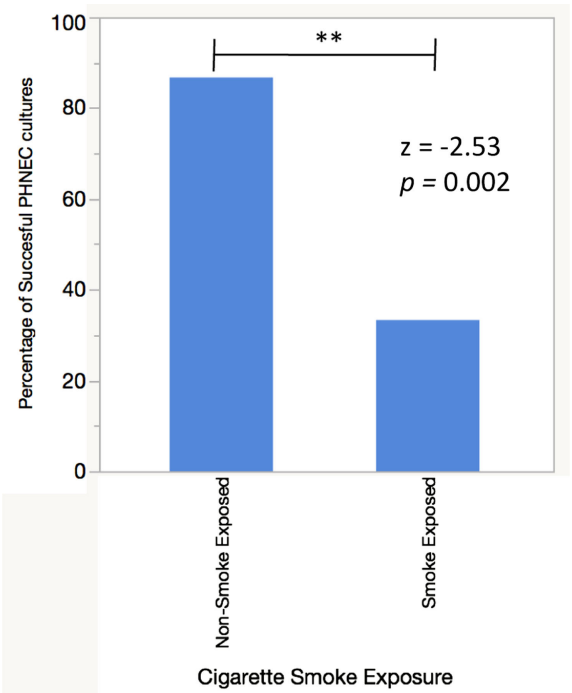

FIGURE 1 | PHNEC sampled from cigarette smoke-exposed individuals fail to grow into confluent ALI cultures more often than PHNEC from non-smoke-exposed individuals. A successful culture was defined as one that grew to confluency in a transwell and differentiated into a ciliated, mucus-producing air–liquid interface culture.

SLPI

Primary human nasal epithelial cell from *in vivo* CS-exposed individuals secreted significantly less SLPI apically and basolaterally than PHNEC from non-CS-exposed individuals. **Figure 3** illustrates this finding by pooling all treatment groups and compares PHNEC SLPI secretion from non-CS and *in vivo* smoke-exposed individuals. For SLPI from cultured PHNEC, there was a highly significant effect of smoking status on SLPI secretion but no significant effect of treatment and no interaction between treatment and smoking status. PHNEC from non-smoke-exposed individuals had much higher SLPI secretions in both the apical ($F_{1,139} = 15.2$, $p < 0.001$) and basolateral ($F_{1,154} = 25.9$, $p < 0.001$) compartments (**Figure 3**). Regardless of the treatment group, PHNEC from smoke-exposed individuals secreted less SLPI apically and basolaterally than that from non-smoke-exposed individuals.

Beta-Defensin 1

Primary human nasal epithelial cell from *in vivo* CS-exposed individuals secreted significantly less BD-1 apically and basolaterally than PHNEC from non-CS-exposed individuals. **Figure 4** illustrates this finding by pooling all treatment groups and compares PHNEC BD-1 secretion from non-CS and *in vivo* smoke-exposed individuals. However, identical to SLPI, analysis of apical and basolateral BD-1 secretion also showed no significant effect of treatment and no interaction between treatment and *in vivo* smoking status. PHNEC cultured from *in vivo* smoke-exposed individuals secreted significantly less BD-1 into the apical ($F_{1,155} = 8.92$, $p = 0.003$) and basolateral compartments ($F_{1,167} = 23.1$, $p < 0.001$) (**Figure 4**).

Time-Course Experiments

CSE Decreased Apical CCL20 Secretion in Unstimulated PHNEC at Hour 1 and LTA-Stimulated Cells at Hour 6 and Also Decreased Basolateral Secretion

Cigarette smoke extract exposure significantly decreased constitutive apical CCL20 secretion at hour 1 (**Figure 5A**), but this difference was not significant at hour 6 (**Figure 5B**). CSE exposure significantly decreased LTA-stimulated apical CCL20 secretion

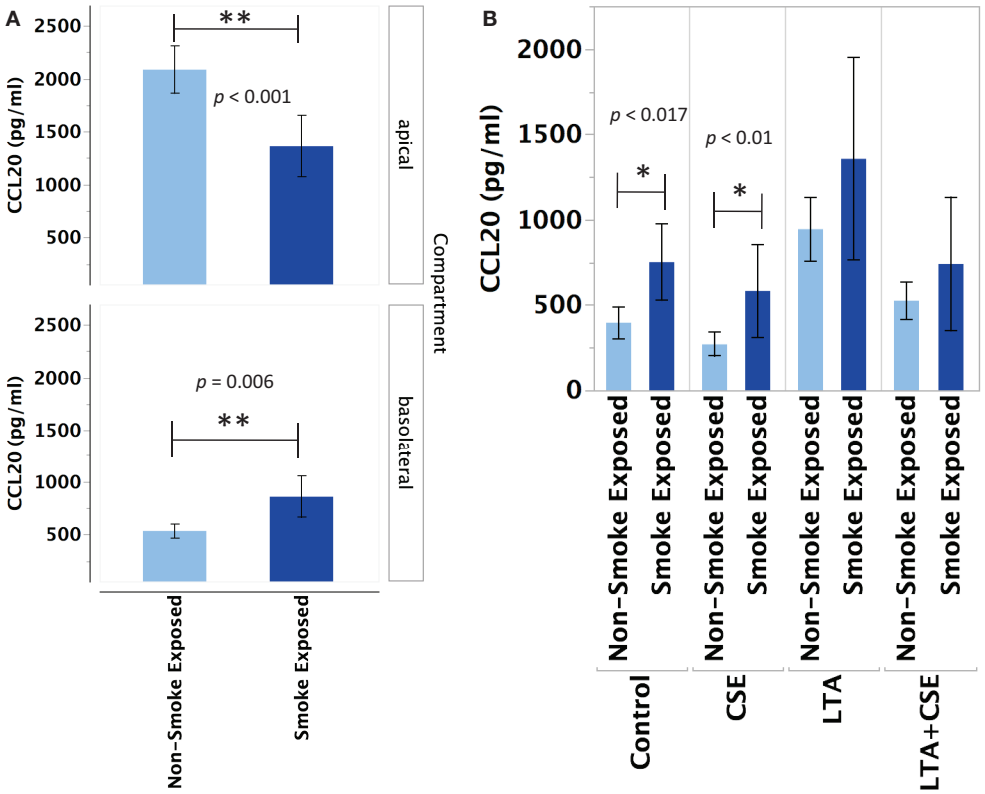

FIGURE 2 | CCL20 secretion is altered in PHNEC from *in vivo* smoke-exposed individuals. Compared to non-smoke-exposed individuals, smoke-exposed individuals had significantly lower apical CCL20 secretion and significantly higher basolateral CCL20 secretion **(A)**. Figure 2A shows secretion data pooled (from all treatments) from every PHNEC secretion sample. CCL20 secretion into the basolateral compartment is significantly higher in PHNEC from *in vivo* smoke-exposed individuals in the control (constitutive) and the CSE-exposed treatments **(B)**.

at hour 6 (**Figure 5D**), but this difference was not significant 1 hour post-LTA stimulation (**Figure 5C**). Basolateral secretion of CCL20 was significantly reduced by CSE exposure at the 6 hour time point in LTA-induced samples (**Figure 6D**), but not at 1-h post-LTA stimulation (**Figure 6C**). Constitutive basolateral secretion of CCL20 was not significantly altered by CSE at the 1-h and 6-h time points (**Figures 6A,B**).

Apical CCL20 secretion was measured in each treatment at 1 and 6 h time points; we observed a significant effect of time point (between subjects, $F_{1,16} = 28.4$, $p < 0.001$), treatment (within subjects, $F_{3,14} = 16.27$, $p < 0.001$), and interaction between time points (within subjects, $F_{3,14} = 11.15$, $p < 0.001$), and treatment for apical CCL20 secretion. Basolateral secretion of CCL20 showed no significant effect of time point but there was a significant effect of treatment (within subjects, $F_{3,11} = 12.77$, $p < 0.001$) and significant interaction between time point and treatment (within subjects, $F_{3,11} = 6.13$, $p = 0.011$).

No Differences Were Detected in BD-1 Secretion at the 1- or 6-h Time Points

No significant differences in apical or basolateral secretion of BD-1 were detected between CSE treated and untreated samples at the 1- or 6-h time point. We observed a significant effect of treatment with apical (within subjects, $F_{3,17} = 5.043$, $p = 0.011$), and basolateral (within subjects, $F_{3,17} = 7.78$, $p = 0.004$) BD-1 secretion. However, in *post hoc* analysis with Bonferroni corrections, no comparisons between CSE treated and untreated samples were significantly different at either time point (data not shown).

CSE Exposure Alters the Expression of Immune Related Genes after 3 h of CSE Exposure Followed by 1 h of LTA Stimulation

In order to further explore gene expression changes from CS exposure a set of 511 immunology-related genes were assayed by Nanostring technology. Total amounts of 20 immunology-related gene transcripts were significantly altered by CSE exposure out of 511 genes assays. This difference was measured in the first hour after LTA stimulation. Three genes had increased number of transcripts and 17 were significantly decreased. By 6 h after LTA stimulation, these differences were minimal and a significant increase in the transcripts of only one other gene was detected (**Table 1**).

DISCUSSION

Our results show that *in vivo* exposure to CS alters the secretion of key antimicrobial peptides from subsequently cultured PHNEC.

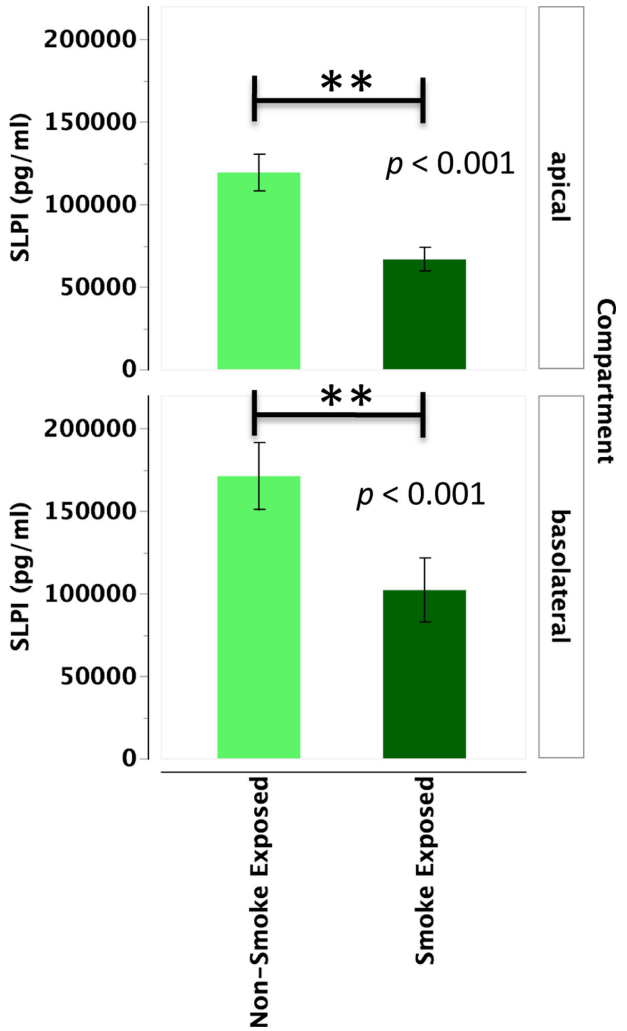

FIGURE 3 | SLPI secretion into the apical and basolateral compartments is significantly lower in PHNEC from *in vivo* smoke-exposed individuals. SLPI secretion was not significantly different among the four treatments in our PHNEC experiments. However, there was a significant effect of smoke exposure status and this figure shows secretion data pooled (from all treatments) from every PHNEC secretion sample.

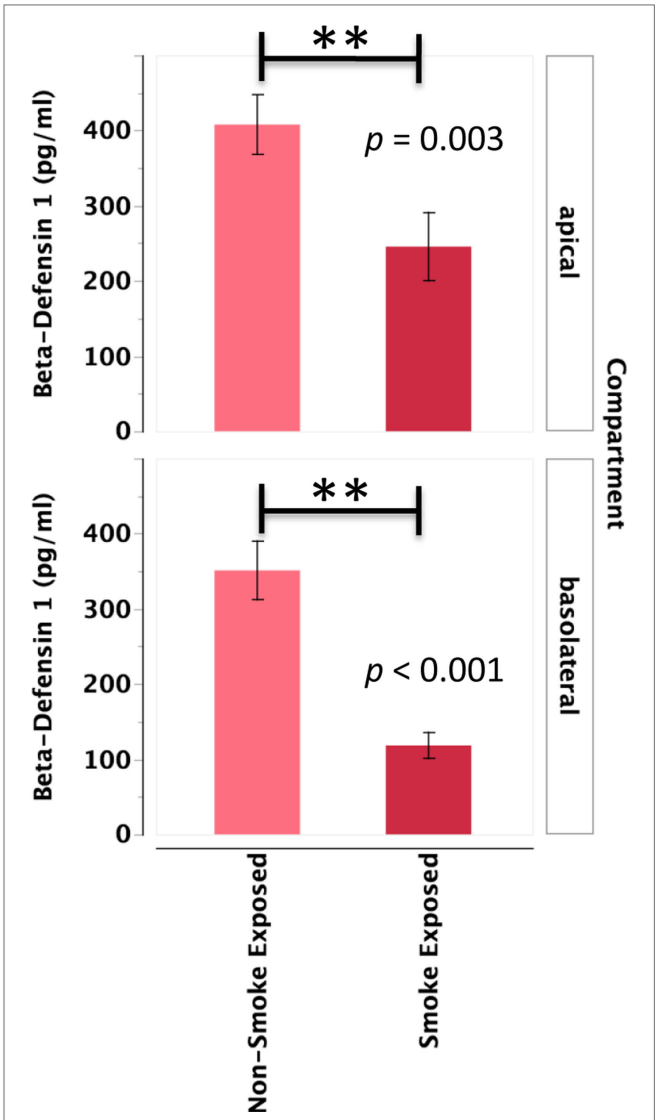

FIGURE 4 | Beta-defensin 1 secretion into the apical and basolateral compartments is significantly lower in PHNEC from *in vivo* smoke-exposed individuals. Beta-defensin 1 secretion was not significantly different among the four treatments in our PHNEC experiments. However, there was a significant effect of smoke exposure status and this figure shows secretion data pooled (from all treatments) from every PHNEC secretion sample.

We observed a significant decrease in apical and basolateral SLPI, and BD-1 secretions as well as a reduction in apical secretions and an increase in basolateral secretions of CCL-20 from PHNEC cultured from CS-exposed individuals. As these are important antimicrobial peptides, it shows that CS may decrease the overall antimicrobial activity of nasal secretions. Previous work from our lab has shown that CCL20 constitutes an important fraction of antimicrobial activity in the secretions of airway (BEAS2B) cells (34). PHNEC from CS-exposed individuals were more difficult to culture than that from non-smoke-exposed individuals. Clearly, the cells that grow out to form PHNEC cultures have modifications that reflect CS exposure *in vivo*. These modifications could be epigenetic marks. This has implications for the time course of recovery of normal resistance to airway pathogens after smoking cessation.

The *in vitro* time-course data of LTA-stimulated PHNEC support other research showing that *in vitro* CS exposure alters the innate immune system or suppresses its ability to respond to pathogens. For example, Manzel et al. (37) showed that CSE suppressed the activation of NF-κB-dependent pathways, reduced the consequent expression of defense genes [IL-8 and intracellular adhesion molecule 1 (ICAM-1)], and decreased cellular secretion of IL-6 and IL-8 in the response to *Haemophilus influenzae* by primary human tracheobronchial epithelial cells. Also, cellular secretion of IL-8 and IL-6 were suppressed (37). Despite the fact that these same molecules did not have reduced expression in our transcription studies, these results in addition

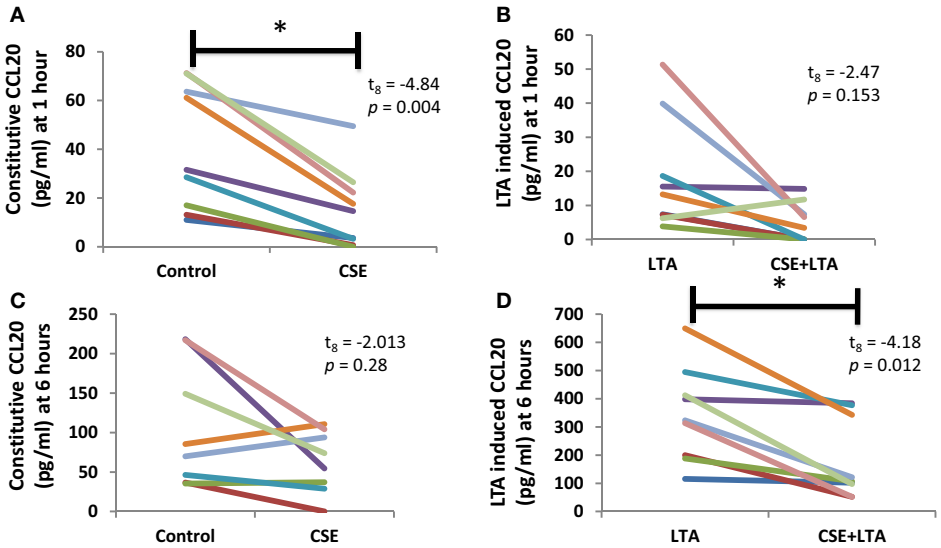

FIGURE 5 | CSE exposure significantly decreased constitutive apical CCL20 secretion at hour 1 (A) and LTA-stimulated apical CCL20 secretion at hour 6 (B). Apical CCL20 secretion from PHNECs from non-smoke-exposed individuals are shown at 1 h **(A,B)** and 6 h **(C,D)** post-LTA stimulation. Primary human nasal cell cultures were exposed apically to cigarette smoke extract in ALI media for 3 h, LTA and control treatments received ALI media only. CSE was then removed by suction and cells were rinsed twice with PBS. Subsequently, LTA and CSE + LTA treatments were stimulated apically with LTA from *B. subtillis* (10 μg/mL) in ALI media by applying 300 uL in the apical compartment. Supernatants were harvested at 1 and 6 h post-LTA stimulation. CCL20 was quantified by ELISA.

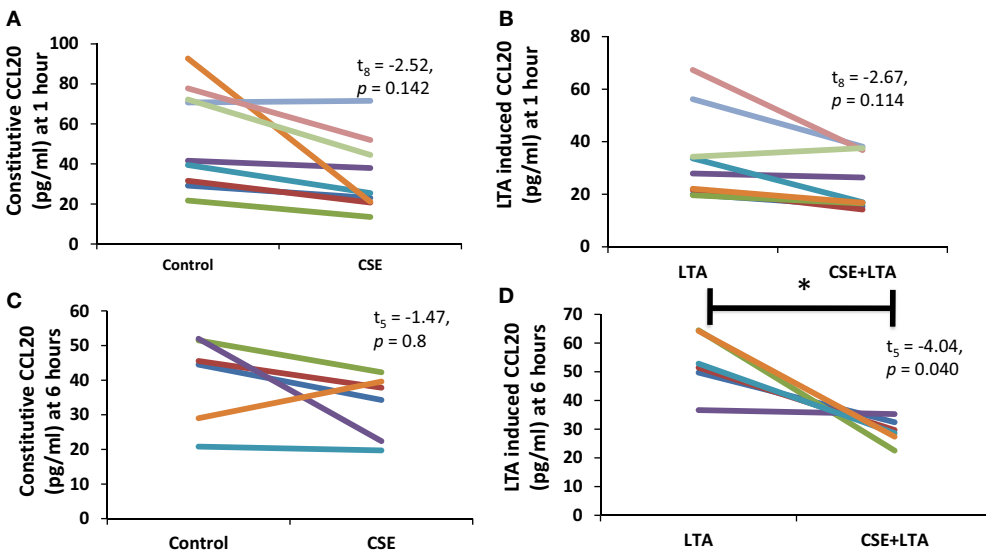

FIGURE 6 | PHNEC basolateral secretion of CCL20 significantly decreased following CSE exposure at the 6-h time point in LTA-induced samples. Depicted in this figure are basolateral CCL20 secretions from PHNECs cultured from non-smoke-exposed individuals at 1 **(A,B)** and 6 h **(C,D)** post-LTA stimulation. PHNECs were exposed apically to cigarette smoke extract in ALI media for 3 h, LTA and control treatments received ALI media only. CSE was then removed by suction and cells were rinsed twice with PBS. Subsequently, LTA and CSE + LTA treatments were stimulated apically with LTA from *B. subtillis* (10 μg/mL) in ALI media by applying 300 uL in the apical compartment. Supernatants were harvested at 1 and 6 h post-LTA stimulation. CCL20 was quantified by ELISA.

to ours suggest that exposure to CSE weakens the innate immune response of the upper airway mucosa to a bacterial invasion. The lack of full correlation between the studies could be due to different anatomical sources of primary epithelial cells, different stimulation conditions, or different culturing techniques.

Examining the list of genes that are significantly decreased in LTA-stimulated PHNEC with CSE exposure reveals that many are involved in immune signaling. Using the KEGG pathways, database shows that four of the genes occur in the neurotrophin signaling pathway (IRAK1, PKCD, MAPK1, and MAPKAPK2),

TABLE 1 | Changes in gene expression in comparisons of cigarette smoke extract and unexposed treatments of LTA-stimulated PHNEC from healthy individuals.

Time point	Gene name	Mean difference with CSE exposure	Change in expression with CSE exposure	*t* ratio	Degrees of freedom	*p*-value
Hour 1	ATG16L1	−26.18	Decrease	−2.83831	8	0.0219
Hour 1	CD59	−652.93	Decrease	−2.87	8	0.0208
Hour 1	CDKN1A	−919.37	Decrease	−2.31452	8	0.0493
Hour 1	CEACAM1	10.83	Increase	3.379778	8	0.0096
Hour 1	GPI	41.24	Increase	3.13942	8	0.0138
Hour 1	IFIH1	−25.28	Decrease	−2.71971	8	0.0263
Hour 1	GUSB	−17.83	Decrease	−4.01185	8	0.0039
Hour 1	IFNAR1	−2.96	Decrease	−2.96357	8	0.0180
Hour 1	IGF2R	−29.66	Decrease	−3.98707	8	0.0040
Hour 1	IRAK1	−49.62	Decrease	−3.57147	8	0.0073
Hour 1	JAK2	−11.71	Decrease	−2.71316	8	0.0265
Hour 1	MAPK1	−51.04	Decrease	−3.09134	8	0.0149
Hour 1	MAPKAPK2	−33.95	Decrease	−2.48863	8	0.0376
Hour 1	MARCO	−15.47	Decrease	−2.62623	8	0.0304
Hour 1	NOD2	25.29	Decrease	2.38436	8	0.0442
Hour 1	NOTCH2	−95.69	Decrease	−3.02181	8	0.0165
Hour 1	PRKCD	−24.65	Decrease	−2.39875	8	0.0433
Hour 1	SLAMF7	−9.59	Decrease	−3.12773	8	0.0141
Hour 1	STAT3	−88.35	Decrease	−2.35686	8	0.0462
Hour 1	IL20	15.61	Increase	3.18543	8	0.0129
Hour 6	BCL3	67.53	Increase	3.040491	7	0.0188

Nanostring quantified RNA transcripts were sampled at 1 and 6 h post-LTA stimulation.

four occur in the chemokine signaling pathway (JAK2, STAT3, MAPK1, and PKCD), two occur in the TLR signaling pathway (IRAK1 and MAPK1), and three occur in the JAK-STAT signaling pathway (IFNAR1, JAK2, and STAT3) (38). Macrophage Receptor (MARCO) is a class A scavenger receptor, probably involved in binding Gram-positive and Gram-negative bacteria (39, 40). SLAMF7 was also detected as a significantly down-regulated gene and SLAM receptors are involved in the fine-tuning of immune cell activation (41). NOD2 expression was increased and this gene is an intracellular receptor involved in the recognition of bacterial peptidoglycans and is involved in activation of NF-Kappa B, cytokine production, and apoptosis (42). Based on the gene expression data, CSE disrupts multiple chemokine, neurotrophin, and immune signaling pathways in PHNEC.

Our data suggest that individuals vary greatly in the amounts of antimicrobial peptides secreted by their PHNECs under baseline conditions. Constitutive and LTA-induced antimicrobial secretions vary greatly among both non-smoke-exposed individuals and *in vivo* CS smoke-exposed individuals. For example, PHNEC from non-smoke-exposed individuals varies approximately 60-fold from 19 to 1223 pg/ml in their secretion of CCL20 under control conditions and approximately 250-fold from 32 to 8000 pg/ml in LTA-stimulated conditions (data not shown).

We had increased difficulty in culturing PHNEC from CS-exposed individuals, suggesting that there are other phenotypic differences as well, perhaps at the level of the stem cells that grow out to form the PHNEC cultures. We could obtain nasal epithelial cells from these individuals, but they would stop dividing and would not proliferate enough to utilize experimentally. This along with our data, showing decreased secretion of antimicrobials from PHNEC from smoke-exposed individuals, suggests some transmissible epigenetic changes in CS-exposed epithelial stem cells from which the PHNEC are derived; these observations warrant further study.

The study was performed with PHNEC samples that had been cultured to a solid monolayer and then differentiated into mucociliary epithelial cells that, like *in vivo* airway epithelial cells, had distinct differences between the apical and basolateral surfaces. Variability in response to secretion of the assayed proteins/peptides between basolateral and apical surfaces was revealed in some conditions in which the same cultures responded differentially between apical and basolateral secretion (for example, see **Figure 2**). This suggests that posttranslational regulation is affected by CS exposure. Since basolateral or apical secretion has different implications for fighting infections, it is likely that the different secretion impacts also play a role in sensitivity of smoke-exposed individuals to airway infections.

Antimicrobial peptides and proteins are important in protecting humans from respiratory pathogens that smoke-exposed people are less able to control. Our results reveal clear suppressive effects of both *in vitro* and *in vivo* CS exposure on some of these defensive peptides and changes in the expression of genes involved in chemokine and neurotrophin signaling pathways. It is not clear why there are disparate effects on different specific proteins and peptides and an understanding of those issues requires further study. This report contributes to the mechanistic understanding of how CS exposure alters the innate immune response and increases an individual's susceptibility to pathogen infection.

AUTHOR CONTRIBUTIONS

JJ was central to this project with involvement in all aspects of research, including cell culture and assays, data analysis and statistics as well as having primary responsibility for figures and manuscript. BG trained JJ and MC-G to harvest tissues from study volunteers and

collaborated on research design. TD did cell cultures and assays. LF helped with assays and data analysis. SF and MC-G mentored research design and development of cell culture protocols and research skills as well as in data analysis and manuscript development.

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OPINION
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The Cognitive Deficits Associated with Second-Hand Smoking

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Keywords: passive smoking, memory impairment, second-hand smoke, everyday memory

Exposure to second-hand smoke (SHS), also known as "passive smoking," refers to a situation where a non-smoker inhales another person's smoke either by sidestream or by mainstream exposure to tobacco smoke. Previous research has suggested that not only is prolonged exposure to SHS associated with a range of health-related problems similar to those found in smokers (1, 2) but is also linked to detrimental effects upon cognitive performance in children, adolescents, and adults. For example, children exposed to SHS show reduced vocabulary and reasoning skills when compared with non-exposed children (3) as well as more general cognitive and intellectual deficits (4). More recently, research using serum cotinine as a biomarker of exposure to SHS found that higher levels of serum cotinine were associated with significant reductions in performance in reading, mathematics, and visual and spatial abilities in children and adolescents (5) indicating that higher levels of SHS exposure is associated with poorer cognitive performance. In adults, exposure to SHS in those who had no history of smoking showed significantly reduced performance in processing speed (how quickly one can process information and perform tasks) and executive function (which includes the ability to organize memory, cognitive flexibility, and problem-solving ability) when compared with non-exposed, never smokers (6, 7). In addition, never smokers who lived with smokers for several decades showed a 30% increase in their risk of dementia (8). Recent work has also revealed everyday memory impairments in never smokers with a history of living with smokers for several years; for example, deficits in everyday prospective memory (memory for future actions), such as remembering to carry out everyday activities, keeping appointments with others, or remembering to post a letter on time (7, 9). What is less clear is the mechanism by which SHS might compromise cognitive performance.

The mechanisms underlying the links between SHS exposure and poorer cognitive performance are far from clear. One potential explanation derives from the notion that the carbon monoxide (CO) in tobacco smoke may interfere with the oxygen being delivered to the brain *via* the blood system. CO binds to human hemoglobin more than oxygen does; therefore, it is feasible that by inhaling tobacco smoke with a high level of CO across a prolonged period of time may diminish the amount of oxygen being carried to the brain, which may, in turn, lead to the range of cognitive impairments observed in the previous research. Although this hypothesis is somewhat speculative, it is a viable explanation and future work could test this by measuring levels of CO in the blood of never smokers who have been exposed to SHS and comparing these with never smokers with no history of such exposure. A second possible explanation stems from recent animal research, which has exposed animals to varying degrees of toxic mixtures of chemicals found in tobacco smoke. This research suggests that tobacco-specific procarcinogens may lead to reduced neuronal mass in specific regions of the brain associated with learning and memory; for example, in the hippocampal region of the brain, which is known to be involved in the mediation of memory and learning (10). Future research should therefore measure levels of procarcinogens, such as NKK, in SHS exposed individuals and whether these are associated with increasing cognitive deficits. A third possibility is that prolonged exposure to SHS may lead to a build-up of cardiovascular disease (CVD), which in turn may lead to a range of health and cognitive problems in later life. There is epidemiological evidence that shows prolonged exposure to SHS is associated with a greater increase in CVD (2, 11, 12). A longitudinal

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design could elucidate this association by observing long-term exposure to SHS and a potential build-up of CVD and how these correlate with performance upon a range of cognitive measures.

Several limitations need to be considered when interpreting research in this area and designing future studies. The exposure to SHS in many studies is based on self-report measures, which may be subject to recall bias and lead to over- or underestimation of exposure. Biological assays could be used to provide more reliable estimates of SHS exposure, for example, cotinine residue levels or nicotine residue in saliva or hair samples. The use of other drugs should also be documented along with the exposure to SHS, because a large body of evidence now exists that documented the health and cognitive risks associated with the use of, for example, excessive amounts of alcohol, ecstasy, cannabis, and other substances. Other factors, such as socioeconomic status and personality variables such as impulsivity and risk-taking propensity, can impact upon health and cognition, so these too should be controlled for in future research.

In summary, the health problems associated with exposure to SHS in individuals who have no history of smoking are fairly well documented. More recently, studies have pointed to a range of cognitive consequences associated with SHS exposure, including everyday memory impairments. Our understanding of what the underlying mechanism(s) are that might account for such cognitive deficits is in its infancy, and there is a great deal of scope for research that looks at the relationship between prolonged exposure to SHS, putative physical and neural damage, and the range of cognitive deficits documented in the literature. Given recent concerns raised by health bodies, such as the World Health Organization, about the risk SHS poses to children and adults around the world, it is clear that this is a topic that warrants further research.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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The Synergistic Impact of Excessive Alcohol Drinking and Cigarette Smoking upon Prospective Memory

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The independent use of excessive amounts of alcohol or persistent cigarette smoking have been found to have a deleterious impact upon Prospective Memory (PM: remembering future intentions and activities), although to date, the effect of their concurrent use upon PM is yet to be explored. The present study investigated the impact of the concurrent use of drinking excessive amounts of alcohol and smoking cigarettes (a "Polydrug" group) in comparison to the combined effect of the single use of these substances upon PM. The study adopted a single factorial independent groups design. The Cambridge Prospective Memory Test (CAMPROMPT) is a test of both time-based and event-based PM and was used here to measure PM. The CAMPROMPT was administered to 125 adults; an excessive alcohol user group ($n = 40$), a group of smokers who drink very little alcohol ($n = 20$), a combined user group (the "Polydrug" group) who drink excessively and smoke cigarettes ($n = 40$) and a non-drinker/low alcohol consumption control group ($n = 25$). The main findings revealed that the Polydrug users recalled significantly fewer time-based PM tasks than both excessive alcohol users $p < 0.001$ and smokers $p = 0.013$. Polydrug users (mean = 11.47) also remembered significantly fewer event-based PM tasks than excessive alcohol users $p < 0.001$ and smokers $p = 0.013$. With regards to the main aim of the study, the polydrug users exhibited significantly greater impaired time-based PM than the combined effect of single excessive alcohol users and cigarette smokers $p = 0.033$. However, no difference was observed between polydrug users and the combined effect of single excessive alcohol users and cigarette smokers in event-based PM $p = 0.757$. These results provide evidence that concurrent (polydrug) use of these two substances has a synergistic effect in terms of deficits upon time-based PM. The observation that combined excessive drinking and cigarette smoking leads to a greater impairment in time-based PM may be of paramount importance, given the key role PM plays in everyday independent living.

Keywords: excessive drinking, smoking, synergistic, prospective memory, CAMPROMPT

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INTRODUCTION

Tobacco and alcohol are two of the most widely used drugs in the Western world and are responsible for a large proportion of harm (1). These two drugs are often used concurrently (2, 3); yet, there remains a paucity of research in relation to their combined effects. The relationship between these drugs is complex and not presently well understood; this is surprising given the synergistic health

risks posed by such polydrug use (4). Previous research suggests that chronic use of large amounts of alcohol and cigarette smoking are independently associated with a variety of cognitive impairments. For example, studies that have examined the effects of alcohol on cognitive performance and have shown that drinking excessively impairs Working Memory (WM), which is responsible for the manipulation and maintenance of information across a short period of time; for example, remembering someone's phone number while driving a car and concentrating on the road ahead (5, 6) as well as Executive Function (EF), which is an umbrella term used to describe a set of resources that are responsible for the management of cognitive functions, including WM and attention; for example, being able to pay attention to a task despite having distractions all around you (7, 8). More recently, excessive alcohol use has been associated with poorer performance in Prospective Memory (PM), which refers to the cognitive ability to carry out planned intentions/actions at a future point in time (9–11). Excessive alcohol drinking is defined as either drinking in excess of the current cut-off limits for safe drinking, which are 14/21 U of alcohol per week for females and males, respectively (12, 13). It should be noted that a UK unit (8 g ethanol) contains 0.343 US fluid ounces of ethanol. Chronic cigarette smoking has also been associated with deficits in these domains, including WM (14, 15), EF (16, 17), and PM (18–20). PM is seen as an important part of everyday remembering, since it is responsible for planning and remembering future activities, such as remembering to meet with friends at a pre-specified time and location, remembering to take an important medication on time, or remembering to turn up for a meeting; in this respect, it is seen as essential for independent living (21).

Prospective memory involves both time-based (an action that is carried out after a specific time period has elapsed; for example, remembering to take an important medication after a specific time has elapsed) and event-based (an action executed as the result of an environmental cue; for example, remembering to pass on a message to someone whom you meet in the street). It has been suggested that the subtypes rely upon different cognitive mechanisms; event-based involves spontaneous retrieval while time-based requires attentional monitoring and as such time-based may be more reliant upon executive resources (22). There is evidence to suggest that event-based and time-based PM are, at least in part, separable. For example, time-based, but not event-based PM deficits were found in a patient with bilateral frontal lobe infarcts (23); whereas Parkinson's disease patients have been found to be impaired on the event-based, but not on time-based, PM tasks (24), suggesting a dissociation between the two processes. In addition, research using Positron Emission Tomography (PET) brain imaging has found evidence of differential involvement of prefrontal regions in time-based and event-based PM (25). Although there is a paucity of research focusing upon the combined effect of alcohol and tobacco use upon cognition and memory, there is some. Evidence in relation to the interactive effect between excessive alcohol use and smoking is evident in other areas of cognition. Cigarette smoking has been found to exacerbate cognitive deficits in those who drink alcohol excessively, including memory deficits, one's ability to think quickly and efficiently, as well as on problem solving tasks (26). Recently, evidence has shown that alcohol-dependant individuals who smoke cigarettes show greater neuropsychological damage than those who do not smoke (27); observing decreased cortical thickness in the polydrug users, with greater thinning in frontal areas of the cortex (a key brain region involved in PM). The combined effect of smoking and alcohol has also recently been linked with faster cognitive decline in such polydrug users, compared with alcohol users alone (28), indicating that cigarette smoking and excessive alcohol use may act in synergy to cause increased cognitive decline. In addition to this greater deficits in EF [believed to rely on the same cognitive processes as PM: (29)] have been found in such polydrug user groups compared to alcohol users alone. Given the cumulative evidence that the combined use of excessive amounts of alcohol and cigarette smoking may damage pre-frontal regions of the brain and may accelerate declines in cognitive processes such as EF, it is possible that the combined use of these two substances may exacerbate declines in PM when compared with the single use of these substances.

The main aim of the study is to explore whether the combined (polydrug) effect of consuming excessive amounts of alcohol and smoking cigarettes is greater than the sum of their independent effects. This will be achieved by comparing the added effects of both single user groups (an excessive drinking group and a cigarette smoking group) with a polydrug group (those who drink alcohol excessively and smoke cigarettes) in order to determine whether there is a significant difference between these with regards PM. This should provide insight as to whether the combination of these two substances has an additive or synergistic impact upon PM function. Since PM involves both time and event-based tasks, the Cambridge Prospective Memory Test (CAMPROMPT) was utilized here as a measure of both time and event-based PM.

MATERIALS AND METHODS

Design

An existing groups design was employed comparing four groups: (1) an "Excessive Alcohol" group who drank excessive amounts of alcohol but who did not smoke cigarettes; (2) a "Cigarette Smokers" group who smoked cigarettes on a regular/daily basis and drank very little alcohol; (3) a "Polydrug" group who drank excessively and smoked cigarettes on a regular/daily basis; and (4) a "Control" group who drank low amounts of alcohol who had never smoked cigarettes. Excessive alcohol drinking was classified as those individuals who drank in excess of the current cut-off limits for safe drinking, which are 14/21 U of alcohol (females and males respectively) per week, as described in the Section "Introduction." The dependent measures included both time-based and event-based CAMPROMPT scores.

Participants

One-hundred and twenty-five unpaid volunteers were recruited as participants through opportunity sampling, which involved taking a sample of people who responded *via* advertisements about the study and who fit the criteria for which the researchers were looking. The inclusion criteria was anyone who fell in to one of the four groupings identified above; therefore any person who was

either an excessive drinker (drinking above the 14/21 U of alcohol per week described above), a regular cigarette smoker who drank very little (ranging from 0 to 7 U of alcohol per week), a polydrug user who drank excessively and smoked cigarettes regularly, or a non-smoker who consumed very little (if any) alcohol. The study was advertised widely around the university, and the inclusion criteria were made clear so that we could ensure recruitment to all of the groups. Anyone who reported using other substances, such as cannabis, ecstasy, heroin, cocaine, "legal highs," etc., were excluded from the study. Anyone who reported having previously suffered from/were currently suffering from, a clinical disorder (such as amnesia, depression, or substance dependence), were excluded from the study. The age range of participants was between 18 and 43 years. Participants were allocated to a group based upon their alcohol and cigarette use. Excessive alcohol was determined by the participant's weekly alcohol usage (regardless of any specific pattern of drinking, such as "binge drinking"), which was whether they exceeded the 14/21 U of alcohol per week for females and males, respectively (12, 13). The Excessive Alcohol group contained 40 participants (25 females) who had never smoked, and their mean alcohol intake per week was 25.9 U (SD 8.60). The Cigarette Smokers group consisted of 20 participants (14 females) who smoked on a regular/daily basis, but did not consume alcohol on a regular basis and drank low amounts of alcohol; they smoked on average 69.3 cigarettes per week (SD 47.7). The Polydrug group contained 40 participants (16 females) who smoked cigarettes on a regular/daily basis and drank excessively; their mean alcohol consumption was 26.5 U per week (SD 6.88), and their mean cigarettes usage per week was 52.5 cigarettes (SD 27.2). The Control group consisted of 25 participants (19 females) who were low-dose alcohol users/non-users who did not smoke; their mean alcohol consumption per week was 1.46 U (SD 2.38). The Excessive Alcohol and Polydrug groups did not differ in terms of the amount of alcohol they consumed per week, nor in terms of the years spent drinking alcohol or their last alcohol use in hours. The Cigarette Smokers and Polydrug groups did not differ in terms of the amount of cigarettes they smoked per week or in terms of their last cigarette use in hours, but the Cigarette Smokers group had been smoking for longer than the Polydrug group. The distribution of male and female participants between the groups did differ significantly. See the Section "Results" for analysis of these non-memory measures.

Measures

The Cambridge Prospective Memory Test (CAMPROMPT) is a valid and reliable measure of time-based and event-based PM (30) and was utilized in the current study as an objective measure of both time and event-based PM. The test consists of three time-based tasks, which require the participant to carry out a task at a specific time; a clock was available for them to monitor the time (for example, "In seven minutes, I would like you to change the pen you are using") and three event-based tasks, which require the participant to carry out a task in response to a cue (for example, "When you come to a quiz question about 'Eastenders' I would like you to give me this book"). The time-based and event-based tasks were to be remembered while completing a set of distracter tasks comprising a set of puzzles. Points were scored for each of the six tasks and the scoring per task ranged from 6 (where the participant completed task unaided) to 0 (where they have failed to complete task), with points between these two on the scale for tasks completed with some prompting from the researcher. Two types of PM scores were obtained: a time-based PM score (out of a maximum of 18) and an event-based PM score (out of a maximum of 18), with the higher score reflecting a more proficient PM.

Alcohol use, smoking, and other drug use were measured using a modified version of the University of East London Recreational Drug Use Questionnaire [RDUQ: (31)]. This questionnaire asked the participant to report their drinking and smoking pattern over a typical week, including quantities and the number of units of alcohol/cigarettes, hours since last use and years spent using alcohol/cigarettes. Participants were further asked to state any other drug use, such as cannabis and ecstasy, and amount and frequency of use.

Procedure

Prior to commencement, the research protocol was approved by the School of Health and Life Sciences Ethics Committee at Northumbria University. All testing was carried out in a laboratory setting, taking approximately 30 min to complete. Participation was voluntary. The CAMPROMPT was administered first, in which participants were asked to complete a set of puzzles and quizzes, while being asked by the researcher to remember to carry out the time-based and event-based memory tasks; this lasted approximately 25 min. Participants were then asked to complete the RDUQ questionnaire, which took only a few minutes. Upon completion, participants were debriefed, any questions they had were answered and they were given the opportunity to withdraw their data from the study (none did so).

RESULTS

In order to identify that the Polydrug user group was appropriately matched to the respective single drug user groups, a series of one-way ANOVAs were applied to the data comparing appropriate groups on alcohol use and smoking. The Excessive Alcohol group and Polydrug group were compared on the amount of alcohol consumed per week, the number of years spent drinking alcohol, and the number of hours since they last drank alcohol (see **Table 1** for the means and standard deviations (SDs) for these measures across the groups). The Cigarette Smokers group and Polydrug group were compared on the number of cigarettes smoked per week, the number of years spent smoking, and the number of hours since their last cigarette was used (see **Table 1** for the means and SDs for these measures across the groups). The analyses revealed no significant differences between the Excessive Alcohol and Polydrug groups in terms of the number of alcohol units consumed per week [$F(1,78) = 0.150$, $p = 0.699$], the number of years spent drinking alcohol [$F(1,78) = 0.153$, $p = 0.697$], and hours since they last drank alcohol [$F(1,78) = 0.414$, $p = 0.522$]. No significant difference was observed between the Cigarette Smokers group and Polydrug Group in terms of the amount of cigarettes smoked per week [$F(1,58) = 3.001$, $p = 0.089$] and number of hours

since their last cigarette [$F(1,58) = 0.034$, $p = 0.855$]; however there was a significant difference in terms of the number of years for which participants had smoked cigarettes [$F(1,58) = 8.624$, $p = 0.005$] – with the Cigarette Smokers group having smoked for longer than the Polydrug group. In summary, there were no significant between group differences between excessive alcohol use and smoking pattern for the potential confounding variables, other than years spent smoking.

A multivariate MANOVA was applied to the data in order to identify whether CAMPROMPT event-based and time-based differences existed between the Polydrug group and the two single user groups. This revealed a significant effect of group on the dependent measures [Wilk's Lambda = 0.636, $F(4,192) = 12.182$, $p < 0.001$; $\eta p^2 = 0.20$]. This analysis indicated a significant effect associated with the time-based CAMPROMPT [$F(2,97) = 24.367$, $p < 0.001$, $\eta p^2 = 0.33$] and a significant effect associated with the event-based measure [$F(2,97) = 10.799$, $p < 0.001$, $\eta p^2 = 0.18$]. Bonferroni adjusted *post hoc* analysis revealed that with regard to event-based PM, the Polydrug group (mean = 11.47; SD 3.34) remembered significantly fewer actions than Excessive Alcohol group (mean = 14.27; SD 2.35), $p < 0.001$, and that the Polydrug users also remembered significantly fewer event-based actions than the Cigarette Smokers group (mean = 13.85; SD 2.52), $p = 0.008$. With regard to time-based PM, the Polydrug group (mean = 9.77; SD 3.99) remembered significantly fewer PM actions than Excessive Alcohol group (mean = 14.67; SD 2.41), $p < 0.001$, and that the Polydrug users also remembered significantly fewer time-based items than the Cigarette Smokers group (mean = 12.30; SD 2.39), $p = 0.012$.

To address the main aim of whether the combined (polydrug) effect of consuming excessive amounts of alcohol and smoking cigarettes is greater than the sum of their independent effects, the CAMPROMPT impairment of the Polydrug Group was contrasted with the combined impairment of the two single user groups (the Excessive Alcohol group and Cigarette Smokers group). Specific impairment of the two single user groups was identified by comparing their mean performance with that of the Control group performance on both CAMPROMPT measures. In the event-based CAMPROMPT measure, the Control mean performance was 16.48 (SD 2.48), and the Excessive Alcohol and the Cigarette Smokers group achieved 14.27 and 13.85, respectively. Thus, the specific impairments for these two user groups were; Excessive Alcohol, 16.48 − 14.27 = 2.21, and Cigarette Smokers, 16.48 − 13.85 = 2.63. The combined event-based impairment level was therefore = 2.21 + 2.63 = 4.84. This combined baseline impairment value is shown in **Figure 1A** below by the horizontal dashed line.

In the time-based CAMPROMPT measure, the Control mean performance was 15.80 (SD 2.00). Thus, the specific impairments for these two user groups were; Excessive Alcohol, 15.80 − 14.67 = 1.13, and Cigarette Smokers, 15.80 − 12.30 = 3.50. The combined time-based impairment level was therefore 4.63, and this combined impairment value is shown by the horizontal dashed line in **Figure 1B** below. In order to identify whether

TABLE 1 | Means (and SDs) for all measures across each drug user group.

	Excessive alcohol ($n = 40$)	Cigarette smokers ($n = 20$)	Polydrug ($n = 40$)
Age	22.30 (4.10)	27.15 (6.80)	22.55 (4.16)
Alcohol units per week	25.90 (8.60)	0.55 (1.42)	26.58 (6.88)
Years drinking alcohol	6.48 (4.39)	1.30 (3.70)	6.84 (3.88)
Hours since last alcohol	90.95 (73.30)	45.60 (107.02)	105.68 (124.83)
Cigarettes per week	0.00 (0.00)	69.30 (47.79)	52.55 (27.23)
Years smoking cigarettes	0.00 (0.00)	10.95 (9.15)	5.99 (3.98)
Hours since last cigarette	0.00 (0.00)	7.70 (12.27)	8.49 (17.01)
CAMPROMPT event	14.27 (2.35)	13.85 (2.52)	11.47 (3.34)
CAMPROMPT time	14.67 (2.41)	12.30 (2.39)	9.77 (3.99)

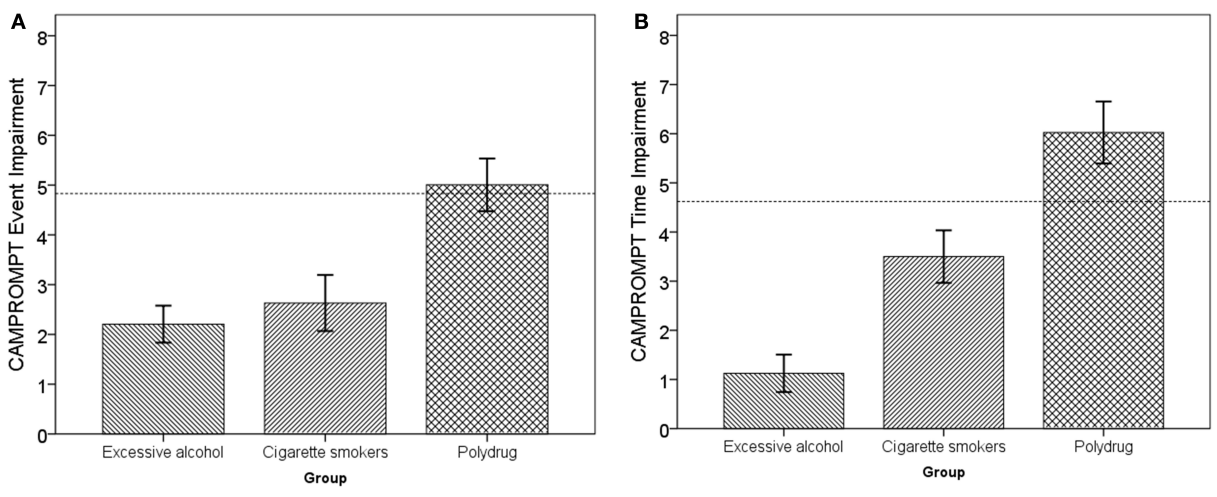

FIGURE 1 | **(A)** CAMPROMPT event-based impairment as a function of user group. **(B)** CAMPROMPT time-based impairment as a function of user group.

the Polydrug impairment (in comparison to the control group performance) was greater than the combined single user deficits, two one-sample *t*-tests were conducted with the respective combined impairments as the criteria. In relation to event-based PM, there was no significant difference between the Polydrug groups' impairment and the combined single user impairment, [$t(39) = -0.312$, $p = 0.757$]. In relation to time-based PM, the Polydrug groups' impairment was significantly greater than the combination of single user impairments [$t(39) = -2.243$, $p = 0.031$].

In summary, significantly more time-based and event-based PM errors were made by the Polydrug user group in comparison to both Excessive Alcohol user group and Cigarette Smoker groups. Importantly, further analysis revealed that the polydrug group also made significantly more PM errors that the combined effect of excessive alcohol and cigarette smoking in time-based PM, although not in event-based PM.

DISCUSSION

The present study explored whether the combined (polydrug) effect of consuming excessive amounts of alcohol and smoking cigarettes is greater than the sum of their independent effects. This was achieved by comparing the added deficits for each of the single user groups (the Excessive alcohol drinkers and cigarette smokers group) with the polydrug group (those who both drink alcohol excessively and smoke cigarettes) to determine whether there was a significant difference between these two in terms of time-based and event-based PM function using the Cambridge Prospective Memory Test (CAMPROMPT) as the main measure of PM. With regards to this aim, the prospective memory (PM) deficits observed in the Polydrug group (those who drank excessive amounts of alcohol and smoked cigarettes) was found to be greater than the combined deficits of the two single user groups (the Excessive alcohol group and the Cigarette Smoker group) in relation to time-based PM, suggesting a synergistic interactive effect rather than an additive interactive effect of these two substances. No such effect was observed in relation to event-based PM. These effects were found after observing no significant differences between these groups in the amount of alcohol consumed per week, the number of years drinking alcohol, the number of hours since they last drank alcohol, the number of cigarettes smoked per week, and in terms of the number of hours since last cigarette. These findings indicate that, using both substances together produces greater deficits than single use of either substance and furthermore, the interaction between excessive amounts of alcohol and cigarettes produces greater deficits upon time-based PM than the sum of their separate effects – suggesting a synergistic effect of combined excessive drinking and smoking upon time-based PM. These findings firstly lend support to the body of research, which has previously found that drinking alcohol and smoking cigarettes separately, is associated with impaired PM (9–11, 18–20), but extends this by observing a synergistic effect of the combined use of excessive amounts of alcohol and cigarette smoking on time-based PM deficits when compared with their single separate use. Although smoking has been found to exacerbate cognitive deficits in excessive alcohol users in the past (26–28), the current study is the first to show this effect for prospective remembering. Given the importance of PM to everyday activities (21), this finding may be important in terms of its suggestion that everyday memory (of which PM is a very good example) may be compromised by the combination of excessive drinking and cigarette smoking in a non-clinical population.

Although this study has demonstrated synergistic effects of excessive drinking and smoking upon PM, the putative underlying damage to the mechanisms underpinning such deficits remains unclear. PM is believed to be a function underpinned by multiple cognitive processes rather than being a single construct in its own right; thus, it is difficult to identify a specific region or mechanism in the brain that may account for the PM deficits caused by excessive alcohol use, smoking, and polydrug use. However, both excessive alcohol use and smoking have been found to impair frontal lobe tasks such as EF (16, 32). Given previous clinical evidence that excessive alcohol users who also smoke show decreased cortical thickness, with greater thinning in frontal areas of the cortex (a key brain region involved in PM) compared with heavy drinking who do not smoke (27), it is possible that the deficits in PM found in the polydrug group in the current study may be the result of frontal lobe dysfunction. Given that the combination of drinking heavily and smoking cigarettes also leads to significant deficits in EF (28), which is heavily involved in frontal lobe resources and is believed to underpin PM (29), this lends further support to the notion that it is the frontal lobe region that is affected by the combined use excessive alcohol drinking and cigarette smoking. This could be explored further by the use of brain imaging (such as PET) alongside a measure of PM (such as CAMPROMPT) in order to observe the degree of frontal lobe activity during the PM task in the polydrug users compared with suitable controls. Again, one must be cautious given the evidence from neuroimaging studies which have also implicated other regions, such as the hippocampus and thalamus in PM (33–35). It is therefore possible that any putative damage as a result of polydrug use may well be indicative of damage that is not confined to the frontal region itself; again, brain imaging techniques could be used in combination with the CAMPROMPT in order to elucidate the links between polydrug use, PM deficits and any underlying neuropsychological damage. The fact that this synergistic effect (i.e., the finding that polydrug user group showed greater PM deficits than the combined deficits of both single user groups) was evident only for time-based PM task is explicable in terms of time-based PM being more reliant upon frontal lobe processes (21), and therefore, if the frontal lobes are being damaged/depleted by the combination of drinking excessively and smoking cigarettes, then one would expect to find this for the time-based tasks and not for the event-based tasks (as was the case in the findings of the current study). This suggests that event-based PM may operate on a different neural network than that of time-based PM, a point that could be pursued in future research. It may also be worth noting that, since nicotine is seen to act as neuroprotective (36), the contributing factor of cigarette smoking to this synergistic effect on PM must come from the toxins contained in cigarette smoke and inhaled by smokers, these toxins combined with excessive alcohol use must act together to damage or deplete those resources in the brain that underpin PM,

future research may wish to explore which of these 70 plus toxins contained in tobacco smoke interact with excessive alcohol use to produce a detrimental impact upon everyday memory.

CONCLUSION AND LIMITATIONS

To the best of our knowledge, this is the first study to examine the synergistic impact of combined excessive alcohol use and cigarette smoking upon everyday PM. The findings revealed that individuals who consumed excessive amounts of alcohol and also smoked cigarettes demonstrated significantly greater deficits in time-based PM than the combined deficits from the single use of either excessive alcohol or cigarette smoking, suggesting a synergistic interactive effect rather than an additive effect of these two substances. It is our hope that the findings uncovered here will help to improve our understanding about the dangers of excessive drinking and smoking beyond the mainly health concerns highlighted in the literature by providing a greater understanding of the cognitive consequences of such polydrug use. Specifically, highlighting the dangers of combined heavy alcohol use and smoking in relation to everyday memory, in this case PM.

There are a number of limitations that should be considered when interpreting the findings form this study. One limitation of the study is the reliance on self-reported drug use, which can be problematic given that it relies upon the honesty and accuracy of the individual. Although the RDUQ (used in the current study to measure substance use) has been used in several studies to measure alcohol, smoking, and other substance use (9–11, 18–20, 31); its utility when compared with other substance use measures has not been tested, which should be considered when considering its use in future studies. Future research should overcome this by utilizing biological drug-screening techniques that provide objective and more accurate measures of alcohol and other drug use, for example the use of blood, urine and hair assays. The study asked anyone who used other substances (such as cannabis, ecstasy, etc.) or who had suffered from/were currently suffering from, a clinical disorder (such as amnesia, depression, or substance dependence), to refrain from taking part in the study as a method of screening participants. However, these were not assessed by biological assays (for measures of drug use) or clinical testing (for clinical disorders). This can be seen as a limitation of the present study, particularly given the fact that there is evidence that polydrug use is associated with greater health risks than single drug use (37), that polydrug use is more prevalent in psychiatric populations (38) and is associated with elevated levels of psychiatric conditions, such as aggression and suicide (39), when compared with single user groups. In addition, given that polydrug users differ from single drug user groups in terms of personality factors, such as exhibiting higher levels of impulsivity and a greater propensity for risk taking (40–42), personality factors should also be taken into account in future work. Therefore, future research should utilize biological methods to more accurately assess substance use, as well as include health, personality, and psychiatric indices to compile a fuller picture of how polydrug users and single drug users differ on these domains and to measure what impact, if any, these domains might have upon everyday memory in the form of PM.

Although the present study has uncovered a synergistic effect of excessive alcohol use and smoking upon PM, future research should attempt to replicate these findings using more ecologically valid PM tasks. This could be achieved by the use of real-world PM tasks such as remembering to carry out an activity after a period of time has passed (e.g., remember to text the researcher 24 h following the completion of the study) or the use of the diary method, both of which have proven useful in measuring PM deficits in clinical populations (43) or the recent use of virtual reality techniques to measure PM (44). Finally, given the frequency with which adolescents drink heavily and smoke, future research should investigate the impact of heavy drinking and smoking in the period of adolescence to upon the developing adolescent brain (45). Since tobacco smoking and alcohol use are two of the most widely used drugs in the Western world; and given the fact that they inflict a great deal of harm upon society and the fact that these two drugs are often used concurrently, a much greater understanding is needed with regards the cognitive consequences of combined cigarette smoking and excessive alcohol use.

AUTHOR CONTRIBUTIONS

A-MM: manuscript, research plan development, data gathering, and analysis. TH: manuscript, administration, and editing. CH: manuscript, editing, and analysis.

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Characteristics of Participants Enrolled in a Brief Motivational Enhancement for Smokers

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Daily smoking is associated with elevated blood pressure, carbon monoxide (CO) toxicity, and impaired pulmonary lung functioning. The benefits of successful smoking cessation are readily apparent, given the health improvements associated with cessation, as well as the reduction of secondhand smoke to which non-smoking coworkers and family members are exposed. Previous literature indicates that providing personalized information to smokers (versus general base rates) without engaging in confrontational pressure to quit smoking, leads to increased interest in quitting smoking and willingness to enter smoking cessation programs. The goal of this study was to examine the pretreatment characteristics of the smokers entering a brief motivational enhancement intervention based on personally tailored health feedback. Participants (N = 28) were 88.2% Caucasian and 59% males, and they were an average of 23 years of age. On average, they smoked 20.08 cigarettes per day for a mean of 6.6 years, a mean Fagerström Test for Nicotine Dependence score of 4.7, and obtained a mean breath CO reading of 19.1 ppm. Smoking-related adverse health outcomes were predictive of stages of change motivation to quit smoking. Implications for cessation programs are discussed.

Keywords: motivational interviewing, smoking cessation, smoking, nicotine, addiction and addiction behaviors

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INTRODUCTION

Despite the widely publicized negative health consequences of cigarette smoking, a quarter of the United States adult population continues to smoke (1). Cigarette smoking remains the leading preventable cause of death in the United States, accounting for more than 430,000 deaths per year (2). Smoking cessation is associated with decreased mortality and morbidity from smoking-related illnesses, such as cancer. For example, former smokers reduce their excess lung cancer risk by 50–80% within 10 years of quitting (3).

Research indicates that effective interventions exist for cigarette smokers (4) and that as many as 60% of current smokers want to quit smoking (5), yet few enter formal smoking cessation treatment programs [e.g., Ref. (6)]. Recent studies have also indicated that among current smokers, nicotine dependence levels tend to be higher than in past years. In addition, the prevalence of comorbid psychopathology, such as depression and anxiety, in smokers is greater now than in previous years, because those without such difficulties have largely been able to quit smoking [e.g., Ref. (7, 8)]. As a result, prevalence for those with psychiatric disorders has remained disproportionately high (9, 10). These smokers tend to experience more difficulty with smoking cessation and maintaining abstinence, and are therefore likely to require and benefit from professional assistance in quitting smoking. This suggests that cessation efforts need to be available and readily accessible (e.g., workplace; primary care settings) in order for more smokers to take advantage of these resources. Smokers

with few resources and of diverse ethnic backgrounds continue to be underrepresented in cessation programs. In a previous study (11), smokers identified transportation and other practical issues among smokers who were socioeconomically disadvantaged. There were also reports of disbelief that smoking was personally harmful to the individual.

Motivational interviewing (MI) (12) is a clinical intervention procedure in which a collaborative, cooperative alliance is formed between the therapist and the individual suffering from an addictive disorder, such as smoking/nicotine dependence. Contrary to the traditional clinical approaches with substance users in which the individual is forced to label him/herself as an addict or be declared in "denial" of his/her addiction, the MI approach elicits motivation from the substance user to change by querying in a non-confrontational manner about adverse consequences the addiction has caused in the individual's life, assists the substance user in weighing the pros and cons of continued use, and offers a variety of intervention suggestions if solicited from the substance user. This non-judgmental, collaborative approach has been shown to be effective in assisting substance users/smokers to become more "ready" to change their substance use behavior. Prochaska and DiClemente (13) discuss this "readiness for change" in health behaviors as occurring in various stages, ranging from precontemplation, contemplation, preparedness, action, and relapse. During each of these stages, individuals are more open or "ready" to accept particular treatment interventions than others. That is, an individual in precontemplation (defined as not believing that his/her substance use is even problematic) would not be receptive to specific strategies for ceasing to use a substance, whereas, an individual in the preparedness or action stage would be receptive to structured suggestions of this type, having already made the decision to stop using the substance and in need of concrete skills. According to contemporary models of high-risk behavior change, in addition to appreciating that risks associated with the behavior apply to him/herself, an individual must view the barriers to quitting that behavior as surmountable, in order to cease participation in that high-risk behavior [e.g., Ref. (14)]. The MI component of offering a variety of intervention suggestions to the individual, if solicited by him/her, could supply this information to the smoker. This would include the fact that there are many efficacious and effective interventions that presently exist for smokers (4).

In the present study, daily smokers were entering an intervention which utilized the feedback component of MI with smokers who were not yet "ready" to quit smoking (13) by agreeing to participate in a paid study entitled, "*Health Screen for Smokers*." We were interested in examining the pretreatment characteristics of these smokers; in particular, what characteristics would be most associated with present motivation for smoking cessation.

MATERIALS AND METHODS

Participants

Participants were Louisiana State University campus employees recruited through fliers on campus and advertisements on their paystubs for a paid study entitled, "*Health Screening for Smokers*." They were randomly assigned to the active treatment condition, in which they obtain personalized information regarding their blood pressure, carbon monoxide (CO) level, and pulmonary lung functioning, or the control group in which they underwent the health screening, but they received no feedback. All participants were offered smoking cessation treatment free of charge at the campus clinic. Outcomes from this motivational intervention are not reported here. Rather, we examined smokers' pretreatment characteristics.

Instruments

Demographic Questionnaire

This form assessed participant demographics, including age, gender, ethnicity, education level, occupation, and income. We also included questions regarding medical insurance status and smoking-related illness.

Smoking Status Questionnaire

This form assessed current and past smoking patterns and included the Fagerström Test for Nicotine Dependence (FTND) (15). The FTND yields scores that range from 0 to 10. This form also included a question regarding previous smoking cessation attempts and the questions, "*Have you experienced any smoking-related health problems*?" and "*If so, please indicate the category that best describes the health problems you've experienced*," with the response options of "respiratory," "circulatory," "cardiac," "cancer," or "other."

Stages of Change Algorithm

The stages of change (SOC) algorithm (16) was used in order to obtain a categorical measure of participants' stage of change. This form comprised a series of mutually exclusive questions: (1) "Are you seriously considering quitting smoking in the next 6 months?"; (2) "Are you planning to quit in the next 30 days?"; and (3) "In the last year, how many times have you quit for at least 24 h?" in order to identify participants as being in precontemplation, contemplation, or preparation. This measure may be viewed as an indication of how ready a smoker is to quit smoking.

Brief Smoking Consequences Questionnaire-Adult

This questionnaire measures smoking outcome expectancies, anticipated rewarding, and punishing consequences from smoking a cigarette (17). The Brief Smoking Consequences Questionnaire-Adult (BSCQ-A) comprises 10 factors derived from principal components analysis: (1) negative affect reduction; (2) stimulation/state enhancement; (3) health risks; (4) taste/sensorimotor manipulation; (5) social facilitation; (6) weight control; (7) craving reduction/addiction; (8) negative physical feelings; (9) boredom reduction; and (10) negative social impression. Scores on each of the 10 scales are calculated by taking the average of the items. Scale scores range from 0 to 9.

BreathCo Carbon Monoxide Monitors (Vitalograph, Inc.)

The BreathCo CO monitors were used to determine expired CO level (ppm). A cutoff of >8 ppm was used to confirm daily smoking status.

Lung Age Meter

A key indicator of chronic obstructive pulmonary disorder (COPD) is a reduced FEV1 compared with predicted FEV1 value. Early identification of a reduction in FEV1 can provide early warning of the damage already suffered by the lungs at the pre-symptomatic stage, when smoking cessation is most effective. The Vitalograph lung age compares a subject's FEV1 with predicted normal values to calculate the subject's "lung age." A high lung age in relation to the subject's chronological age can illustrate the likely negative impact of continued smoking on lung function and encourage smoking cessation.

Procedure

Participants called in response to an advertisement for paid research for smokers in a study entitled, "*Health Screen for Smokers*." They completed a brief phone screen with a member of the research team to determine basic eligibility [>18 years of age; self-report of current smoking rate >20 cigarettes per day (CPD) for at least 1 year], listened to a brief description of the study, including the provision of health-related feedback on CO and lung functioning, and were told that they would receive $50.00 for completion of the study. They were scheduled for a session at which they were told their smoking would be biochemically verified. They were greeted by a research assistant when they arrived and were randomly assigned to the active treatment condition, in which they obtain personalized information regarding their CO level and pulmonary lung functioning, or the control group in which they underwent the health screening, but they received no feedback. All participants were offered smoking cessation treatment free of charge at the university campus clinic. Participants completed the questionnaires and the research assistant obtained breath samples for the CO reading and for the lung age meter reading. Participants were paid $50 for their participation. All study procedures, including the ethical treatment of human subject participants, were reviewed and approved of by the Institutional Review Board of Louisiana State University.

RESULTS

Participant Characteristics

Participants ($N = 28$) were 88.2% Caucasian and 59% males, and they were an average of 23 years of age. On average, they smoked 20.08 CPD for a mean of 6.6 years, a mean FTND score of 4.7, and obtained a mean breath CO reading of 19.1 ppm. Participants' responses to the SOC measure indicated that 47% were in precontemplation and 35.3% were in the contemplation stage.

Forty-three percent of participants endorsed having a diagnosed smoking-related health problem, of which 75% were identified as respiratory and the rest as other than respiratory, circulatory, cardiac, or cancer. The mean lung age meter reading among participants was 65.9 (SD = 29.6), and the mean scale score on the BSCQ-A for health risks was 8.3 (SD = 5.6).

Prediction of SOC Readiness to Change

We used linear regression analyses with health indices as predictors and SOC readiness to change as the dependent variable. Currently, having a smoking-related health problem significantly predicted SOC readiness to change, such that smokers with a smoking-related health problem reported themselves as more "ready" for smoking cessation ($\beta = 0.35$), $F(1, 27) = 3.56$, $p = 0.035$; R-squared 0.125, adjusted R-squared 0.09. Regression analyses with BSCQ-A health risk scale scores and lung age were not significant in predicting SOC readiness to change.

DISCUSSION

Participants in the present study were part of a larger study in which personally tailored health feedback was provided to daily smokers utilizing MI techniques. In the present study, we were interested in examining pretreatment characteristics among daily smokers, including their smoking patterns, smoking outcome expectancies, and smoking-related health information. This included their CO levels and lung age related to risk for COPD.

Smokers in this study were predominantly Caucasian, young adults. On average, they smoked one pack per day of cigarettes for approximately 7 years and were moderately dependent on nicotine, as indicated by their FTND scores. They endorsed strong beliefs regarding the health risks associated with smoking, as reflected on their scores obtained on the health risks scale of the BSCQ-A. In fact, these health risk scores were sufficiently high at pretreatment that a ceiling effect may preclude detection of pre- to posttreatment increase in the tailored feedback condition of the larger intervention study. A similar finding in a smoking expectancy challenge with health risk expectancies suggests that current smokers identify the health risks associated with smoking, but that these beliefs can be increased by providing health information, and that this increase in expectancies may be accompanied by increased motivation to quit smoking (18). In addition, in the development study for the Smoking Consequences Questionnaire-Adult (SCQ-A), health risk expectancy scores significantly distinguished between smokers entering cessation treatment and those not currently interested in cessation (19). These findings within the context of previous literature therefore support the utility of targeting health risk expectancies in attempts to increase motivation for smoking cessation.

The present study suggests that recruitment of precontemplators was successful, in that almost half (47%) of the smokers who agreed to participate were in the precontemplation stage of readiness to quit smoking. This goal was consistent with the larger study's goal of increasing motivation to quit smoking (e.g., progression from precontemplation to contemplation) by providing tailored, smoking-related health feedback to participants. Participants who were already motivated to quit smoking in the present study were more likely to endorse having a smoking-related health problem. Given this finding, it will be interesting to see whether tailored health feedback to participants regarding their CO level and pulmonary functioning/lung age is associated with increased motivation for cessation. Such results would be consistent with interventions that increase progression in stage of change, according to Prochaska and DiClemente (13). That is, an individual in precontemplation would be receptive to strategies that increase his/her awareness of the adverse effects of smoking. Further, the MI component of providing treatment information

should assist the individual in viewing the barriers to quitting smoking as surmountable. According to Reyna and Farley's model (14) of high-risk behavior change, this is a critical step in addition to the individual appreciating that risks associated with the behavior apply to him/herself.

There are several limitations to the present study that should be noted. First, the study is cross-sectional and therefore all results are correlational in nature. We have explicitly stated that the purpose of this brief study was to examine and describe pretreatment characteristics, and it was anticipated that these characteristics may assist in informing cessation interventions. The present study was conducted as a portion of a larger experimental study, which includes a motivational intervention for daily smokers based on tailored health feedback. Second, the sample size is small, which may limit generalizations that can be made to other populations of daily smokers. Finally, the participants in the present study were young adults with an average age of approximately 20 years, which may further compromise the study's external validity in that typical smoking cessation treatment participants are older and may differ significantly from the present sample in other important ways as well. Indeed, the study was designed with mature, adult smokers in mind, and the adverse health information provided to participants would likely be more severe if this population had been recruited into the present study.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Community-Based Screening, Brief Intervention, and Referral for Treatment for Unhealthy Tobacco Use: Single Arm Study Experience and Implementation Success in Rural and Semi-Rural Settings, South-West Nigeria

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Objective: To determine whether screening, brief intervention, and referral for treatment can reduce the prevalence of tobacco use in rural and semi-rural settings.

Method: *Design and participants:* A non-randomized clinical trial with assessments at baseline and post-intervention assessments at 3 and 6 months was conducted in a rural and semi-rural district in South-West of Nigeria. A representative sample of 1203 persons consented to the study and had alcohol, smoking, and substance involvement screening test (ASSIST) administered to them by trained community health-care extension workers between October 2010 and April 2011. Follow-up participation was more than 99% at all points. *Intervention:* Participants received a single ASSIST-linked brief intervention (BI) and referral for treatment (RT) at entry, and a booster ASSIST BI and RT at 3 months. *Main outcomes and measures:* The primary outcome was self-reported scores on ASSIST.

Results: At baseline, out of 1203 respondents, lifetime prevalence and current prevalence of any tobacco products were 405 (33.7%) and 248 (20.6%), respectively. Of the current users, on the ASSIST, 79 (31.9%) scored 0–3 (low health risk), 130 (52.4%) scored 4–26 (moderate risk), and 39 (15.7%) scored 27+ (high risk). At 3 months, out of 1199 respondents, prevalence of current users was 199 (16.5%) and out of 1195 respondents, was 169 (14.1%) at 6 months. Prevalence of tobacco use reduced significantly at 3 months $Z = -3.1$, $p = 0.01$ and at 6 months when compared with baseline $Z = 4.2$, $p = 0.001$, but not at 6 months compared with at 3 months, $Z = 2.1$, $p = 0.09$. Multivariate analysis revealed that age at initiation of tobacco use, gender, marital status, setting of dwelling, and socioeconomic status were the only variables that were associated with current tobacco use at baseline, 3 and 6 months.

Conclusion: A one-time BI with a booster at 3 months had a significant effect on tobacco use in persons living in community settings. This finding suggests a need for promoting the adoption of this intervention for tobacco use in rural and semi-rural community settings.

Keywords: screening, brief intervention, community, tobacco, smoking

INTRODUCTION

While globalization of the use of cigarette and other products of tobacco is a major threat to public health worldwide (1, 2), studies have noted the decline in tobacco use in high-income countries and an increase in use in middle- and low-income countries (3, 4). For instance, Guindon and Boisclair reported that from 1970 to 2000, per capita cigarette consumption reduced by 14% in the Western world while it increased by 46% in the developing nations (3).

The growth of cigarette use in developing countries might be linked to the marketing efforts of tobacco companies, loose restrictions of tobacco control policies (5), and poor surveillance of smoking prevalence in these countries (3, 6, 7). For instance, in Nigeria, cigarette imports have increased over the years from 20 million sticks in 1970, to 198 million in 1990, and 2966 in 2000 (8). In addition, Nigeria ranks third among the largest tobacco markets in Africa after Egypt and South Africa. Despite this increase, the lifetime prevalence of tobacco use is 17% (9), and the overall prevalence of tobacco use in Nigeria is 8.9% (10), which is comparatively low compared with the US prevalence rate of 16.8% (11). Thus, Nigeria appears to be in early stages of cigarette epidemic. Estimate of deaths from smoking-attributed causes in sub-Saharan Africa reaches only 5–7% for men and 1–2% for women (12). Yet, this status and the consequences of cigarette use are not likely to respond to a quick change, partly due to weak government restrictions on tobacco use or sales.

In Nigeria, the infrastructure for tobacco control is poor despite the country's ratification of the WHO Framework Convention on Tobacco Control (13). Nigeria has not implemented regulations such as age verification for sales, sales to minors, misleading information on packaging, the amount of tar and nicotine, product constituents as confidential information, product constituents as public information, constituent disclosure by brand, and constituent disclosure by aggregate, smoking in restaurants, nightclubs, and bars (14). Specifically, the Tobacco Control Bill, which was passed in the National Assembly about a decade ago, was yet to be adopted (15). Hence, the unbridled widespread use of tobacco in Nigeria may jeopardize future improvements in longevity that could be gained by curbing the impact of AIDS, starvation, and violence (16). Of note is the fact that attributable mortality to tobacco use is rising and if the current epidemic continues, more than 70% of these deaths are expected to occur in developing countries including Nigeria (17).

Therefore, there is a need to find other strategies that might work while efforts are ongoing to firm up tobacco restrictions at the government level in Nigeria. Screening, brief intervention, and referral for treatment (SBIRT) is a public health model that is used to screen for substance abuse and also for the delivery of low-intensity substance abuse treatments in a primary health-care setting (18). SBIRT for unhealthy drug use has been described in the scientific literature for over 50 years (19), and there has been relatively robust evidence for its effectiveness for substance use, particularly unhealthy alcohol use (20). However, some studies have noted that evidence for the effectiveness of SBIRT is much more limited for other drug use and in settings other than primary care (21).

In the Western world, the provision of SBIRT as a form of smoking cessation intervention is generally toward treatment seeking smokers (22). In developing countries such as Nigeria, where the majority live in rural settings, and access to medical care is limited to urban centers, smoking cessation treatment may not be offered until tobacco-related disease is detected or until the smoker expresses interest in quitting. Within this context, SBIRT will be particularly well suited for non-treatment seeking smokers of cigarette. In other words, while much attention has focused on primary care as a setting to promote smoking cessation, community settings could be uniquely positioned for tobacco intervention efforts in resource poor settings, such as Nigeria, where access to hospital care is limited. However, given the studies that reported inefficacy or mixed results of SBIRT, SBIRT intervention should not be taken as universally effective.

Therefore, in the developing countries, such as Nigeria, where it has not been evaluated before, there is a need for evaluative studies to guide policy and interventions. The current investigation was a single arm study designed to examine the prevalence of tobacco use and evaluate the effectiveness of SBIRT in semi-rural and rural communities. We hypothesized that participants who receive SBIRT intervention would demonstrate a decrease in cigarette consumption.

MATERIALS AND METHODS

Study Area

Nigeria is a developing country in West Africa. Nigeria was ranked 191st among 194 member states of the World Health Organization in terms of overall health attainment. The study site was in Ibadan, Oyo State. Ibadan is the capital of Oyo state, Nigeria, and it is the third largest city in Nigeria. The city is located in the southwestern part of the country. It has a population of over 3.5 million people and 11 local government areas (23).

Ethical approval for the study was obtained from the Ethical Review Committee of the Ministry of Health, Oyo State, Nigeria. Assent was obtained from participants between 15 and 18 years and informed consent from 18 years and above.

Study Design

A systematic stratified sampling method was used to select two local governments in Ibadan between October 2010 and April

2011. In the first stage, all 11 LGA were classified into rural or semi-rural based on government fund allocation. In the second stage, one local government was randomly chosen from each group, and in the third stage, four enumeration areas were systematically selected as clusters. The fourth stage involved the mapping and numbering of all buildings in each of the selected enumeration areas. All households within each building were serially listed in the Form specifically designed for the purpose. After getting the list of the households, simple random sampling was used to identify the households that fell within the sample. Regular households were distinguished from institutional households. All eligible respondents, who were 15 years and above in each household, were selected and were interviewed using the questionnaires including alcohol, smoking, and substance involvement screening test (ASSIST) after they gave consent/assent. The inclusion criteria for the study were both male and female tobacco users of age ≥15 years and permanent residents of study areas. The exclusion criteria were non-users of tobacco of age <15 years, not willing to get tobacco cessation intervention, and not a permanent resident of the study areas.

Intervention Training in SBIRT and Quality Control

To increase the possibility of an effect while observing a real-world feasibility in a resource poor setting, brief interventionists were recruited from community health-care extension workers (CHEWs) in participating primary health-care clinics ($n = 18$). The CHEWs had been involved with previous surveys and agreed to adhere to the study protocol. All interventionists were trained by Victor Olufolahan Lasebikan using a group workshop followed by individual feedback on audio-taped role plays with up to five standardized patients over the course of 5 weeks (24). Treatment fidelity was assessed by scoring of 5 audio-taped role plays using Motivational Interviewing Treatment Integrity coding system (MITI 3.0) (25). Mean global scores ranged from 4.35 to 4.64 which were well above the proficiency benchmark of 4.0. All interventionists met basic motivational interviewing proficiency training goals on at least one practice role play.

A 3 days of debriefing and review of all protocols were carried out, after a pilot survey in each of the study local governments. Each interviewer had conducted two pilot interviews in the field. All questionnaires were reviewed for completeness by field coordinators. The pilot studies were carried out in a ward unit as enumerated during the National population census in each of the study local governments. This was to assess applicability of the instruments of data collection and research adherence.

Instruments

1. A sociodemographic *pro forma* was specifically designed for this study to elicit the sociodemographic characteristics of the respondents. The questionnaire contained items such as age, age at initiation, frequency of tobacco use, marital status, socioeconomic class, and years of education. For the purpose of this study, tobacco use was synonymous with smoking.
2. The ASSIST was developed mainly to screen for drug use, but can be used for other substances, including alcohol and tobacco as well, particularly in high prevalence settings (26). The ASSIST is an eight-item instrument that screens for use of all substance types [tobacco products, alcohol, cannabis, cocaine, amphetamine-type stimulants (ATS), sedatives, hallucinogens, inhalants, opioids, and "other" drugs] and determines a risk score ("lower," "moderate," or "high") for each substance (27). The risk scores are generated from item questions 2–7 for tobacco. The responses are scored from 0 to 6 based on the frequency of use of the specific substance. The risk scores are recorded on the ASSIST feedback report card which is used to give personalized feedback to clients by presenting them with the scores that they have obtained, and the associated health problems related to their level of risk. Asking clients if they are interested in viewing their scores allows the health worker to commence a discussion (brief intervention) with the client in a non-confrontational way and has been found to be a successful way of getting clients at moderate risk, in particular, to change their substance use (27). Scoring: for tobacco use, 0–3 (low risk), 4–26 (moderate risk), and 27+ (high risk). This instrument was used to score a representative sample of 1203 adolescents and adults (15 years and over) for the risk of hazardous and harmful consequences of tobacco and other substances.

The lifetime prevalence of tobacco use was obtained from Q1: "In your life, which of the following substances have you ever used (non-medical use only)?" We obtained current prevalence of tobacco use from Q2: "In the past 3 months how often have you used the substances you mentioned?" Responses were "never," "once or twice," "monthly," "weekly," and "daily/almost daily." For the purpose of this study, use in the past 3 months was considered to be current use.

Procedure

For those who screened positive for unhealthy tobacco use, ASSIST-Linked SBIRT was conducted as appropriate.

Intervention

The intervention for those who had a low risk of tobacco use (score of 0–3) was general health advice, for those with moderate risk (score of 4–26), was brief intervention and a leaflet containing information about tobacco use, and those with high risk tobacco use (score of 27+) had brief intervention, information leaflet on tobacco use and were offered referral to a specialist hospital for further assessment and treatment.

The information leaflet about tobacco use contained facts about the consequences of unhealthy tobacco use, tips for reducing the risk of tobacco-related harm, and sources of support for tobacco problems (e.g., contact details of services available in the local health district). Respondents, who had an unhealthy tobacco use, were followed up and reassessed at 3 and 6 months.

A booster brief intervention and referral to treatment was given at 3 months. Interviewers used eight anchor community members to maintain contact with members of the household, while interviewers maintained contact with these anchor persons in between interviews. Tobacco use in this study is defined as cigarette smoking.

Evaluation of the Intervention

The outcome of the intervention was assessed by evaluating changes in the mean number of cigarettes smoked/day at 3 and 6 months post-intervention and the mean ASSIST scores at 3 and 6 months. This is in accordance with the application of ASSIST instrument in following up clients over time. The use of mean ASSIST score has been specifically found to be highly valuable in assessing changes in ASSIST scores over time (28).

Data Analysis

For our univariate analysis, the association between sociodemographic variables and current tobacco use was determined using the Pearson's chi square statistics. Using the current prevalence rates at baseline, the Wilcoxon Signed-Rank test and paired *t*-test were used to determine significant changes in the proportion of tobacco users and the mean ASSIST scores at 3 and 6 months. For categorical data, all Chi squares were Yates corrected for all two levels comparisons and Bonferroni corrected for comparisons more than two levels. Following Bonferroni corrections, multiple pairwise comparisons were carried out using Chi square statistics.

Multivariate analyses were carried out using variables that were significant during univariate analysis to determine the association with tobacco use. This was carried out using binary logistic regression. To facilitate the interpretation of odds ratio, a reference category was always chosen for the independent variables with which other independent variables could be compared with tobacco use. This was done for the data at baseline, at 3 and 6 months. Analysis of data was carried out using the Statistical Program for Social Studies SPSS version 13.0.

RESULTS

Enrollment and Screening

The interventionists identified a total of 1329 community dwellers as potentially eligible, of whom 1213 underwent screening. Of them, 10 were excluded because of the presence of severe general medical conditions, giving a response rate of 91.3%. The final analysis was carried out for 1203 questionnaires at baseline. At 3 months, analysis was carried out on 1199 respondents and on 1195 participants at 6 months.

Participants' Characteristics

The mean age of respondents at baseline was 24.45 ± 9.23 years, 51.8% were males, 66.2% were married, 47.4% had at least some secondary education, and 49.7% were of low-average socioeconomic group. Current tobacco use was more significant among males, $\chi^2 = 55.2$, $p < 0.01$, unmarried, $\chi^2 = 9.6$, $p = 0.002$, and low socioeconomic group, $\chi^2 = 11.1$, $p < 0.001$ (**Table 1**). Mean age of initiation into smoking was 17.83 (3.23) years.

Intervention Effects

At baseline, overall lifetime prevalence and current prevalence of any tobacco products was 33.7 and 20.6%, respectively. At 3 months, prevalence of current tobacco use was 16.5% and was 14.1% at 6 months. The prevalence of tobacco use reduced significantly at 3 months $Z = -3.1$, $p = 0.01$ (RR: $0.009 < 0.04 > 0.071$) and at 6 months when compared with baseline $Z = -4.2$, $p = 0.001$ (RR: $0.034 < 0.065 > 0.095$), but not at 6 months compared with at 3 months, $Z = -2.1$, $p = 0.09$ (RR: $-0.004 < 0.025 > 0.0536$) (**Table 2**).

Of the current users, 79 (31.9%) scored between 0 and 3 on the ASSIST (at low health risk), 130 (52.4%) scored between 4 and 26 on the ASSIST (at moderate health risk), and 39 (15.7%) scored 27+ on the ASSIST (at high health risk). The mean ASSIST score significantly reduced at 3 and 6 months, compared with baseline measure, $t = 5.0$, $p < 0.001$ and $t = -5.7$, $p < 0.001$, respectively (**Table 2**).

Referral to Treatment and Engagement

Thirty-nine (15.7%) participants had ASSIST scores ≥27 and were referred for treatment. At 3 months follow-up, 21 (10.6%) participants were referred for treatment and 20 (11.8%) at 6 months follow-up (**Table 2**).

TABLE 1 | Sociodemographic and other characteristics of respondents at baseline (T0) *N* = 1203.

Variation	Total (*N* = 1203)	Current use (*n* = 248)	%	χ^2	df	*p*
Age (years)						
<25	508	122	28.0	8.8	(5)	0.1
25–34	256	54	21.1			
35–44	158	25	15.8			
45–54	120	22	18.3			
55–64	111	17	15.3			
>64	50	8	16.0			
Age at initiation (years)						
<25	508	239	47.0	372.2		<0.001
>25	695	9	1.3			
Gender						
Male	623	181	29.1	55.2	(1)	<0.01
Female	580	67	11.6			
Setting						
Urban	487	78	16.0	10.1	(1)	<0.01
Rural	716	170	23.8			
Marital status						
Married	796	143	16.0	9.6	(1)	0.002
Not married	407	105	26.0			
Education (years)						
0	119	26	21.8	1.2	(3)	0.7
1–6	431	91	21.1			
7–12	570	111	19.5			
>12	83	20	24.1			
Socioeconomic group[a]						
Low	513	173	33.7	11.1	(3)	<0.001[BS]
Low average	598	68	11.4			
High average	63	6	9.5			
High	29	1	3.4			

[a]*Based on monthly wages in local currency: low – <18,500 Naira (government minimum wage), low average – 18,500–75,000 Naira; high average – 75,000–150,000 Naira, high – >150,000 Naira.*

BS, significant following Bonferroni correction.

TABLE 2 | Tobacco use and associated factors: prevalence and effect of brief intervention.

Variables	Baseline (*N* = 1203)	3 months (*N* = 1199)	6 months (*N* = 1195)	Baseline vs. 3 months	Baseline vs. 6 months	3 vs. 6 months
				Statistics *p*	Statistics *p*	Statistics *p*
Lifetime prevalence, *n* (%)	405 (33.7)	405 (33.7)	405 (33.9)			
Current use, *n* (%)	248 (20.6)	199 (16.5)	169 (14.1)	-3.1^{z}, $p = 0.01$	-4.2^{z}, $p = 0.001$	-2.1^{z}, $p = 0.09$
Male, *n* (%)	230 (19.1)	192 (16.0)	164 (13.7)			
Low risk tobacco use[a], *n* (%)	79 (31.9)	114 (57.3)	107 (63.4)	7.4^{z}, $p < 0.001$	6.6^{z}, $p < 0.001$	1.3^{z}, $p = 0.89$
Moderate risk tobacco use[a], *n* (%)	130 (52.4)	64 (31.7)	42 (24.9)	-8.9^{z}, $p < 0.001$	-10.1^{z}, $p < 0.001$	-3.5, $p < 0.01$
High risk tobacco use[a], *n* (%)	39 (15.7)	21 (10.6)	20 (11.8)	-5.8^{z}, $p = 0.001$	-5.7^{z}, $p = 0.001$	1.1^{z}, $p = 0.99$
High risk tobacco + moderate or high risk alcohol use[a], *n* (%)	36 (92.3)	16 (76.2)	13 (66.6)	-8.1^{z}, $p < 0.001$	-8.8^{z}, $p < 0.001$	1.2^{z}, $p = 0.88$
Moderate risk tobacco + moderate or high risk alcohol[a], *n* (%)	65 (50.0)	27 (42.2)	16 (38.1)	-5.2^{z}, $p < 0.001$	-5.5^{z}, $p < 0.001$	1.3^{z}, $p = 0.89$
Mean ASSIST score (SD)	20.11 (5.56)	16.12 (3.23)	15.45 (3.1)	-5.0^{t}, $p < 0.001$	-5.7^{t}, $p < 0.001$	1.2^{t}, $p = 0.96$
Mean (SD) daily cigarette smoking	23.76 ± 13.53	18.56 ± 10.09	17.98 ± 9.76	−7.85, $p < 0.001$	−19.8, $p < 0.001$	-0.8^{t}, $p = 0.79$

[a]*Level of risk was based on ASSIST scores.*

Z, Wilcoxon Signed-Rank Test; t, paired t-test.

Of the 39 current users at high risk of health problems, 36 (92.3%) were also at either moderate or high risk of alcohol. Sixty-five (50.0%) of the 130 current users at moderate risk of health problems were either at high or moderate risk of health problems from alcohol (**Table 2**).

There was a significant reduction in the mean number of cigarettes smoked per day at 3 months compared with baseline, $t = -7.85$, $p < 0.001$ and also at 6 months compared with baseline, $t = -19.8$, $p < 0.001$ (**Table 2**).

At 3 months, a significantly higher proportion of respondents whose age of initiation into tobacco use was <25 years were current users compared with those whose age at initiation into tobacco use was ≥25 years, $\chi^2 = 309.4$, $p < 0.001$. Also, a higher proportion of respondents, who were of male gender were current tobacco users compared with the female gender, $\chi^2 = 200.0$, $p < 0.001$. A significantly higher proportion of respondents who were rural dwellers were current tobacco users compared with the semi-rural dwellers, $\chi^2 = 27.4$, $p < 0.001$. A significantly higher proportion of respondents who were unmarried were current tobacco users compared with those who were married, $\chi^2 = 27.1$, $p < 0.001$. There was also a significant difference in the prevalence of current tobacco use across the socioeconomic status of these respondents $\chi^2 = 8.01$, $p = 0.005$. *Post hoc* multiple comparisons show that this difference was due to a higher current tobacco use among the low socioeconomic group, compared with the low average group $\chi^2 = 28.2$, $p < 0.001$, on the one hand, a higher current tobacco use among the low socioeconomic group compared with the high average group FE $p < 0.001$ and a higher current tobacco use among the low socioeconomic group compared with the high socioeconomic group FE $p < 0.001$, on the other hand.

At 6 months, a significantly higher proportion of respondents whose age at initiation into tobacco use was <25 years were current users compared with those whose age at initiation into tobacco use was ≥25 years, $\chi^2 = 265.1$, $p < 0.001$. Also, a higher proportion of respondents who were of male gender were current tobacco users compared with the female gender, $\chi^2 = 160.0$, $p < 0.001$. A significantly higher proportion of respondents who were rural dwellers were current tobacco users compared with the semi-rural dwellers, $\chi^2 = 48.2$, $p < 0.001$. A significantly higher proportion of respondents who were unmarried were current tobacco users compared with those who were married, $\chi^2 = 52.7$, $p < 0.001$. There was also a significant difference in the prevalence of current tobacco use across the socioeconomic status of these respondents $\chi^2 = 8.2$, $p = 0.004$. *Post hoc* multiple comparisons show that this was due to a higher current tobacco use among the low socioeconomic group compared with the low average group $\chi^2 = 59.3$, $p < 0.001$, on the one hand, a higher current tobacco user among the low socioeconomic group compared with the high average group FE $p < 0.001$ and a higher current tobacco user among the low socioeconomic group compared with the high socioeconomic group FE $p < 0.001$, on the other hand (**Table 3**).

Multivariate analysis reveals that at baseline, significant factors that remained associated with current tobacco use were age at initiation into tobacco use OR = 0.03, 95% CI (0.001–0.05), $p < 0.001$, female gender OR = 0.28, 95% CI (0.18–0.46), $p < 0.01$, being unmarried OR = 2.96, 95% CI (1.12–5.23), $p < 0.01$, high socioeconomic status OR = 0.32, 95% CI (0.19–0.59), $p = 0.001$, high average socioeconomic status, OR = 0.69, 95% CI (0.23–0.71), $p = 0.003$, low average socioeconomic status, OR = 0.65, 95% CI (0.18 = 0.78), $p = 0.01$, and being a rural dweller OR = 3.05, 95% CI (2.00–4.14), $p = 0.001$.

At 3 months, significant factors that remained associated with current tobacco use were age at initiation into tobacco use OR = 0.04, 95% CI (0.003–0.07), $p < 0.001$, female gender OR = 0.18, 95% CI (0.009–0.37), $p < 0.001$, being unmarried OR = 2.07, 95% CI (1.28–4.22), $p < 0.01$, high socioeconomic status OR = 0.31, 95% CI (0.002–0.37), $p = 0.001$, high average socioeconomic status, OR = 0.69, 95% CI (0.35–0.73), $p = 0.002$, low average socioeconomic status, OR = 0.54, 95% CI (0.17–0.61), $p = 0.006$, and being a rural dweller OR = 2.83, 95% CI (1.54–4.02), $p = 0.002$.

At 6 months, significant factors that remained associated with current tobacco use were age at initiation into tobacco use OR = 0.02, 95% CI (0.003–0.04), $p < 0.001$, female gender

TABLE 3 | Sociodemographic correlates of tobacco use.

	3 months					6 months				
Variation	**Total (*N* = 1199)**	**User (*n* = 199)**	**%**	**χ^2**	***p***	**Total (*N* = 1195)**	**User (*n* = 169)**	**%**	**χ^2**	***p***
Age (years)										
<25	507	110	21.7	2.9	0.09	506	102	20.2	3.0	0.08
25–34	255	44	17.3			254	39	15.4		
35–44	157	17	10.8			156	12	7.7		
45–54	119	14	11.8			118	9	7.6		
55–64	111	10	9.0			111	5	4.5		
>64	50	4	8.0			50	2	4.0		
Age at initiation (years)										
<25	479	191	39.9	309.4	<0.001	477	164	34.4	265.1	<0.001
≥25	720	8	1.1			718	5	0.7		
Gender										
Male	604	192	29.0	200.0	<0.001	602	164	27.2	160.0	<0.001
Female	595	7	4.0			593	5	0.8		
Setting										
Semi-rural	479	46	9.6	27.4	<0.001	477	26	5.4	48.2	<0.001
Rural	720	153	21.2			718	143	19.9		
Marital status										
Married	791	99	12.5	27.1	<0.001	790	70	8.9	52.7	<0.001
Not married	408	100	24.5			405	99	24.4		
Education (years)										
0	118	20	20.3	1.2	0.7	116	24	20.7	0.5	0.4
1–6	431	80	18.6			431	65	15.1		
7–12	567	95	16.8			566	68	12.0		
>12	83	10	12.0			82	9	11.0		
Socioeconomic group[a]										
Low	511	144	28.1	8.01	0.005[BS]	511	134	26.2	8.2	0.004[BS]
Low average	596	51	8.6			596	33	5.5		
High average	62	3	4.8			62	2	3.2		
High	29	1	3.4			25	1	4.0		

[a]*Based on monthly wages in local currency: low – <18,500 Naira (government minimum wage), low average – 18,500–75,000 Naira; high average – 75,000–150,000 Naira, high – >150,000 Naira.*

BS, significant following Bonferroni correction.

OR = 0.12, 95% CI (0.03–0.29), $p < 0.001$, being unmarried OR = 3.54, 95% CI (2.02–7.423), $p = 0.001$, high socioeconomic status OR = 0.32, 95% CI (0.002–0.57), $p = 0.001$, high average socioeconomic status, OR = 0.57, 95% CI (0.35–0.73), $p = 0.002$, low average socioeconomic status, OR = 0.63, 95% CI (0.49–0.81), $p = 0.005$, and being a rural dweller OR = 2.99, 95% CI (1.03–8.23), $p < 0.001$ (**Table 4**).

DISCUSSION

This study is most probably the first in sub-Saharan Africa that aimed to determine in semi-rural and rural community settings, the prevalence and correlates of tobacco use, as well as the effectiveness of ASSIST-Linked SBIRT in unhealthy tobacco users among these communities dwellers.

The lifetime prevalence of tobacco use among our participants was 33.7% and this is not much lower than the 44% lifetime use among those 15 years and older in Canada (29). We also found that the current prevalence of tobacco use was 20.6% at baseline. This is higher than the 17% prevalence reported among Nigerian adults in 2007 in a nationally representative sample (9), but is similar to the 16 and 20% prevalence of tobacco use among persons who were 15 years and above in Canada and America, respectively. It is also similar to the overall tobacco use prevalence of 21% in India (30). However, compared with our estimates, the 2012 Global Adult tobacco Survey (GATS) that was conducted in 16 countries found higher current tobacco use prevalence in North America, Europe, and South Asia (31). Nonetheless, our finding suggests that tobacco use might be assuming epidemic proportions in people who live in rural and semi-rural settings in Nigeria.

Another key finding in our study is the group of correlates of tobacco use among current users. Those who used tobacco were more likely to have started around 17 years of age, to be males, unmarried, of low socioeconomic status and live in a rural setting. With respect to the age at initiation of tobacco use, this is similar to the age at smoking initiation that is before the age of 18 years in Western countries (32). We acknowledge that the average age at tobacco initiation varies by country, income, education, and age cohort (31, 33–35). In addition, we note that differences in age at initiation by country may reflect variation in stages of tobacco use epidemic between countries or the

TABLE 4 | Odd ratio for current tobacco use.

	Baseline			3 months			6 months		
Variation	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
Age at initiation (years)									
<25	1			1			1		
>25	0.03	0.001–0.05	<0.001	0.04	0.003–0.07	<0.001	0.02	0.003–0.04	<0.001
Gender									
Male	1			1			1		
Female	0.28	0.18–0.46	<0.01	0.18	0.009–0.37	<0.001	0.12	0.03–0.29	<0.001
Marital status									
Married	1			1			1		
Not married	2.96	1.12–5.23	<0.01	2.07	1.28–4.22	0.01	3.54	2.02–7.42	0.001
Socioeconomic group									
Low	1			1			1		
Low average	0.65	0.18–0.78	0.01	0.54	0.17–0.61	0.006	0.63	0.49–0.81	0.005
High average	0.69	0.23–0.71	0.003	0.69	0.35–0.73	0.002	0.57	0.35–0.73	0.002
High	0.32	0.19–0.59	0.001	0.31	0.002–0.37	0.001	0.32	0.002–0.57	0.001
Setting									
Urban	1			1			1		
Rural	3.05	2.00–4.14	0.001	2.83	1.54–4.02	0.002	2.99	1.03–8.23	<0.001

complexity of tobacco control measures implemented (36). We cautioned above that tobacco use in the rural and semi-rural settings in Nigeria might be assuming epidemic proportions. In line with this, we situate our current finding of lower age at initiation of tobacco use as highlighting a significant problem that could be faced in the future of the health consequences of tobacco use and dependency on tobacco.

From the foregoing, one could justifiably ask "what could make youths especially in rural settings use tobacco at an earlier age?" Our data deductively serve to guide and stimulate additional research. In addition, priority needs to be given to the development of country specific tobacco control programs for adolescents and youths. Furthermore, given the public health importance of tobacco-related diseases such as CVD and other CVD risk factors (e.g., diabetes, hypertension) (37, 38), it is critical to track tobacco use within specific contexts (i.e., rural vs. semi-rural settings) in Nigeria and to characterize tobacco use patterns in terms of populations that could be vulnerable to tobacco use.

Concerning other correlates, our study aligns with prior research (30, 38–40). In both India and Pakistan, some predictors of tobacco use are male gender, low socioeconomic status, and rural geographic location (30, 41). We confirm that male gender is a correlate of tobacco use in low-income countries, in contrast to middle- and high-income countries where the male preponderance is blurring (42). Similar to the male gender contrast, our findings as regards the association between tobacco use and rural dwelling is in line with low-income countries (43) but dissimilar to findings in middle- and high-income countries where tobacco use correlates with dwellers in large cities (44). However, our result with respect to a positive relationship between tobacco use and low socioeconomic status is in agreement with Western findings (44) as well as findings from other developing nations (30, 38, 39). It is possible that unawareness of the health risks of tobacco use might be the factor that ties these associations together. Further research is needed to tease out the possible moderating influence of lack of information on the health risk of tobacco use.

Our observation of an associated alcohol-related health risk among respondents with tobacco-related health risks is illustrative of the co-use of both tobacco and alcohol. Studies have found that smokers are much more likely to use alcohol and vice versa (45). Also, tobacco and alcohol share a similar psychological mechanism as subjective mood altering chemicals that are socially learned and are used by some individuals as coping mechanisms (46). Moreover, repeated use acts as positive reinforcement (47); the pharmacological dependence of nicotine usually increases the probability of alcohol use, usually in social settings because of the social acceptability of alcohol.

A major finding in this study is that it underscores the usefulness and applicability of SBIRT in the hands of CHEW and the positive impact of SBIRT delivered through CHEW on tobacco use as well as unhealthy use in a semi-rural community setting. This current study is important in three ways: (1) it focused on tobacco, and not alcohol, (2) SBIRT was deliverable by CHEW rather than by clinicians only, and (3) it enrolled people in the community with poor access to orthodox medicine and who might not seek treatment rather than those who went to the hospital or were admitted in emergency settings.

In rural and semi-rural community settings, we investigated the usefulness of a single session of brief intervention with a booster session in reducing tobacco use. A major finding in this assessment was that the rate of tobacco use reduced significantly between baseline and 3 and 6 months, respectively. There were significant shifts from high risk to moderate and low risk use of tobacco.

Our study was limited by a number of factors. First, we did not stratify the users into different stages of change. In other

words, we could not assess the impact of different stages of change in unhealthy tobacco use in our study population. This is very relevant considering reports indicating that psychosocial interventions, that target behavioral change often do not yield a significant effect (48). We therefore recommend further studies to explore the possible influence of stages of change in tobacco use reduction. Second, we did not assess tobacco cessation and tobacco cessation in relation to SBIRT. The main objective of SBIRT being cessation from substance use. Future studies are required to access tobacco cessation following SBIRT, because the reduction in the tobacco use rate does not equate cessation. Third, we did not include a control group. This has greatly limited the interpretation of the effect of the intervention. Fourth, all our analysis was based on self-reports. Future works require, including a toxicological screen to their methodology. Fifth, we also did not allocate any diagnosis to the tobacco users; therefore, it was difficult to determine if the effect of the intervention was on sparing users or long-term users.

In conclusion, while tobacco use is reaching epidemic proportions in rural and semi-rural settings in Nigeria and is associated with male gender, early age at initiation into use, low socioeconomic status, and living in rural areas, SBIRT promises to have implementation potentials in delivering the intervention for the reduction of tobacco use and unhealthy tobacco use in semi-rural community in Nigeria.

AUTHOR CONTRIBUTIONS

VL conceived the idea and was responsible for study design, analysis, and manuscript writing. BO was responsible for data collection and was also involved in manuscript writing. Both authors gave a substantial contribution to the study.

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Meditative Movement as a Treatment for Pulmonary Dysfunction in Flight Attendants Exposed to Second-Hand Cigarette Smoke: Study Protocol for a Randomized Trial

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A study protocol is presented for the investigation of meditative movement (MM) as a treatment for pulmonary dysfunction in flight attendants (FA) who were exposed to second-hand cigarette smoke while flying before the smoking ban. The study will have three parts, some of which will run concurrently. The first is a data gathering and screening phase, which will gather data on pulmonary and other aspects of the health of FA, and will also serve to screen participants for the other phases. Second is an exercise selection phase, in which a variety of MM exercises will be taught, over a 16-week period, to a cohort of 20 FA. A subset of these exercises will be selected on the basis of participant feedback on effectiveness and compliance. Third is a 52-week randomized controlled trial to evaluate the effectiveness of a digitally delivered form of the previously selected exercises on a group of 20 FA, as compared with an attention control group. Outcome measures to be used in all three parts of the study include the 6-min walk test as a primary measure, as well as a range of biomarkers, tests, and questionnaires documenting hormonal, cardio-respiratory, autonomic, and affective state. This study is registered at ClinicalTrials.gov. Identifier: NCT02612389.

Keywords: meditative movement, Qigong, flight attendants, second-hand cigarette smoke, pulmonary dysfunction, COPD, autonomic nervous system, inflammation

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BACKGROUND

COPD and Its Co-Morbidities in FA

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide; the primary cause of COPD is exposure to cigarette smoke. Those exposed to second-hand cigarette smoke (SHCS) are at increased risk for respiratory, cardiovascular, and other organ system disease that are similar to those suffered by smokers (1). Many flight attendants (FA) who flew before the ban on smoking in commercial aircraft, and who were thus exposed to SHS, have abnormal pulmonary function consistent with mild COPD, despite not meeting the GOLD criteria of reduced FEV1/FVC ratio (2). They also show increased rates of many of the co-morbidities associated with COPD, such as chronic bronchitis, cardiovascular disease, skin cancer, and depression and anxiety (3). The co-morbidities of COPD exacerbate and are exacerbated by the COPD (4).

Thus, consideration of the co-morbidities of COPD is an essential aspect of its treatment (4). Significant co-morbidities include cardiovascular disease (4); depression and anxiety (5); muscle weakness, osteoporosis, skin and lung cancer, and diabetes (4); as well as autonomic disturbance (6), systemic inflammation and cachexia (4); frequent hypoxia, dyspnea, and disturbed breathing patterns (7); and disturbed posture and movement (8); see **Figure 1**.

The Autonomic Nervous System in COPD

Autonomic dysfunction (AD) is known to be associated with COPD and most of its co-morbidities (6, 9–15); see **Figure 2**. Most of the diseases known to be co-morbidities of COPD may be caused or exacerbated by AD; for instance, cardiovascular disease (15, 16); hypoxia (17, 18); disturbed respiratory patterns (7, 19, 20); disturbed posture and movement patterns (21, 22); diabetes (17); immune function (4); airways restriction (6, 23); and anxiety and depression (12).

Meditative Movement

Qigong, a traditional Chinese health practice, has been used in China for hundreds of years in treating those with respiratory disease (24). Recently, a case has been made for a novel category of exercise "meditative movement," (MM) of which Qigong, Tai Chi, and Hatha Yoga are examples (25). Significantly, MM is hypothesized to act via its effect on the autonomic nervous system (ANS) (26, 27), as well as on neuromuscular control (28, 29), musculoskeletal condition (30), and mental state (31).

Meditative movement has been shown to be an effective intervention for COPD (30, 32–36) and equivalent or superior to conventional pulmonary rehabilitation in benefiting several symptoms of COPD (37).

Meditative movement may also be of benefit in many co-morbidities of COPD, such as heart disease risk factors (38, 39), hypertension (39, 40), depression and anxiety (26, 41), diabetes (42), osteoporosis (43), and muscle weakness (44), as well as reduced immune function (45, 46), inflammation (47, 48), and autonomic imbalance (49). Many of these beneficial effects may happen via the influence of MM on the ANS (26, 50); see **Figure 3**.

Study Aims

To evaluate COPD-related health factors in FA exposed to SHCS while flying, and to determine the effects of digitally delivered MM training on these factors.

There are three sub-aims, relating to the three main aspects of the study:

(1) To investigate the hypothesis that COPD-related pulmonary and other symptoms in SHCS-exposed FA are part of a syndrome of related disorders.
(2) To determine that MM practices are most suited to and effective for ameliorating pulmonary dysfunction and related health problems in FA exposed to SHCS.
(3) To conduct a randomized controlled trial (RCT) to determine whether this specifically adapted MM training can be

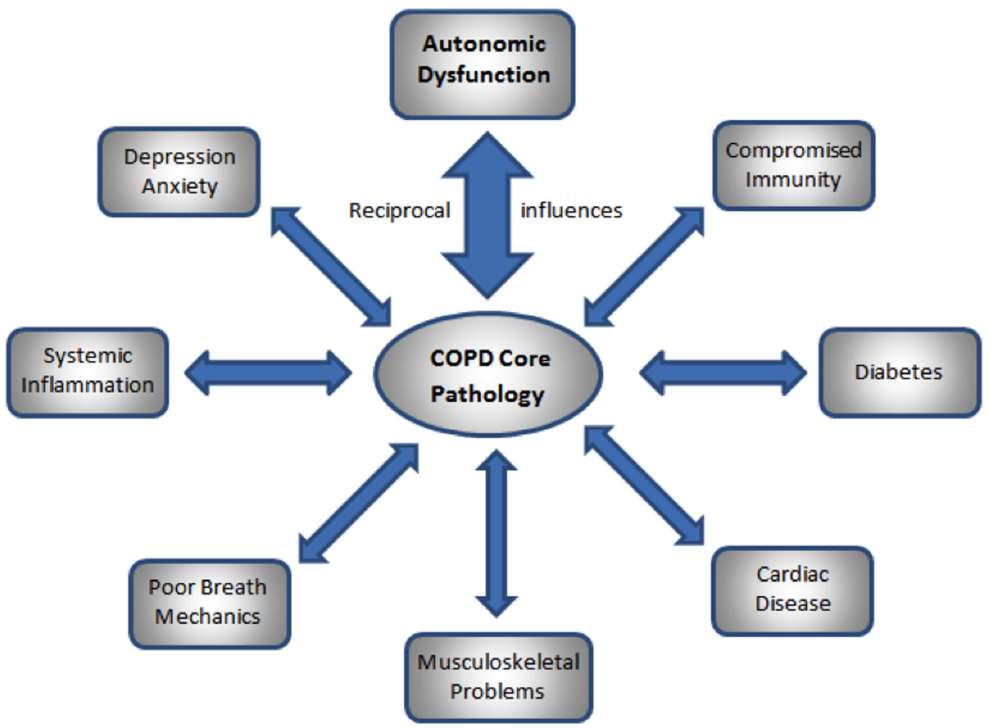

FIGURE 1 | Reciprocal influences of core COPD pathology and the co-morbidities of COPD.

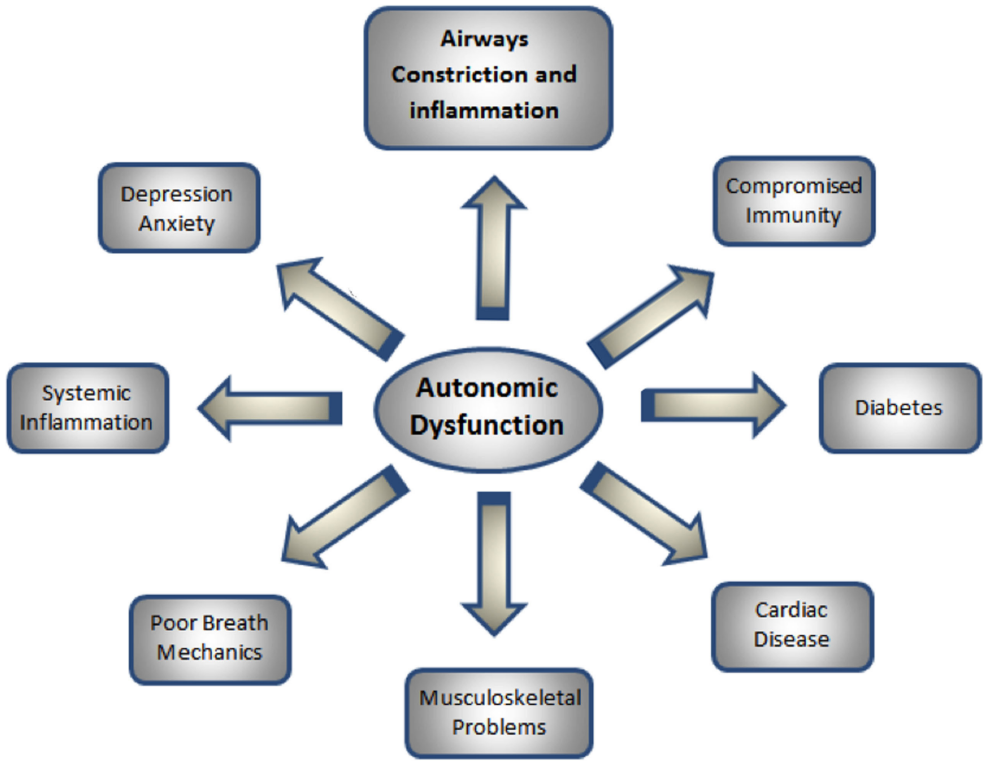

FIGURE 2 | Relationships of autonomic dysfunction to the co-morbidities of COPD.

effectively delivered digitally, providing the benefits of MM without face-to-face instruction being necessary.

METHODS

Overall Structure of the Study

This study has three stages: Screening, Selection, and RCT. Screening will continue throughout the study and will gather data as well as serving to provide participants for the other Selection and RCT stages. The RCT will follow the Selection stage. All aspects of this study have been approved by the Dartmouth IRB. Additionally, this study is registered at ClinicalTrials.gov Identifier: NCT02612389. https://clinicaltrials.gov/ct2/show/NCT02612389/

Screening

To screen a cohort of FA exposed to SHCS for a range of biomarkers and symptoms to determine whether symptom clusters exist that suggest a syndromal entity.

Rationale

Flight attendants exposed to SHCS have been observed to display a wide range of respiratory, functional, inflammatory, autonomic, cardiovascular, affective, and other symptoms. The possible relationship between these symptoms has not previously been explored. We hypothesize that these symptoms may be correlated and form a syndromal entity.

Selection

To determine which of a variety of possible MM exercises are most effective in improving specific outcome measures, most enjoyable, and most likely to be practiced in a cohort of FA exposed to SHCS and having a degree of pulmonary dysfunction. This is done by conducting a 16-week in-person MM training program that is held in the Northeastern region of the US. Participants provide extensive feedback on the exercises.

Rationale

Meditative movement offers an inexpensive intervention that could benefit pulmonary symptoms and other related symptoms, through its effect on the ANS, cardiovascular, and immune systems. However, there is a large body of possible MM exercises, and there has been no investigation of the relative effectiveness, acceptability, and compliance among FA for the many MM exercises that are traditionally recommended for COPD-related symptoms. To our knowledge, no other study has attempted to evaluate a wide range of MM practices to determine which are most appropriate for the population being studied.

Randomized Controlled Trial

To develop a DVD or other digital form for delivering the selected exercises that have proven to most appropriate for this population, and to test by RCT the effectiveness of this digital format in improving specified outcome measures in a group of FA with

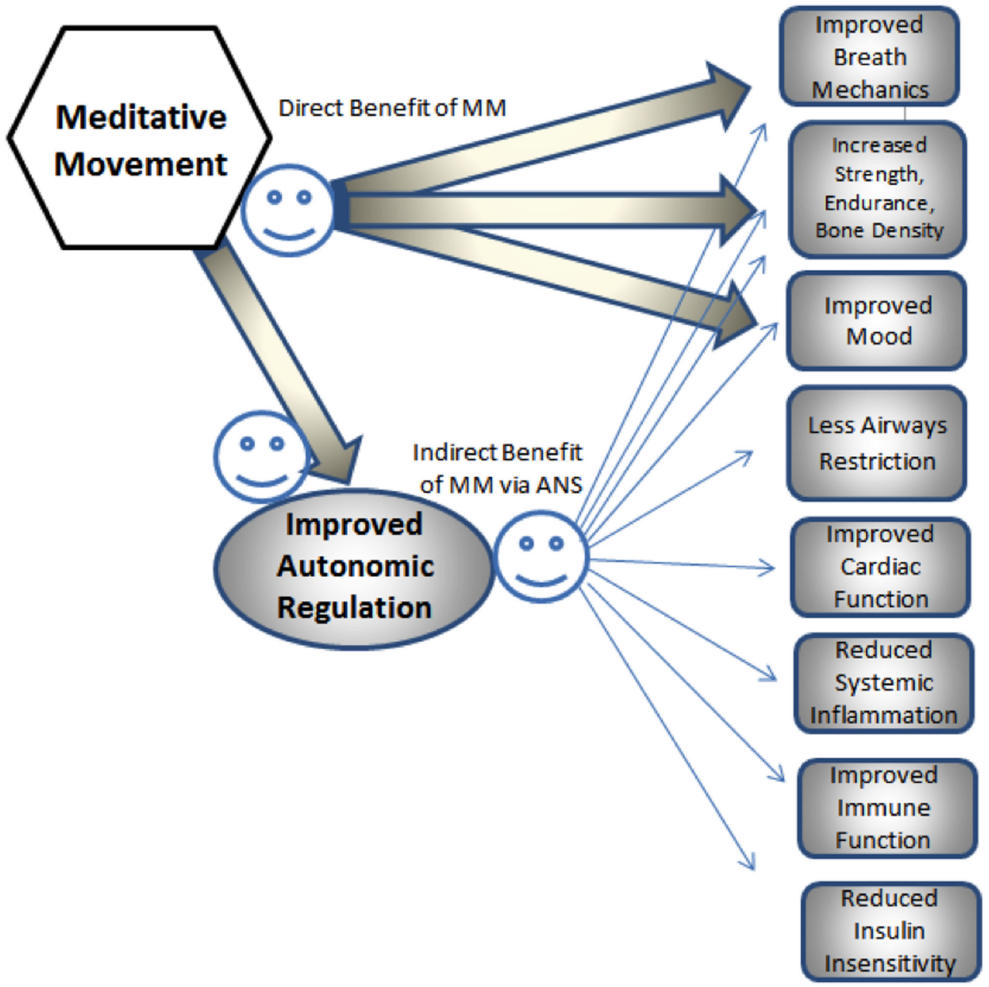

FIGURE 3 | Hypothesized pathways of direct and indirect influence of MM on co-morbidities of COPD.

pulmonary dysfunction and exposed to SHCS, as compared to a matched attention control group who will watch educational digital material of similar length.

Rationale

Selecting appropriate exercises and providing face-to-face instruction in MM offers significant difficulty due to the lack of trained teachers in most areas of the country and the practical demands of attending classes. A digitally delivered format would make this intervention much more accessible, and an RCT will evaluate the effectiveness of this intervention.

Participants

Recruitment

Flight attendants were recruited from the Northeastern region and other areas of the US for the Selection stage; the Screening and RCT stages will involve recruitment throughout the continental US in locations convenient to data collection points. Primary outreach is through existing FA organizations and networks, social media, and FA publications, as well as in conjunction with the Harvard Flight Attendant Health Study. Social media support is also provided by Department of Health Behavior at the Roswell Park Cancer Institute in Buffalo, NY, USA.

Randomization and Blinding

The Screening and Selection stages of this study do not use a control group; blinding and randomization are, therefore, not necessary. In the RCT, participants will be stratified by gender, age, and severity of pulmonary dysfunction and randomized to an intervention groups and an attention control, using the covariate adaptive randomization method (51). This method accommodates recruitment and testing over an extended period of time. Full blinding of participants to intervention is not possible under these circumstances; however, all scoring of tests will be done by assistants blinded as to participant group.

Inclusion Criteria

The Screening stage of the study acts both to collect participant baseline data and to determine eligibility for the Selection and RCT stages.

All participants must be current or former non-smoker FAs employed by a US carrier for at least 5 years while smoking was permitted in the aircraft cabin. They must have a score of above 5 on the COPD assessment test (CATest); this will eliminate those with no detectable pulmonary issues. A score of 5 is a low score, indicating minimal level of pulmonary dysfunction.

Many FA exposed to SHCS have detectable pulmonary abnormalities despite not meeting Gold criteria for mild COPD (2); we wish to include this population, since they are likely at risk for the future development of COPD or its co-morbidities. We will eliminate any participants not meeting at least one of the following spirometric criteria: ratio of forced expiratory volume in 1 s to forced vital capacity (FEV1/FVC) < 0.70; OR forced expiratory flow (FEF) at 25–75%, volume <80% of predicted normal; OR FEF at 50%, volume <80% of predicted normal; OR FEF at 75%, volume <80% of predicted normal. We will follow procedures as described by Arjomandi et al. (2) for determining normal FEF values.

Participants must be lifetime non-smokers, defined as having smoked <100 cigarettes in their lifetime. They must have a device on which to listen to audio or watch video instruction and be willing to do so; for the RCT portion of the study, capacity to watch online or DVD videos is required. Those who are pregnant, or who plan to become pregnant during the study, are excluded. Patients with cognitive impairment, severe emotional problems, or who are unable physically to perform the exercises are excluded. Participants will be asked not to modify their lifestyle significantly during the study period apart from the practice required by the study. Participants must all sign informed consent forms.

Sample Size

The Screening stage of this study has no limit on number of participants. For the Selection and RCT stages of the study, we determined that, based on a predicted difference of 46 m in the 6-min walk test (6MWT) and an assessment of previous similar studies of MM for COPD, this study required a sample size of 20 participants in each group to achieve a statistical power of 80% at a significance level of 5%. To compensate for anticipated dropout, we recruited a total of 27 participants into the Selection group. On the basis of our experience of a high dropout and non-compliance rate in the Selection stage (~40%), we intend to recruit 120 participants for the RCT, 60 for each group.

Interventions

The Screening stage of the study involves no intervention. In the Selection stage, the MM intervention is given in person in small groups (between 3 and 7), in 3-h classes, spaced 2–4 weeks apart, in locations convenient to the participants, for a total of 18 h of contact time over 4 months. Participant feedback will be solicited as to the subjective effectiveness, enjoyability, and likelihood of regular practice of each specific exercise.

The MM classes will be taught by one of the authors, Peter Payne, a Qigong teacher with over 40 years experience. In the classes, rather than teaching rote movements, the purpose and philosophy behind each exercise is explained. Suggestions are made as to how to set up a regular practice and how to integrate the exercises into everyday life. The participants are encouraged to ask questions and make comments on the exercises; all questions and comments are recorded in writing at the time. Individual attention is given to the needs of each participant, and written and audio explanations are also available to participants online. (Please see the Supplementary Material for the description of exercises from which we draw.)

The RCT stage of the study will involve the design of a digitally delivered intervention based on the exercises determined in the Selection stage to be most helpful. The intervention may be delivered on a DVD or through a web site. The goal will be to simulate as closely as possible the experience of attending a class through the development of innovative methods, such as computer animation, branching path selection, and user-controlled sequencing. Participants in the RCT will also have access to audio and written materials. Attention control subjects in the RCT will receive equivalent digitally delivered health education materials.

Safety Considerations

Very few studies of MM have reported significant risks (52) and we do not anticipate any ill effects. Nevertheless, participants are instructed to discontinue the exercise immediately if they experience any of the following: dizziness, rapid or irregular heart-beat, sudden or excessive dyspnea, chest pain, significant pain anywhere in the body, and headache. In addition, during the classes we carefully monitor the participants for signs of distress, including facial paleness or redness, rapid breathing, sweating, or jerky movement. Any data gathered that suggest possible need for medical attention are drawn to the attention of the participants.

Outcomes

These are the outcome measures to be used in the Screening stage of the study as well as to evaluate the effects of the MM practices in the Selection and RCT stages. The broad range of outcome measures will allow us to examine correlations between a wide range of variables representing a number of different physiological and psychological systems that may be involved in COPD and its co-morbid conditions.

Primary Outcome Measures

Six-minute walk test
Change in high sensitivity C-reactive protein (hs-CRP)

Secondary Outcome Measures

Blood pressure (BP) (pre- and post-6MWT),
Heart rate (HR) (pre- and post-6MWT),
Blood oxygen saturation,
Spirometry FVC, FEV1, FEF 25–75,
Compass 31 (Autonomic function self-report),
Zung Depression Inventory,
Zung Anxiety Inventory,
COPD Assessment Test (CATest),
FA Health Questionnaire,
Multidimensional Assessment of Interoceptive Awareness (MAIA),
Borg Dyspnea Scale,
Heart Rate Variability (HRV),

2 Ewing tests,
Urine analysis (melatonin and cortisol),
Saliva analysis (diurnal cortisol), and
Blood (fingerprick) analysis.

All tests are administered by trained researchers. Blood, urine, and saliva analysis provided by ZRT Laboratory, Beaverton, OR, USA.

Outcome Measurement Details

General Health

Flight Attendant Health Questionnaire

This was developed by Dr. Eileen McNeely at the Harvard School of Public Health. Its use provides us with a health and FA-related occupational history and enables us to compare our study results with those of Dr. McNeely.

Functional Ability

Exercise endurance and tolerance is evaluated by the 6MWT followed by the Borg Dyspnea inventory. This is used as our principal outcome measure since it is a well-recognized measure of overall functional ability. The walk is conducted on an indoor level surface free of obstructions. Research assistants are on hand to assist participants with any difficulties. Maximum distance walked in 6 min is recorded (53–55). The difference in scoring from baseline to the end of study is calculated for each participant. *The Borg Dyspnea Inventory*, a well-established instrument for measuring respiratory distress, is administered following the 6MWT. The 6MWT is subject to variation due to motivation; the addition of the Borg instrument increases the reliability of the test.

Respiratory Health

The COPD Assessment Test

The CATest is used as an initial recruitment screening as well as an outcome measure. Volunteers must score above 5 for inclusion in the study. Its main purpose is to eliminate those with no appreciable respiratory dysfunction.

Spirometry

FEV1/FVC and Flow/Volume Curves

Forced expiratory volume in 1 s to forced vital capacity and Flow/Volume Curves are used as part of the inclusion criteria (see above under inclusion criteria), and to determine the degree of pulmonary dysfunction. We use specific validated spirometric cut points (56). We record FEV1/FVC ratio and Flow/Volume Curves using the EasyOne Plus Frontline spirometry system. FEV1/FVC is a standard measurement used to determine the GOLD grade of COPD. The Flow/Volume Curves indicate the degree of pulmonary dysfunction; participants with no detectable pulmonary dysfunction are excluded from the study. The differences in scoring from baseline to specific intervals are calculated for each participant.

Cardiovascular Measures

Blood Pressure and Heart Rate

Blood pressure and HR are measured at the beginning of each testing period and before and after the 6MWT by a trained technician using standard clinical instruments. *Cardiac R-R intervals* are recorded over the entire testing period (about 1½ h) using a commercially available system, the Holter myPatch 24-h single channel AMS3000 from DMS-Services, Los Angeles, CA, USA. This is a non-intrusive device, using two electrodes. Due to problems with availability, this measure was not included with some participants during the Selection stage of the study only. Blood oxygen saturation is also measured, using a Choicemmed Oxywatch Fingertip Pulse Oximeter.

Autonomic Condition

Heart rate Variability

Subjects are asked to sit quietly for 10 min. In post-analysis, the HR data collected during these periods is used to calculate HRV. HRV data will be analyzed using software provided by the manufacturer: CardioScan II, version 12.4.0054a, from DMS Software, Los Angeles, CA, USA. Time domain (SDNN, RMSSD) and frequency domain [power spectral density (PSD)] analysis methods will be used. Dr. Phyllis Stein, Director of the Washington University School of Medicine HRV Lab, will serve as a consultant on HRV analysis and interpretation. HRV measurements are only available for a subset of participants in the Selection stage due to delayed availability of HR monitors; monitors will be fully available during the RCT stage.

Ewing Tests

The Ewing tests evaluate cardiac autonomic function by measuring the response of HR or blood pressure to a physical challenge. We will administer two of the battery of five Ewing Tests: deep breathing heart rate challenge (DBHR) and lying to standing blood pressure challenge (LSBP). DBHR has been determined to be the most significant of the Ewing tests, carrying 80% of the significance. To measure this, participants will be asked to breathe deeply for three 1-min periods at a rate of six times a minute; post-analysis using recorded R-R intervals will enable evaluation of HR response to challenge, which is a measure of the integrity of the sympathetic branch of cardiac autonomic control. To measure LSBP, the systolic blood pressure change from supine to standing is measured. This gives an indication of cardiac parasympathetic function. Comparing the results from HR, BP, HRV, DBHR, and LSBP will allow a more accurate evaluation of the cardiorespiratory autonomic system than any one of them taken alone. Full Ewing test results are only available for a subset of participants due to problems with availability of the heart monitors.

COMPASS 31

In addition, we administer the COMPASS 31, a questionnaire for detecting dysfunction in many aspects of the ANS.

Affective State

Zung Anxiety and Depression Inventories

These evaluate the degree of affective disturbance. They are both well validated and widely used instruments. Since depression and anxiety are significant co-morbidities of COPD, and since we speculate that MM intervention will have an impact on them, the Zung inventories are relevant.

Interoceptive Awareness

The Multidimensional Assessment of Interoceptive Awareness (MAIA)

The Multidimensional Assessment of Interoceptive Awareness (MAIA) is a questionnaire that evaluates the degree of a person's awareness of interoceptive cues, as well as his or her degree of comfort with these cues. We believe this is relevant to a person's ability to benefit from the MM intervention.

Biomarkers

Biomarkers include urine, saliva, and blood analysis.

Blood Analysis

Capillary blood drops will be taken from the finger following fingerprick with a lancet, and deposited onto a blood spot card used specifically for dried blood spot (DBS) collections. Blood spots are dried on the filter card for at least 2 h before closing the cover and then further dried overnight at room temperature before storing the cards for shipment to ZRT Laboratory. Six-millimeter disks of blood spots are punched into 96-well plates and the blood extracted with buffers. Extracts are assayed for C-reactive protein (CRP), a marker of inflammation, as well as HbA1c (a marker of metabolic function), thyroid stimulating hormone, and Vitamin D.

Urine Steroid Hormone and Element Testing

Participants will be asked to provide first morning and then a bedtime urine samples: these are collected onto an absorbent paper strip and dried. They will be instructed in correct procedures for this. The sample will later be analyzed at ZRT Lab to determine by LC-MS/MS levels of urinary free cortisol, free cortisone, and melatonin, and by ICP-MS levels of iodine, bromine, selenium, arsenic, cadmium, and mercury (all normalized to creatinine).

Salivary Steroid Hormone Testing

Participants are instructed to provide four saliva samples over the course of a day. These samples will be stored frozen and batch-shipped to ZRT Laboratory and analyzed to determine diurnal cortisol levels as well as first morning estradiol, progesterone, testosterone, and dehydroepiandrosterone (DHEA) by LC-MS/MS.

Dried blood spots, dried urine (DU), and saliva samples are mailed to the researchers and stored at −70°C until they can be shipped in bulk to ZRT Labs for testing.

This extensive range of biomarkers will allow us to examine correlations between levels of function of a wide range of physiological systems and pulmonary dysfunction.

During the Selection and RCT stages of the study, feedback will be gathered from participants through written logs, and a questionnaire on Survey Monkey. Copies of the forms for the written logs and the Survey Monkey questionnaire are available as Supplementary Material.

Statistical Methods

Descriptive statistics, including mean, SD, and frequency, will be used to describe and summarize baseline data. We will also compare distributions between intervention and control groups in the RCT stage using Wilcoxon rank-sum tests and chi-square tests at baseline. An intent-to-treat analysis will be carried out where missing data will be handled by multiple imputation approach, and linear regression models will be used to compare the intervention group with the control group in terms of the primary outcomes.

Stepwise Methods

Stepwise methods are detailed in **Figure 4**.

Anticipated Results

In the Selection and the RCT stages of this research, it is anticipated that subjects in the study will increase endurance as demonstrated by an increase in the 6MWT by margins that meet or exceed statistical significance. A general marker of inflammation, hs-CRP, is expected to decrease significantly. Consistent with evidence that MM may benefit the ANS, we expect to see a decrease in blood pressure. With the larger number of subjects in the RCT stage of the study, secondary measures are expected to provide data suggesting or discounting relationships between ANS function, mood, pulmonary, and immune system functions.

Pitfalls, Artifacts, and Troubleshooting

A potential pitfall in this study is the difficulty in recruiting and retaining subjects. Our extensive network of contacts among FA will facilitate this process, as will our outreach through FA-connected social media. The recruitment process can be extended in time to assure adequate numbers. In addition, the researchers are prepared to travel to locations throughout the country as necessary.

Retention of subjects in the second and third stages is another concern. It is anticipated that retention will not exceed the 40% of the Selection stage of the study, as the exercises to be taught are simple and enjoyable. However we are allowing for up to 60% drop-out rate. Should dropout be more than anticipated, we will need to recruit more subjects, which should prove possible. We have maintained good communication with all subjects.

In the Selection stage of the study, any difficulties participants have had with the training, including safety-related concerns, were explored as a source of information concerning appropriate selection of exercises; this was one of the aims of this portion of the study.

The smoking status of study participants is queried during the recruitment and further during testing for each stage of this study. However, additional details of a smoking history have, in a few cases, emerged when other data had already been collected on these subjects. Outcomes from these subjects will be sequestered and reported separately from those who smoked less than 100 cigarettes in a lifetime.

Due to limited availability of equipment, the wearable ECGs were not used in the majority of pre-testing in the Selection stage. Changes in HRV are, therefore, not documented. However, other measures of cardiovascular autonomic function were made, including HR, BP, and changes in these measures in response to challenge. These will provide some information about possible autonomic changes.

In the Selection stage, we do not use a control group. Since the purpose of this portion is to determine that MM exercises

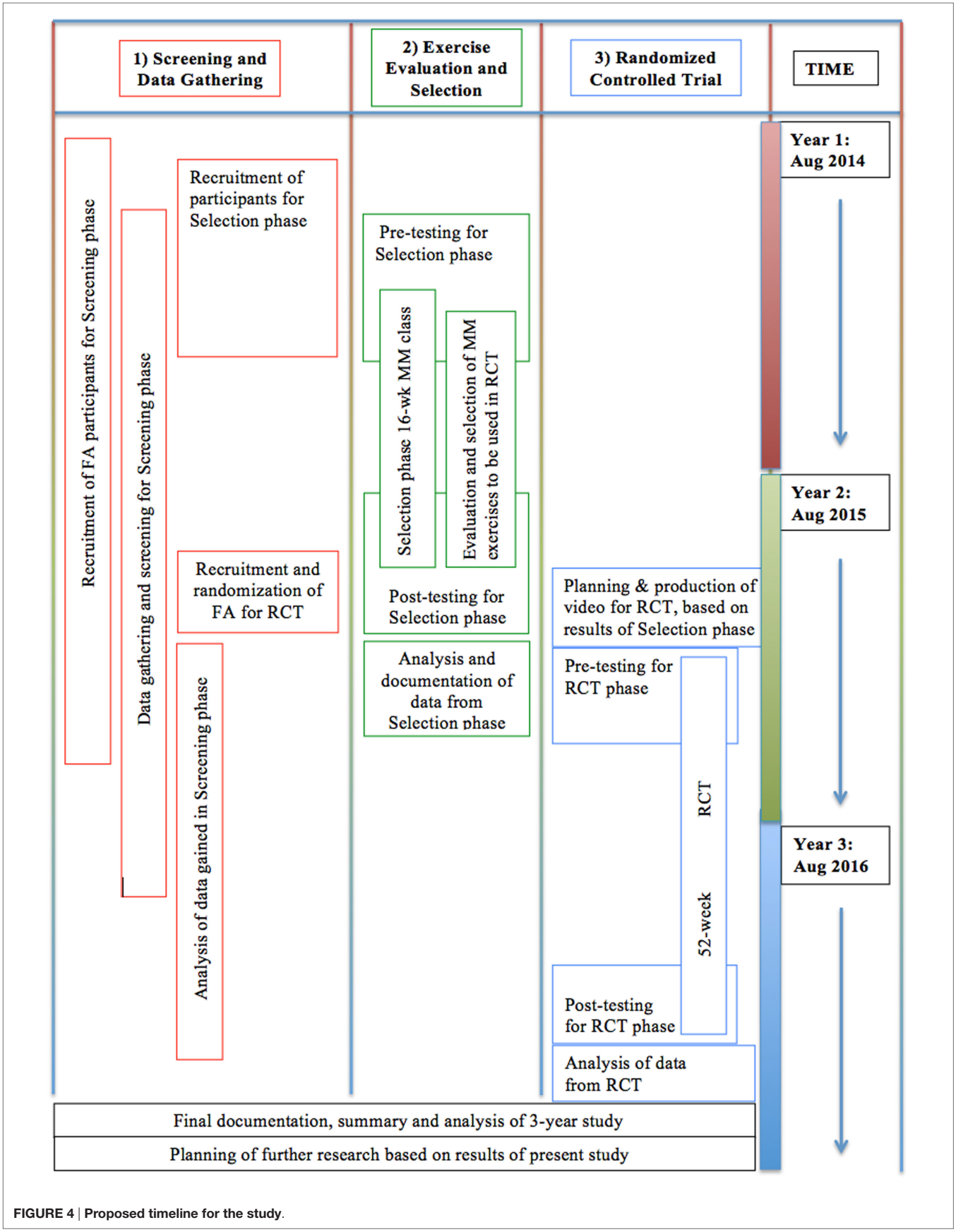

FIGURE 4 | **Proposed timeline for the study**.

are most effective and best tolerated, use of a control group would not be appropriate. This diminishes the power of results from the pre-and post-intervention tests in this portion as it will not be possible to determine with certainty whether results are due to attention, seasonal or other factors. However, the final RCT stage of the study, using an attention/health education control group, will provide a well-controlled validation of the effects of the selected MM exercises. It is, however, possible that positive results from the testing in the Selection stage, followed by negative results from the RCT stage, would reflect the ineffectiveness of digital delivery rather than the ineffectiveness of the intervention. In this case, an RCT testing in-person delivery would be indicated.

The 6MWT is known to be influenced by motivation. The use of the Borg Dyspnea Inventory is used as a control on this, as it indexes amount of effort, and every precaution has been taken by the researchers to standardize the administration of the test. Nonetheless, in the Selection stage, subjects may be more highly motivated in the post-test situation, and this could skew results. As mentioned above, the use of a control group in the RCT stage will provide a check on these results.

In the RCT stage, compliance is expected to be an issue. We plan to be in contact by email, phone, and digital questionnaire with all subjects on a weekly basis. We will follow up quickly any lapses in communication or reporting of difficulties, so as to minimize this issue. Such difficulties arising in this stage will also provide further information for future refinement of the delivery method and the selection of exercises.

DISCUSSION

We believe that the ANS may be a link between the various co-morbidities of COPD.

We speculate that COPD-related pulmonary and other co-morbid conditions appearing in FA exposed to SHCS may be part of a syndrome of interacting conditions related to disturbed autonomic function. The Screening stage of this study will provide data to test this speculation.

In addition to our primary outcome measures, we are gathering a wide range of data, including hormonal biomarkers, affective, and autonomic measures. This may allow for the formulation of hypotheses for future testing concerning the mechanisms of action of MM on pulmonary dysfunction and other health conditions. In preliminary data, we are seeing patterns of subclinical morbidities that may provide an improved picture of the health care needs of this unique group of workers. It, however, remains to be seen whether these patterns are simply age related or unique to this group and their exposure to SHCS.

Since MM has been shown to have beneficial effects on many of the co-morbid conditions of COPD, possibly by way of its regulating effect on autonomic functioning, this suggests that a single approach (MM) might target not only COPD-related respiratory symptoms but also many other COPD-related conditions. Future studies could determine whether MM could slow the progression of pre-COPD conditions to clinical COPD.

Previous trials of the effects of MM have relied on a preselected set of exercises, in some cases chosen for their traditional relevance to the condition being treated (57) but in most cases a traditional general set (30, 32–36). Our study is the first to involve a test period in which a wide range of MM exercises is evaluated for a specific population. We have already observed that participants have trouble setting aside time for regular practice. Our approach to MM includes a number of practices that can be integrated with the activities of daily life (rather than having to be practiced at a certain time), and we have found that this form of practice is more acceptable to this group of FA.

We experienced an unexpected level of dropout during the Selection stage of the study. Given that the average age of participants is 69, this should have been anticipated. We have found that the provision of exercises that can be integrated into daily life, such as simple standing, sitting, walking, and breathing practices, increases compliance and appears to reduce dropout. We believe that the emphasis on this kind of practice can make Asian practices more accessible and appealing to Westerners with their busy lifestyles.

Our study explores the possibility of administering an MM intervention by digital means. To our knowledge, no other study of MM has tested this possibility. Information gained from this aspect of the study may provide information about the most effective ways of conveying MM training digitally, as well as validating the effectiveness of MM for COPD-related pulmonary dysfunction. Should our principal hypothesis be supported, it would not only open the possibility of a safe, cost-effective intervention for those with pulmonary dysfunction, but also support the further exploration of digitally delivered MM for a wide range of conditions, especially those with a significant autonomic component.

Trial Status

Recruitment for the Screening and evaluation portion of the study began in August 2014 and will continue for the duration of the study, until mid 2017. Comprehensive data have been gathered on more than 60 participants to date, and preliminary analysis has begun.

Recruitment for the MM exercise selection portion of the study began in October 2014 and will continue through October 2015. While 27 participants have been recruited so far into the Selection stage, there is concern for assuring that a statistically significant number will complete this stage of the study. No adverse events have been reported. Post-intervention testing began in July 20 2015, and is on going.

Preparations for the development of a digitally deliverable MM intervention are presently underway, involving developing video footage and planning. Recruitment for the RCT portion of the study began in October 2015.

AUTHOR CONTRIBUTIONS

PP: manuscript, research plan development, and MM instruction. DZ: data reporting and analysis, supervision of sample analysis,

and manuscript. SF: manuscript, research plan, and supervision. MC: manuscript, administration, research plan development, and implementation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpsyt.2016.00038.

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APPENDIX

ACRONYMS

6MWT	Six-minute walk test	A standard test for assessing functional ability, the distance walked in 6 min
ANS	Autonomic nervous system	Portion of the nervous system controlling those physiological functions outside normal voluntary control
BP	Blood pressure	
COPD	Chronic obstructive pulmonary disease	Chronic disease involving obstruction of the small airways
CRP	C-reactive protein	Biomarker of inflammation
DBHR	Deep breathing heart rate	One of the battery of 5 Ewing tests for cardiac autonomic dysfunction; the most significant of the 5
DVD	Digital video disk	Standard vehicle for presenting video
EAC	Education activity control	A control group engaging in educational activity, in this case watching educational videos
FA	Flight attendants	
FAMRI	Flight attendant medical research institute	The foremost organization promoting research into medical problems affecting flight attendants
FEF	Forced expiratory flow	Spirometric measurement, flow of air during forced exhalation
FEV1	Forced expiratory volume one	A measure of lung function: the maximum volume of air breathed out in a forcible exhalation in 1 s
FVC	Forced vital capacity	A measure of lung function: the total volume of air breathed out in a complete forcible exhalation
GOLD	Global initiative on obstructive lung disease	A research and information group that is the principal global authority on the nature and treatment of COPD
HR	Heart rate	
HRV	Heart rate variability	Degree of variability over time (especially in synchrony with the breathing) of the R-R, or beat-to-beat, heart rate; since this variation of heart rate is under control of the parasympathetic nervous system, HRV offers a way of evaluating this aspect of ANS function
LSBP	Lying standing blood pressure	A Ewing test of the blood pressure response to going from lying to standing
MAIA	Multidimensional assessment of interoceptive awareness	A questionnaire designed to evaluate several dimensions of interoceptive awareness
MM	Meditative movement	A newly identified form of exercise characterized by a meditative state of mind, deep relaxation, movement, and attention to the breathing. Qigong and Yoga are the best known examples
NHANES	National health and nutrition examination survey	
PI	Principal investigator	
PSD	Power spectrum density	A measure of frequency distribution used to evaluate HRV
QOL	Quality of life	A general measure of the quality of a patients daily life
RCT	Randomized controlled trial	The premier form of research study in which participants are randomly assigned to control or intervention group for the purpose of objective assessment of an intervention
RMSSD	Root mean square standard deviation	A statistical method used in this study for the analysis of HRV data
SDNN	Standard deviation	A statistical method used in this study for the analysis of HRV data
SHCS	Second-hand cigarette smoke	Cigarette smoke inhaled by someone other than the smoker; ambient smoke not inhaled directly through the cigarette

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Reversion of AHRR Demethylation Is a Quantitative Biomarker of Smoking Cessation

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Smoking is the largest preventable cause of morbidity and mortality in the world. Although there are effective pharmacologic and behavioral treatments for smoking cessation, our inability to objectively quantify smokers' progress in decreasing smoking has been a barrier to both clinical and research efforts. In prior work, we and others have shown that DNA methylation at cg05575921, a CpG residue in the aryl hydrocarbon receptor repressor (AHRR), can be used to determine smoking status and infer cigarette consumption history. In this study, we serially assessed self-report and existing objective markers of cigarette consumption in 35 subjects undergoing smoking cessation therapy, then quantified DNA methylation at cg05575921 at study entry and three subsequent time points. Five subjects who reported serum cotinine and exhaled carbon monoxide verified smoking abstinence for the 3 months prior to study exit averaged a 5.9% increase in DNA methylation at cg05575921 ($p < 0.004$) over the 6-month study. Although the other 30 subjects did not achieve smoking cessation at the 6-month time point, their self-reported reduction of cigarette consumption (mean = 6 cigarettes/day) was associated with a 2.8% increase DNA methylation at cg05575921 ($p < 0.05$). Finally, a survey of subjects as they exited the study demonstrated strong support for the clinical use of epigenetic biomarkers. We conclude that AHRR methylation status is a quantifiable biomarker for progress in smoking cessation that could have substantial impact on both smoking cessation treatment and research.

Keywords: DNA methylation, epigenetics, aryl hydrocarbon receptor repressor, cg05575921, diagnostics, smoking cessation

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INTRODUCTION

Smoking is the largest cause of preventable morbidity and mortality in the United States. Each year, nearly a half-million Americans die secondary to the effects of smoking (1). Still, nearly one in every five US adults currently smoke (2). Currently, three pharmacological agents, bupropion, varenicline, and nicotine replacement therapy (NRT), are commonly used for smoking cessation (3). By themselves, each of these medications is modestly effective and recent clinical trials suggest that the combination of varenicline and NRT is most effective in achieving cessation (3, 4). Nevertheless, the efficacy of these treatments in actual clinical practice has been less than optimal (5).

Although many barriers to the effective implementation of these and smoking cessation interventions exist, one of the more difficult hurdles to overcome is our current inability to quantify decreases in smoking and the success of cessation therapy. In epidemiologic studies, self-report is generally accurate, however, in clinical populations, it is much less reliable (6–8). Currently, two biological methods are commonly used to determine the success of therapy and corroborate self-report: exhaled carbon monoxide (CO) and cotinine levels. Exhaled CO levels are perhaps the easiest assessments to perform. But this measure is only capable of detecting smoking in the past 3–4 h and is not useful in qualifying changes in smoking at these levels because it is relatively insensitive to light-to-moderate smoking (9, 10). By contrast, assessments of cotinine, which has a serum half-life of 15 h, are much more sensitive and can detect smoking in the past 48–72 h (11). However, because false positives can arise from other forms of tobacco consumption (second-hand smoke, e-cigarettes, and ironically, NRT use) its clinical utility in monitoring decreased smoking and abstinence is limited. In fact, since over one-fourth of all patients who successfully quit smoking using NRT remain on NRT for at least 1 year after smoking cessation (12), the efficacy of employing cotinine levels to guide smoking cessation in clinical settings is minimal.

The development of quantitative continuous dose–response measures of decreases in smoking in therapy could significantly advance smoking cessation efforts in much the same way that the introduction of hemoglobin A1C (HbA1c) levels to assess the need and effectiveness of diabetes management has revolutionized the treatment of Type 2 diabetes (T2DM) (13). The use of HbA1c assessment, which is a measurement of the level of the acetylation of hemoglobin by serum glucose, allows clinicians to not only diagnose T2DM but also objectively quantify the progress of diabetic therapy. The latter is particularly important because numerous studies have shown that patients, in particular those who are at the highest risk, do not accurately report treatment compliance (14). The same challenges confront clinicians dealing with smoking cessation, suggesting that if a similar tool for measuring smoking intensity could be developed, it is possible that clinicians could use that assessment to detect changes in smoking, and modify treatment strategies during therapy.

DNA methylation assessments may provide a tool that can accurately assess amount and changes in smoking status in order to track the trajectory of smoking initiation and cessation. Over the past 3 years, at least 20 studies have confirmed the initial findings that methylation at cg05575921, a CpG residue in the aryl hydrocarbon receptor repressor (AHRR), is the most sensitive indicator of smoking status at all levels of smoking (15, 16). In particular, in a recent clinical trial, methylation status at this locus was employed to classify the smoking status of adult subjects, and was shown to be extremely accurate with a receiver operating characteristic (ROC) area under the curve (AUC) of 0.99 (15). Whereas these and other studies clearly indicate that DNA methylation can be used to track changes in smoking, they have not addressed the question of whether DNA methylation could be used to guide smoking cessation therapy.

Several genome-wide studies, including work from Zeilinger and colleagues and Tsaprouni and colleagues have compared smokers' and non-smokers' methylation at specific loci and concluded that the smoking-induced DNA methylation signature reverts as a function of long-term abstinence with cg05575921 being one of the most prominent loci demonstrating reversion (17, 18). In addition, however, Zeilinger et al. estimated that the speed of that reversion was relatively slow, with a change of approximately 7% occurring over a course of 7 years. If this is correct, this would suggest that DNA methylation changes relatively slowly and could not be used for monitoring smoking cessation.

However, recent evidence has suggested that the speed of reversion of DNA methylation at cg05575921 may be significantly faster than that estimate. In our recent examination of alcoholic inpatients, those who smoked prior to admission but were either completely or partially deprived of cigarettes during their stay, averaged a 1.7% increase in CG05575921 over 25 days (19). Second, both the Zeilinger and Tsaprouni studies were cross-sectional studies that employed self-report without biochemical verification of smoking status. Since the reliability of retrospective recall of smoking cessation is poor (20) and their study design did not allow before and after comparisons of individual subjects, the speed of methylation reversion in Zeilinger and Tsaprouni reports may be an underestimate. In this study, we directly examine the relationship between cigarette consumption status and DNA methylation at cg05575921 in a cohort of subjects undergoing smoking cessation therapy under the direction of their personal physicians.

MATERIALS AND METHODS

All methods and procedures used in this study were approved by the University of Iowa Institutional Review Board. In brief, the subjects were recruited by direct advertising and word of mouth from University of Iowa affiliated clinical operations. Inclusion criteria for the screening of potential subjects for the study included the following: being a current active smoker who was getting ready to begin smoking cessation within 4 days of the intake appointment, and abstinence from any nicotine-containing product, including e-cigarettes. Please note that the rationale for exclusion of those subjects using other forms of nicotine-containing products was to allow the team to use cotinine assays to detect surreptitious smoking. Non-combustionable forms of tobacco consumption do not have an effect on cg05575921 levels (15, 21). Other exclusion criteria included use of any medication thought to interfere with DNA methylation, such as methotrexate, and any active form of substance use with the exception of alcohol.

At intake, all subjects were interviewed with the Semi-Structured Assessment for the Genetics of Alcoholism, Version 2 (SSAGA-II) modified for use in our studies (22). Notably, the Fagerstrom Test for Nicotine Dependence (FTND) (23) is embedded within the interview. In addition, substance consumption over key time frames was interrogated by a tailored substance use questionnaire described previously (19). Exhaled CO was assessed using a Tabataba CO Tester (Depisteo, France). Phlebotomy was then performed by a trained research assistant with sera being immediately separated via centrifugation, then stored at −80°C until use. Whole blood DNA was prepared

using cold protein precipitation, quantified with a NanoDrop photometer (ThermoFisher, Holtsville, NY, USA) and stored at −20°C until use (24).

Subsequently, each subject was assessed in person at 1, 3, and 6 months after study intake. In addition, they were contacted via phone or e-mail at 2, four, and 5 months after study intake. At the in person visits, each subject was re-interviewed with the substance use questionnaire, interval health, and medication use, including the use of any nicotine-related products, and exhaled CO were assessed, and phlebotomy was performed. During the phone or e-mail contacts, subjects were interviewed with the substance use questionnaire. DNA and sera were prepared from the in person visits as described above.

DNA methylation status at cg05575921 was determined using quantitative PCR (qPCR) as previously described (25). In brief, whole blood DNA was bisulfite converted using Fast 96 Bisulfite Conversion kits (Qiagen, Valencia, CA, USA) according to manufacturer's direction. Subsequently, cg05575921 methylation status of each sample was measured in quadruplicate using an ABI 7900HT Genetic Analysis System (Applied Biosystems, foster city, CA, USA), qPCR reagents (both assay and standards) from Behavioral Diagnostics (Iowa City, USA), and standard. The SD of replicate measurements was 0.23 cycles. The average methylation value for each sample was then determined by interpolation against the standard curve (25).

Serum levels of cotinine and hydroxy tetrahydrocannabinol (THC-OH) was determined using kits from AbNova (Taiwan) according to manufacturer's directions. Because the THC-OH kit does not come with internal standards suitable for the assessment of serum samples, a series of dilution of a methanol solution containing (±)-11-nor-9-carboxy-delta-9-THC (T-010, Sigma, Ronkonkoma, NY, USA) was used to quantify the extent of cannabis use.

Genotype at rs16969968 was determined using a primer probe set and a 2X polymerase master mix from Applied Biosystems (Foster City, CA, USA) per our usual protocols (26).

All regression analyses were conducted using JMP Version 10 (SAS Institute, Cary, SC, USA). For the main analysis, which examined the relationship between DNA methylation and smoking cessation status, a least squares regression model stipulating DNA methylation as the independent variable and subject, time since initial quit date, and a subject × time interaction term as the dependent variables, was used.

RESULTS

A total of 47 subjects passed the initial screening for inclusion in the study. Subsequently, four of those subjects were disqualified from further continuation of study for revealing information, such as active cannabis use, during the intake interview that was incompatible with continuation in the study. In order to ascertain substance use and increase study retention, we attempted to contact all remaining subjects monthly with the in person visits also serving as an opportunity to perform biochemical verification of smoking status. By and large, this strategy was successful in retaining 35 of the 43 (81%) subjects eligible to continue in the study participating in the 6-month visit.

The clinical characteristics of the 35 subjects who completed the 6-month study are given in **Table 1**. The subjects are mostly female (60%) of northern European ancestry (75%) and have an average age in their early 40s. They reported smoking an average of 11 cigarettes/day and had an average history of 16 pack years of smoking. Five reported use of bupropion; the remaining 30 attempted to quit smoking without pharmaceutical assistance.

At each in person contact point, serum cotinine and exhaled CO were assessed. In keeping with prior findings, exhaled CO assessments were less sensitive than cotinine levels for detecting smoking. For example, at the 6-month exit time point, nine subjects had CO of <10 ppm but still reported continued smoking and had serum cotinine levels supportive of continued smoking. Six of these nine reported daily smoking (between one and six cigarettes/day), while the three others reported continued periodic smoking (i.e., every other or every third day). In addition, one subject who reported 59 days of abstinence had an undetectable level of cotinine registered a reading of 19 ppm, which is suggestive of a false positive.

Active cannabis use and/or continued use were both exclusion criteria for the study. To examine the reliability of subjects with respect to this inclusion criterion, serum THC-OH levels, at intake and 1-month time points, were assessed. Two subjects, both of whom reported continued tobacco use at all study time points, had markedly positive serum THC-OH levels at the intake and 1-month study time points.

We defined successful smoking cessation as having self-reported smoking cessation, and both negative serum cotinine and exhaled CO levels at the 3- and 6-month time points. Using these criteria, four subjects [tobacco cessation (TC) 24, 31, 41, and 46] successfully quit smoking with a fifth (TC 28) having had only two cigarettes since quitting at study inception, 180 days prior, with all subjects giving serum and exhaled CO levels consistent with those reports. In addition, one subject who reported 59 days of abstinence had a negative cotinine at study exit, but not at the 3-month time point. Finally, five other subjects reported smoking cessation at study exit. Unfortunately, each of those had exhaled CO levels >10 ppm and high levels of serum cotinine inconsistent with cessation.

TABLE 1 | Clinical characteristics of the study completers.

Age	42.3 ± 13.5 years
Gender	
Male	14
Female	21
Ethnicity	
White	26
African Amer.	4
Hispanic	1
Other	3
Consumption at intake	
Lifetime	16.3
Past month	11.3 cigarettes/day
rs16969968 genotype	
GG	24
AG	15
AA	5
FTND score	3.7 ± 2.5

We then analyzed the relationship between smoking cessation and cg05575921 methylation for the five subjects who had negative cotinine and CO levels at the 3- and 6-month interview visits using least squares regression. **Figure 1** illustrates the results of those analyses. Not surprisingly, subject consumption history had the greatest effect on methylation levels ($p < 0.0001$) with TC 28 and 31 having the highest initial methylation levels, the lowest levels of current smoking and the least history of smoking, 2 and 4 pack years, respectively. In fact, the methylation level of TC 28 returned to the range consistent with a lifetime history of non-smoking by the end of the study (95%), while the level of TC 31 at study exit was nearly 89%. By contrast, although the methylation levels of TC 24 (15 pack years), TC 41 (20 pack years), and TC 46 (15 pack years) increased as a function of TC, their values remained lower than those of the subjects with less smoking history, with methylation levels of 75, 47, and 77%, respectively. Still, the effect of time of cessation was clearly significant, i.e., increasing time since study intake being associated with increasing cg05575921 methylation ($p < 0.004$).

In addition to the promising results shown for those in full cessation, examination of the DNA methylation from those subjects whose cotinine and exhaled CO data were not consistent with complete cessation at 6 months were also promising. In total, 29 subjects did not have negative cotinine and CO levels at the 3- and 6-month time points. In fact, with the exception of the subjects listed above, only two other subjects had a negative cotinine level at either time (both at 3 months; but not at study exit). Still, as a whole, these subjects reported an average decrease in cigarette consumption of 5.8 cigarettes/day (11.3 cigarettes/day at intake and 5.5 cigarettes/day at 6-month exit). This decrease in smoking was accompanied by an increase in methylation from an average of 66.7 to 69.5% over the 6 months (Adj. $R^2 = 0.14$, $p < 0.05$).

Prior work by ourselves and others has shown that cg05575921 is associated with the quantity of cigarette consumption. To better understand how this consumption is linked to other factors, such as nicotine craving and key genetic variables, we conducted a series of regression analyses with methylation as the independent variable, and total history of smoking (pack years), current smoking, FTND, and rs16969968 genotype. Consistent with prior analyses, cg05575921 methylation at intake was associated both with current consumption (Adj. $R^2 = 0.37$, $p < 0.0002$) and history of consumption (Adj. $R^2 = 0.34$, $p < 0.0004$). In addition, a regression model that included FTND score and rs16969968 fitted to cg05575921 methylation was highly significant (Adj. $R^2 = 0.38$, $p < 0.001$) with significant effects of FTND score ($p < 0.0002$) and a trend ($p < 0.06$) for an interaction between FTND and rs16969968 genotype but no main effect of rs16969968 genotype.

One critical question for the field is whether the use of epigenetic biomarkers will be accepted by patients. To examine this question, we conducted a voluntary exit survey of the attitudes of subjects after their sixth visit (Supplemental Table 1). Thirty three subjects agreed to fill out the survey. As a group, the subjects reported a high degree of commitment to smoking cessation with nine indicating more modest commitment and one subject reporting a complete ambivalence to quitting smoking. Supporting prior assertions that dysfunctional patient-provider interactions may interfere with therapy, 10 of the 33 subjects indicated previous discomfort in answering physicians' questions about their smoking habits. Finally, when queried with a 6-point Likert scale as to their interest in receiving data from a test that could inform them on their success in smoking cessation and risk for adverse cardiovascular outcomes, the response to receiving epigenetic feedback was overwhelming positive with all but one subject, indicating moderate to great interest (the average score on 0–5 scale was 4.3) in receiving epigenetic feedback.

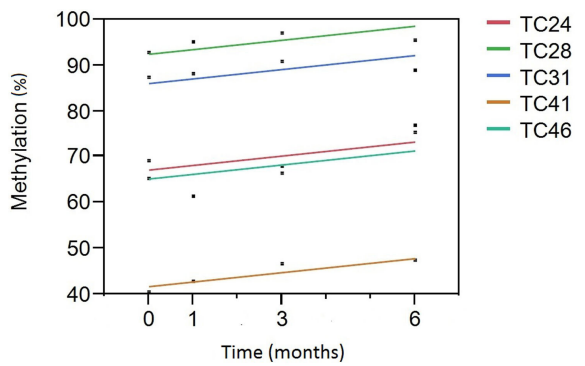

FIGURE 1 | A Plot of cg05575921 methylation as a function of time from smoking cessation intake/quit point. Percent methylation, as indicated by the qPCR assay, is given on the *Y* axis. Time (in months) of the blood draw relative to the inception of the subject into the study and hopefully their efforts to reduce smoking is given on the *X* axis. Each of the subjects had negative cotinine and exhaled CO levels at the 3- and 6-month time points. The linear fit of the reversion curve for each subject is denoted by the color in the figure legend. For example, the best fit line for tobacco cessation (TC) subject 31 (TC31) is shown in blue.

DISCUSSION

Before discussing these results, it is important to note some important limitations of this study funded under a National Institutes of Health pilot mechanism. First of all, the study cohort is small, largely White and drawn from the clinics of a tertiary care hospital. Further examinations using larger numbers of subjects of all ethnicities and more representative treatment settings are required to demonstrate the generalizability of the findings. Second, although the proportion of subjects in this study using pharmacotherapy aid in their smoking cessation efforts is in keeping with that in the general clinical population, in order to most rapidly advance the usefulness of this technique, examination of patient attitudes toward epigenetic biomarkers in state of the art treatment paradigms would be desirable to optimize potential impact of this technology.

Still, if the current results are replicated and extended, the clinical implementation of an epigenetic monitoring tool could have substantial impact on the exorbitant toll that smoking exerts upon the healthcare system. In actual clinical practice, only 13% of physicians routinely refer smokers for treatment with 33% reporting a lack of confidence in their ability to monitor treatment (27). As a result, millions of smoking-induced cases of heart disease, diabetes, and cancer occur that could otherwise be prevented costing hundreds of billions of dollars and untold human misery.

Before this goal can be realized, it will be important to better understand the dynamic relationship of cg05575921 response to smoking. To accomplish this task and avert the potential impact of recall bias in cross-sectional studies, prospective longitudinal studies of subjects as they enter and exit periods of smoking will be necessary to fully understand the response characteristics at AHRR. For example, although for the sake of simplicity, we have modeled the methylation reversion curve as linear, careful scrutiny of the points in **Figure 1** will show that this may be an oversimplification. Indeed, in our unpublished results from a 2013 examination of 19-year-old subjects, there was a trend for an overcorrection or hyper-methylation of cg05575921 to occur after smoking cessation in these young subjects (28). Additionally, it should be clear from the present work and the prior work of several groups that both current and past history of smoking does not fully explain the magnitude of the demethylation response at cg05575921 (17, 25, 29). Therefore, in order to adjust therapy in the first 2 months of smoking cessation therapy, which is the portion most critical to cessation efforts, it is absolutely essential to gather additional data points during this period of treatment and more fully understand the environmental, behavioral, and genetic factors that can influence the rate of cg05575921 change.

This study replicates and extends prior findings showing that self-report is an unreliable method for determining smoking cessation success (7). Our exit study confirms prior data showing that patients often feel uncomfortable when discussing their smoking habits with their physicians. This was borne out in our objective analyses. In our study, 11 subjects reported cessation of smoking at the 6-month time point, but only six had confirmatory serum cotinine and CO levels. Since each of the subjects was compensated for their efforts whether or not they achieved their personal treatment goals, there was no financial incentive for reporting cessation. In fact, as part of the consent process, it was carefully explained to the subjects that we would be checking CO status at each appointment. Since the bogus pipeline effect would predict that conducting CO testing should reduce false reporting (30), the rate of false report in general practice may be even higher.

Developing relatively fool proof methods of detecting smoking and changes in smoking patterns may be particularly important for efforts to increase the success rate of cessation programs by using financial rewards. In controlled trials, these incentive plans can increase the rate of smoking threefold up to 16% (31, 32). If these paradigms could incorporate the most effective currently available pharmacological treatment approach, combined varenicline and nicotine replacement, the rate of quitting could be even higher. However, to optimally achieve the full impact of financial incentives, reliably rewarding cessation early in the course of treatment, including among those ~27% of ex-smokers who remain on nicotine replacement long-term (12), is critical. Unfortunately, because CO monitoring is insensitive to light smoking (9, 10) and the nicotine used in NRT is metabolized to cotinine, the two leading approaches to objectively quantifying cessation are not useful. However, because nicotine itself does not affect cg05575921 methylation status (15, 21) and it is possible to quantify partial responses, the use of DNA methylation assessment could provide a useful yard stick for determining financial reward in contingency-based smoking cessation paradigms.

It is likely that this approach would be acceptable to most patients. In previous work, Hetherington and colleagues showed that the use of CO monitoring feedback was not only accepted by patients but also increased the odds of smoking cessation by fourfold (33). In our post study survey, subjects were extremely receptive to the use of this technology to assess both smoking cessation success and the impact of the success on their personal health outcomes. This positive attitude toward NextGen technology suggests methylation assessments may be a new avenue through which to engage patients in their personalized healthcare. These assessments may include other health outcomes such as the F2RL3 residue referred to as cg03636183, cg05575921 methylation is linked to adverse cardiac and cancer related outcomes (34, 35). Through simultaneously measuring methylation at AHRR as well as a panel of other loci linked to important health outcomes, such as diabetes and obesity (36, 37), it may well be that patients will gain additional motivation to collaborate with their healthcare providers in optimizing their well-being.

The full facilitation of this clinical engagement will not occur in the absence of patient education. Like all humans, patients are less likely to accept what they do not understand. In that respect, the basis of CO monitoring is readily understood because patients understand that tobacco smoke contains CO. By contrast, the fundamental mechanisms by which smoking influences DNA methylation are not well understood by many even in the healthcare community. Furthermore, because DNA methylation technologies may be able to measure a wide variety of outcomes of potential interest to patients, more in-depth analysis of the perceived health care needs of current and potential patients could be beneficial. Therefore, patient engagement and education should be a part in any future clinical approaches.

In summary, in this communication, we show that methylation status at cg05575921 can be employed to track progress in the process of smoking reduction and cessation, and suggest that periodic assessment of changes in methylation and feedback to patients may be useful in facilitating smoking cessation therapy. Future research designed to incorporate the use of this epigenetic tool into treatment is likely to be fruitful.

AUTHOR CONTRIBUTIONS

RP participated in all phases. TO, MG, FG, SB and KW participated in the analysis of data and writing. SE, SW, KV, NH, and EA participated in sample acquisition, processing, and experimentation. They also edited the manuscript. All authors approved the final draft.

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SUPPLEMENTARY MATERIAL

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OPINION
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Regulatory issues surrounding audit of electronic cigarette charge composition

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Clinical Case Triggering Attention to the Issue

A 29-year-old patient who was treated with buprenorphine for 2 years without recurrence attends regular psychiatric controls that include screening for psychoactive substances. During one of the visits, when he was accompanied by his parents, a test for psychoactive substances was positive for opiates. The patient denied that he used illegal substances, and his parents claimed so as well. During this period, the patient lived fairly in isolation, with the members of his family. The only new substance that he used was a new cartridge for e-cigarette. The cartridge was bought at the market in another place, at a pretty low price. Since the father used the same cartridge, he offered to take the test on PAS. The test was positive for opiates (father had never used psychoactive substances in his life). This inspired us to do the following experiment: we poured the content of the cartridge into a glass with water, and the test was again positive for opiates. In this case, neither the outcome of the test of our patient and/or his father nor naive experiment (with the help of immunochromatographic test for the simultaneous qualitative detection of drugs and their metabolites in the urine) or experimental method that is highly dubious was important, but the questions (i.e., more questions) that open after these clinical experiences.

The questions posed are: does any authority regularly control the composition of e-cigarettes, and if so, which one, under what circumstances, and whether it is regulated by law?

Let us start from the known premise: electronic cigarettes (e-cigarettes) are battery-powered devices that allow nicotine intake with chemicals that have different tastes by inhalation, and they are substitutes for smoking ordinary cigarettes. It is known that there are over 250 different brands of e-cigarettes currently on the market. It is estimated that the number of users of e-cigarettes in the world is rapidly growing. In Europe, there are about 7 million users of e-cigarettes. In France, there are about 1.5 million e-cigarette users, while, for example, in the UK, the number of e-cigarette users has tripled since 2012 (from 700,000 to over 2 million) (1).

The tobacco industry is investing a huge sum of money into the development of e-cigarettes, and according to the researchers from HSPH's Center for Global Tobacco Control (CGTC), Department of Social and Behavioral Sciences, it is very important to identify the subpopulation that will probably use them more than others and determine the implications for public health. This research has shown that millions of people – including many young people and smokers who want to stop smoking – try e-cigarettes. This also points to the fact that the importance of determining the potential harm (or benefit) is being underestimated (2). Each e-cigarette contains the following components: batteries (mainly lithium ion) that can be automatic or manual; electronic atomizer spray (responsible for controlling the operation of the device and the release of nicotine vapor during inhalation); and tank in which the liquid for e-cigarette is poured (newer versions have atomizer and tank in one unit, and such a device is called clearomizer). The first e-cigarette appeared on the market in China in 2004, and since then, it is marketed as a healthier alternative to smoking.

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The biggest advantage of e-cigarette is, as stated in the advertisements, that the smoker inhales only a controlled dose of nicotine vapor that fulfills his need for smoking but does not inhale tar, carbon monoxide, remnants of metal, mercury, and many other harmful substances existing in each real cigarette. Also, according to advertising, "it protects and preserves the health of passive smokers, children also."

Are e-Cigarettes Safe?

E-cigarettes contain nicotine and other potentially dangerous substances. Except for nicotine, which is known to be highly addictive substance, other found chemicals, such as formaldehyde and acetaldehyde, are toxic and carcinogenic. In inhaled air from e-cigarette, nanoparticles of metal were found. Health consequences after consuming e-cigarettes are not known (3).

Users of e-cigarettes use cartridges with different concentrations of nicotine (and other substances), and so, potentially, they may be exposed to toxic concentrations of the same. The concentration of nicotine was ranked in the range from 0 to 34 mg/mL, but recent studies show a discrepancy between the indicated concentrations of nicotine on the bottle with filling and measured concentrations (4). E-cigarettes produce aerosol, which contains nicotine, and since that aerosol is heated (temperature depends on the design of the e-cigarette), it affects the aerosolization of the nicotine and its activity (5). Nicotine affects the peripheral nervous system and the central nervous system and represents the primary addictive ingredient with non-nicotine substances, such as anabasine, nornicotine, and acetaldehyde, which also affect the addiction to tobacco. FDA analysis showed the presence of anabasine in several types of e-cigarettes (6). In 20 different models of the e-cigarette, the presence of alkaloids similar to nicotine, such as nornicotine and anabasine, was found (7). Given that e-cigarettes are so different in design and content of nicotine, it makes it difficult to compare and assess the pharmacological properties of content, and therefore, the addictive and toxic potential.

Do Regulatory Authorities Conduct Periodical Audit of Composition of e-Cigarette Cartridges?

Until recently, in most European countries, e-cigarettes were not treated either as a tobacco product or as a medical agent. This means that the only law that controls them is the national Consumer Protection Act that deals exclusively with the technical characteristics of products. Many contemporary laws on tobacco in the EU did not mention e-cigarettes as a tobacco product, and so they were not subject to stricter regulations. European Parliament in February 2014 approved that the products that have nicotine concentrations up to 20 mg/mL can be considered as tobacco products and that those with a higher concentration or used for therapeutic purposes can be considered a medicinal agent (8). However, this decision is left to the member countries themselves. This Directive insists on the health warning, mandatory information regarding the ingredients, and the side effects that package must contain.

Cartridges that can be refilled are allowed if their volume does not exceed 2 mL (or if at least three member countries estimate that they are potentially dangerous to health, they may be prohibited by the European Commission). In the UK, although there were indications that the e-cigarette will be declared a medical agent that is used for smokers who want to stop this habit, this has not happened still. E-cigarettes are treated as a "consumer product." Restrictions relating to the marketing of e-cigarettes have not yet been introduced (9). The US Department of Health and Human Services (FDA) has proposed in April 2014 a set of regulatory rules relating to the control of e-cigarettes. Just e-cigarettes that are used for therapeutic purposes are currently regulated by the FDA (Center for Drug Evaluation and Research – CDER).

According to the FDA reports, voluntary reports of adverse effects of e-cigarettes, which include reports of consumers, medical professionals, and public, are regularly arriving. Adverse events that required even hospitalization described in these reports are pneumonia, congestive heart failure, disorientation, epileptic seizures, hypotension, and other health problems (10). E-cigarettes are manufactured in China and quality control is variable (11). Users can modify many products and even use other substances (e.g., marijuana) via e-cigarette.

State-of-the-Art Clinical Research on e-Cigarettes

A relatively small number of clinical studies investigate the effect of e-cigarettes on health of people. Some of them show that e-cigarettes can "deliver" a similar amount of nicotine as traditional cigarettes (12, 13). Others show that the utilization of nicotine from e-cigarettes depends on the user experience and habits in use (7). Bahl et al. tested for cytotoxicity in 41 e-cigarettes produced by four different companies, using three types of cells: lung fibroblasts, embryonic stem cells (both of human origin), and neural stem cells of a mouse. Cytotoxicity varied among the products from highly toxic to non-toxic. Nicotine did not cause toxicity but other components did. It is important to point out that what was non-toxic to lung fibroblasts was extremely toxic to both types of stem cells (14). The research done by Schober and his associates measuring pollution in the room where three people smoked e-cigarette over a period of 2 h is interesting. The increase in the concentration of nicotine, 1,2-propanediol, aluminum, glycerin, and seven polycyclic aromatic hydrocarbons were classified as probably carcinogenic by the International Agency for research on Cancer (15). Propylene glycol and glycerin are the main ingredients of cigarettes. Exposure to propylene glycol can lead to irritation of the eyes and lungs, and repeated inhalation leads to effects on the CNS, behavior, and results in damage to the spleen (16). The conclusion is that a very small number of studies investigate the direct health effects, but some suggest that aerosols from e-cigarettes have biological effect. Long-term biological effects are still unknown, given that e-cigarettes are a relatively new invention.

Let us go back to the beginning of the story: it is obvious that we do not know the composition of e-cigarettes, even when we think it is known and controlled by the competent regulatory authorities. The health effects of "known" components

of aerosols emitted by e-cigarettes are unknown. There are not enough research/clinical studies on the topic of the impact/risk to human health.

Increasingly, these electronic devices are used for the enjoyment of other PAS. Marijuana is mostly used in liquid form or in the form of wax. It is perfect for users since there is no characteristic odor that occurs when smoking marijuana. This way, unimpeded by police or similar services, illegal drugs can be used or smuggled. This is certainly a new reason to think about.

Concern regarding the quality control and health outcomes is justified. It will be necessary to evolve legal framework to regulate the production and circulation of e-cigarettes and determine their actual effect on health. Anyway, one of the first steps is regulation that will allow finally that e-cigarettes come under the scrutiny of the professional public for its initial market access stage. Thus, in the upcoming years, full clinical potential as well as room to avoid key adverse events would become better known to the consumers and addictologists as well.

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GENERAL COMMENTARY
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The psychobiological problems of continued nicotine dependency in E-cigarette 'vapers'. Commentary: "Electronic Cigarettes"

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Electronic cigarettes were originally introduced as devices to facilitate smoking cessation, but their efficacy in this regard has not been established. Instead, they are often used to facilitate continued cigarette smoking in adults, and are common entry devices for nicotine dependency in children and adolescents (1). The aim of this commentary is to emphasize these problems since far from improving health, E-cigarettes may paradoxically be damaging physical/psychological health. Furthermore, while this is certainly an issue for active smokers, it may become an increasing problem for those who are "passively vaping" E-cigarettes.

E-cigarettes are battery-operated devices, which deliver hits of nicotine via the inhalation of an aerosol spray, which contains a mixture of nicotine, flavorings, and other chemicals (1–3). The E-cigarette was originally introduced as an aid to facilitate smoking cessation. It followed the successful development of earlier nicotine substitution devices, such as nicotine chewing gum, nicotine transdermal patch, and the inhaler (4–6). However, these earlier products were only partially effective at nicotine delivery, with 2 mg gum producing around 25% of the psychophysiological effects of a nicotine cigarette and 4 mg nicotine gum producing around 40% of its physiological effects (7). Yet, despite their comparative inefficacy at nicotine delivery, they partially assuaged nicotine cravings, which allowed them to double the success rates for smoking cessation (4). Crucially, this was achieved *without* making these early nicotine substitution devices attractive to non-smokers.

E-cigarettes were consciously designed to make them attractive, but unfortunately this can now be seen as a mistake since it has encouraged their use as a facilitator for continued smoking in adults, and has introduced many youngsters into nicotine and nicotine dependency. Grana et al. (1) note that they were designed to look like cigarettes; also they were given "kid-friendly flavors, including grape, chocolate, bubble gum, and gummy bear"; furthermore, they have also been marketed "aggressively" often using simplistic and misleading messages. Since first introduced into the USA and Europe in 2006, their usage has risen dramatically, with sales doubling every year [Wells Fargo Internet report cited in Ref. (3)] so that instead of being used to aid tobacco cessation, they are often being used to facilitate continued cigarette smoking. Grana et al. (1) noted that many adult smokers continue to smoke tobacco in private, but vape E-cigarettes where smoking is banned. Furthermore, E-cigarettes are providing a simple introduction into nicotine

TABLE 1 | The adverse effects of nicotine dependency in cigarette smokers, and the similar psychobiological problems predicted for E-cigarette users.

Mood fluctuation: smokers typically experience positive moods on smoking, followed by negative moods on nicotine withdrawal. This mood vacillation is rapid, with moods going up and down every day in parallel with cigarette/nicotine intake (9, 11). Similar types of mood fluctuation are likely to be found in regular E-cigarette "vapers"
Psychophysiological vacillation: the repetitive mood changes of smokers are just one element of a core psychobiological fluctuation. Hence, alertness goes up and down over the day, in parallel with the changes in mood state. Cognitive skills and memory abilities may also fluctuate (13). Similar core psychobiological fluctuations may also develop with E-cigarette users
Addiction potential: psychobiological fluctuation provides an essential rationale for nicotine's strong addiction potential. Similar basic processes underlie the addiction potential of cocaine (10) and other CNS stimulants, such as methamphetamine, MDMA, and mephedrone (8). To the extent that E-cigarettes are effective at nicotine delivery – they will also show addiction potential
Wider psychobiological problems: nicotine dependency in cigarette smokers leads to greater daily stress, depression, cognitive deficits, worse memory, poorer sleep, lower self-efficacy, and many other deficits (12–15). Similar psychobiological problems are predicted to develop in regular uses of E-cigarettes

dependency for children and adolescents. Grana et al. (1) reported that youth use of E-cigarettes had increased from 3.3% in 2011 to 6.8% in 2012. They further noted that around a third of adolescent E-cigarette users have never smoked a tobacco cigarette. Hence, another major concern is the potential progression to higher self-dosing with nicotine – readily achieved by progressing onto cigarette smoking.

Grana et al. (1) noted several of health problems associated with E-cigarettes: the noxious cancerous chemicals in the aerosol vapors, the dangers of passive vaping (viz: as with passive cigarette smoking), and several other problems. However, they did not describe one of the most important problems – that nicotine is highly addictive drug, and that regular nicotine usage has many damaging psychological consequences. In neuropsychobiological terms, nicotine is a powerful CNS stimulant, with many basic similarities to other CNS stimulants, such as cocaine and methamphetamine. Hence, the acute effects of increased heart rate, greater alertness, and mood intensity are similar to every other CNS stimulant drug. All drugs in this class generate brief mood gains, but they are soon followed by negative moods (feeling tired and stressed), during the post-drug recovery period [for review, see Ref. (8)]. With nicotine, this mood fluctuation can be very rapid, with moods fluctuating up and down in parallel with their nicotine intake [viz: every 20–30 min in regular smokers; see Figure 1 in Ref. (9)]. This rapid mood fluctuation helps explain its high addiction potential. Indeed, nicotine is one of the most addictive of all drugs due to its very rapid onset and consequent downturn. Hence, nicotine is similar to cocaine in its psychobiological effects and addiction profile (10). Furthermore, as with every CNS stimulant drug, nicotine can damage the integrity of the HPA axis. Hence, all stimulant drugs can lead to deficits in homeostasis, with disrupted sleep, disrupted circadian rhythms, altered cortisol levels, and other neurohormonal deficits [for review, see Ref. (8)]. Regular nicotine users can also suffer from neurocognitive and other psychobiological deficits, which I have outlined in earlier reviews (11, 12). These included daily mood fluctuation, increased stress, heightened depression, poorer memory, and neuroimaging data indicative of other neurocognitive deficits (6, 13–15). Finally, these psychobiological problems tend to be greater in disadvantaged individuals with a propensity for distress, making nicotine and other stimulant drugs particularly damaging vulnerable individuals (12).

In summary, nicotine is a powerful CNS stimulant with a wide range of adverse effects. Its addictive potential may be widely recognized, but there is far less realization about its damaging psychobiological and health effects – on heart rate, mood stability, alertness, neurocognitive skills, sleep, the HPA axis, cortisol, and stress (12). These psychobiological deficits are likely to be found in regular users of E-cigarettes, even in those who use them alone (i.e., without smoking tobacco cigarettes in parallel; see **Table 1**). The main aim of this commentary is to emphasize that all these core functions, which are crucial for human well-being, need to be empirically studied in E-cigarette users. My core prediction is that these damaging effects of nicotine in cigarette smokers will be replicated in users of E-cigarettes.

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