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ALTERATION OF THE NASAL RESPONSES TO INFLUENZA VIRUS BY TOBACCO SMOKE

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Abstract

Purpose—The purpose of this review is to highlight recent data regarding the impact of exposure to tobacco smoke on influenza virus infection. This is timely because of the continuing pattern for influenza to cause epidemics and pandemics.

Recent findings—Experimental animal studies suggest that tobacco smoke severity of respiratory disease with influenza. The interaction is complex and dependent on dose and chronicity of both virus and smoke exposure. Smoke-induced oxidant stress and suppression of innate immunity are mechanistic factors leading to worse disease. Experiments using human respiratory cells show that tobacco smoke increases viral replication through mechanisms including suppression of antiviral pathways and altered cytokine patterns in cell types with central roles in mucosal innate immunity, such as epithelium, dendritic cells and natural killer cells. Studies also suggest a role for antioxidant strategies in reducing risk. Human volunteer studies using live attenuated influenza virus as a model appear to corroborate many of these findings.

Summary—Exposure to tobacco smoke remains extremely prevalent worldwide. While avoidance of exposure is a primary goal, it is important to understand the mechanisms underlying increased infection risk with tobacco smoke and other pollutant exposures, so that novel preventive or treatment strategies can be developed.

Keywords

Influenza; tobacco smoke; interferon

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Conflicts of interest:
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INTRODUCTION

Influenza virus is a common human respiratory pathogen resulting in annual epidemics and periodic pandemics due to antigenic shift, such as the most recent one in 2009 [1]. Individuals with chronic respiratory and other diseases are at high risk for complications from influenza, but the potential for catastrophic events like the 1918 pandemic makes it important to mitigate risk factors relevant even to healthy populations. The purpose of this brief review is to highlight recent data from our laboratory and others, regarding the impact of exposure to tobacco smoke on influenza infection.

EPIDEMIOLOGY

Exposures to environmental pollutants such as cigarette smoke have been associated with increased susceptibility to influenza infections. Epidemiological studies in the past have shown that smokers are more susceptible to influenza virus infections than non-smokers [2,3], but the mechanisms mediating this effect are not clear. In a 16-year mortality follow-up of nearly 300,000 US veterans, influenza-related mortality (pneumonia and influenza deaths combined) was higher in cigarette smokers than in nonsmokers [4]. A cohort study of female military recruits showed that smoking was a risk factor for severe influenza-like illness during an outbreak of influenza A (H1N1) subtype infection [3]. Similarly, an influenza outbreak in the Israeli army demonstrated that smokers were more susceptible to influenza virus infections than nonsmokers, although influenza-specific antibody levels were not decreased [3,5,6]. A recent retrospective study based on Thailand's National Avian Influenza Surveillance system showed an increased risk of fatal influenza outcomes in current smokers [7]. To summarize, cohort studies indicate that smoking increases susceptibility and severity of influenza infection, and available evidence suggests that the effects of cigarette smoke on susceptibility to influenza infection may involve elements of early or innate host defense against viruses. However, the mechanisms underlying this observation have remained unclear.

EXPERIMENTAL ANIMAL MODELS

In mice, chronic exposure to mainstream cigarette smoke can alter influenza virus-induced primary antiviral and inflammatory responses, but adaptive immune responses, as marked by influenza-specific antibody production, appear to be unaffected. Exposure of C57BL/6 mice for 3-5 months to mainstream tobacco smoke caused suppression of inflammatory responses to low-dose influenza in BALF (neutrophils, mononuclear cells) but no change for levels of cytokines IL-6, TNF-α, or MIP-2 [8]. In contrast, in the case of high-dose influenza, smoke exposure was associated with heightened IL-6 and TNF-α responses. These experiments suggest that the effect of tobacco smoke on host response to influenza is complex and partly dependent on chronicity of exposure and on infection dose. Alveolar macrophages from chronically smoke-exposed mice had reduced LPS-stimulated cytokine production (IL-6, TNF-α, RANTES) as well as reduced nuclear translocation of NF-κB and AP-1 [9]. In another study, mice were exposed to cigarette smoke and then infected with influenza A by intranasal infection. Cigarette smoke exposure inhibited lung T-cell production of IFN-γ during infection with influenza A virus via decreased phosphorylation of transcription factors, and resulted in increased weight loss and mortality [10]. The Nrf2-mediated antioxidant system is essential to protect the lungs from oxidative injury and inflammation. Yageta et al. [11] investigated the role of Nrf2 in protection against influenza virus-induced pulmonary inflammation after cigarette smoke exposure and found induction of antioxidant genes in lungs of wild-type mice but not those of Nrf2-deficient mice after cigarette smoke exposure. Cigarette smoke-exposed Nrf2-deficient mice showed higher rates of mortality than did wild-type mice after infection, with enhanced peribronchial inflammation, lung

permeability damage, and mucus hypersecretion. Lung oxidant levels and NF-κB-mediated inflammatory gene expression in the lungs were also enhanced in Nrf2-deficient mice, indicating that Nrf2-dependent antioxidant pathways are pivotal for protection against influenza-induced pulmonary inflammation and injury under oxidative conditions.

In summary, experimental animal studies suggest that exposure to tobacco smoke increases risk of severe respiratory disease with influenza. The interaction is complex and dependent on dose and chronicity of both virus and smoke exposure. Smoke-induced oxidant stress and suppression of innate (but not acquired) immunity are mechanistic factors leading to risk of worse disease.

EXPERIMENTAL STUDIES WITH HUMAN CELLS *IN VITRO*

The respiratory epithelium is the main target of respiratory viruses and plays an important role during the initial antiviral defense responses. Viral infection normally induces the production of type I interferons (IFN), which in turn activate the synthesis of interferon-stimulated genes (ISGs) and as a consequence limit viral replication [12]. Some experimental studies of the impact of tobacco smoke and oxidant pollutants on human respiratory epithelium *in vitro* have found a stimulatory effect on inflammatory cytokine production [13-18]. However, it is possible that the predominant early producers of IL-6 in respiratory mucosae are resident non-epithelial innate host defense cells. There is evidence in both animal models and human studies for cigarette smoke-induced suppression of some functions of these cell types [8,19-21]. A common pathophysiologic effect of many tobacco smoke components may be to increase cellular oxidant stress, which can alter host defense and virus clearance [22]. Beyond direct effects of tobacco smoke, it is possible that inflammatory responsiveness to subsequent microbial stimuli could be suppressed after prior smoke exposure. Kulkarni et al. [23] recently reported that cigarette smoke extract suppressed IL-8 and IL-6 responses of human and murine respiratory epithelial cells after bacterial stimulation, an effect that was abrogated by antioxidants.

Recent *in vitro* data from our laboratory suggest that tobacco smoke directly inhibits epithelial antiviral pathways. Nasal epithelium in long term differentiated cell culture from smokers responded to influenza infection with greater cytotoxicity, IL-6 release, and viral shedding than cells from non-smokers, effects associated with increased DNA methylation of the interferon regulatory factor 7 (*IRF7*) gene, a key transcriptional factor in the context of a viral infection [16]. Previous studies demonstrated that hypermethylation of *IRF7* results in decreased ability of type I IFNs to induce gene expression [24]. IRF7 induction after influenza was suppressed both *in vitro* in long-term differentiated cultures of nasal epithelium, and in freshly biopsied nasal epithelial cells obtained from smokers after inoculation with LAIV [16] (Figure 1). Expression of IRF7 is critical for amplification of the type I interferon response [25]. Thus, it is conceivable that suppressed epithelial type I IFN signaling contributes to enhanced viral replication in smokers and second-hand smoke (SHS)-exposed individuals. Interestingly, our observations appear to be in contrast to a previous study which demonstrated that mice exposed to cigarette smoke for 2 weeks and subsequently stimulated with poly I:C had increased levels of type I IFNs in bronchoalveolar lavage fluid [26]. Similarly, airway epithelial cells exposed to cigarette smoke condensate and infected with RSV showed greater expression of IRF7 [27].

We also established a model using differentiated nasal epithelial cells (NEC) from nonsmokers and smokers, co-cultured with peripheral blood monocyte-derived dendritic cells (mono-DC) from nonsmokers [28]. We observed that both NEC and mono-DC co-cultured with NEC from smokers exhibited suppressed IRF-7, Toll-like receptor-3, and retinoic acid inducible gene-1 (Figure 2), likely because of suppressed NEC production of

IFNα. Furthermore, NEC/mono-DC co-cultures using NEC from smokers exhibited suppressed concentrations of the T-cell/natural killer (NK) cell chemokine interferon gamma-induced protein 10 (IP-10) after infection with influenza. In contrast, NEC/mono-DC co-cultures using NEC from smokers contained increased influenza-induced concentrations of the Th2 chemokines thymic stromal lymphopoeitin (TSLP) and thymus and activation-regulated chemokine (TARC). Thus, in this model, in the context of influenza infection smokers' NEC create an overall cytokine microenvironment that suppresses the interferon-mediated Th1 response and enhances the TSLP-TARC-mediated Th2 response, with the potential to modify underlying susceptibilities to respiratory viral infections, and the likelihood of acquiring allergic diseases.

Wu et al. [29], using a human lung organ culture model, found that 2-20% cigarette smoke extract (CSE) inhibited influenza-induced IP-10 protein and mRNA expression, IFN-β mRNA, and RIG-I mRNA and protein expression. Inhibition of viral-mediated RIG-I induction by CSE was prevented by the antioxidants N-acetyl-cysteine and glutathione. We also have observed that the induction of Nrf2 via the antioxidants sulforaphane and epigallocatechin gallate (EGCG) inhibits viral entry and replication in cultured respiratory epithelium, and increased expression of RIG-I, IFN-β, and MxA at baseline in the absence of infection [30].

In summary, experiments using human respiratory cells (epithelial and other relevant cell types) *in vitro* generally show that tobacco smoke increases viral replication. Many studies, including our own using human nasal epithelial cells, show that tobacco smoke suppresses antiviral pathways; this may be linked to epigenetic modification of key transcription factors and may be mitigated by antioxidants. Like in animal models, effects of smoke exposure on specific pathways *in vitro* are somewhat model-specific, and our co-culture data demonstrate the potential importance of interactions between epithelial and other mucosal cell types with central roles in innate immunity, such as DC and NK cells.

STUDIES IN HUMAN VOLUNTEERS

Given the model-specific variability and complexity noted above, we sought to develop a protocol for the study of human airway responses to virus in the intact human respiratory tract. Nasal delivery of live attenuated influenza virus (LAIV) vaccine results in transient viral shedding and induces the full range of host immune responses similar to a natural influenza infection without inducing serious adverse effects [31-34] and therefore is a potentially useful tool to study influenza infections in humans *in vivo*. To test how cigarette smoke exposure alters inflammatory and antiviral responses to influenza in the intact human respiratory tract, we serially sampled nasal secretions in an observational cohort study comparing local nasal mucosal responses to LAIV among healthy young adults who are active smokers, nonsmokers exposed to secondhand smoke, and unexposed controls [35]. This was an observational cohort study comparing nasal mucosal responses to LAIV among young adult active smokers (n=17), nonsmokers exposed to secondhand smoke (SHS, n=20), and unexposed controls (n=23). Virus RNA and inflammatory factors were measured in nasal lavage fluids (NLF) serially after LAIV inoculation. The time course of rise and decline of mediators in NLF after LAIV inoculation in normal volunteers is shown in Figure 3. For key endpoints, peak and total (area under curve) responses were compared among groups. Compared with controls, NLF IL-6 responses to LAIV (peak and total expressed as area under curve) were suppressed in smokers (Figure 4A). There were significant associations between urine cotinine and NLF IL-6 responses (negative correlation) or virus RNA in NLF cells (positive correlation) for all subjects combined. Virus RNA in NLF cells was significantly increased in smokers (Figure 4B), as were IP-10/virus ratios.

While it is also possible that smokers shed more viral sequences for reasons unrelated to innate immunity (e.g. altered mucociliary clearance), we hypothesize that increased replication is the mechanism here due to the extended time course for increased virus in smokers and our *in vitro* experience in which replication is enhanced [16]. In general our study's results thus seem to most closely correlate with the inflammatory suppression effects associated with chronic smoke exposure and "low dose" virus in murine models described above. In the context of influenza infections, IL-6 is a key cytokine important for regulating the shift from innate to adaptive components of the antiviral immune responses [36] including proliferation of T cells and influenza-specific T memory cells [37]. Therefore, suppressed induction of IL-6 expression after infection with influenza could have an impact on T cell-dependent adaptive immune responses, though we did not measure T cell responses in the present study. There was also evidence that IFNγ is suppressed in smokers. Nonsmokers exposed regularly to SHS also had suppressed total IL-6 responses after LAIV, and generally appeared to have results which were intermediate between controls and active smokers. Along with a statistically significant correlation between urine cotinine levels and virus quantity, this suggests that the factor(s) responsible for these changes are present in SHS as well as in mainstream cigarette smoke. This observational cohort study thus suggests that either active or second hand exposures to tobacco smoke may have a measureable impact on early innate respiratory mucosal host defense responses to influenza virus.

In a subsequent observational cohort study, we used a similar protocol to characterize non-epithelial immune cells in NLF after LAIV, using flow cytometry [38]. We observed that NK cells were present in NLF and constituted a significant portion of NLF immune cells. NK cell activation during influenza infection is dependent upon secretion of cytokines and chemokines such as RANTES and IP-10 from cells in the respiratory mucosa [39,40] through binding to chemokine receptors CCR5 and CXCR3, respectively. Upon activation by pathogens and inflammatory mediators such as Type I IFNs, IL-12, and IL-15, NK cells can become more cytotoxic as they reduce CD56 expression and acquire CD16 expression [41]. In our study, cytotoxic (CD56+CD16+) NK cells, which release granules containing perforin and granzymes to induce apoptosis in influenza infected cells [42], increased in normal volunteers after LAIV, but these responses were significantly suppressed in smokers (Figure 5). Peripheral blood NK cell activation was unaffected by smoking status. Thus a further mechanism for increased susceptibility of smokers to influenza may be suppression of the normal activation of mucosal cytotoxic NK cells after infection.

SUMMARY AND CONCLUSIONS

As pointed out by van Zyl-Smit et al. [43], at the beginning of the 21st century, we are facing the convergence of several epidemics including tobacco smoking and influenza, and these epidemics interact by way of increasing disease susceptibility and worsening outcomes. Smoking and exposure to second hand tobacco smoke remain extremely prevalent worldwide, and their effects on susceptibility to infection could be shared by other common oxidant particulate pollutants such as biomass burning or diesel exhaust. Exposure to all of these factors is likely to increase with increasing global urbanization. It is therefore important that we begin to understand the mechanisms underlying increased infection risk with tobacco smoke exposure. Data from recent experimental studies point toward suppression by tobacco smoke of several specific antiviral and innate host defense pathways, both epithelial and non-epithelial, at the level of the respiratory mucosa. Studies also suggest a possible role for antioxidant strategies in reducing risk. Further investigation of these mechanisms is warranted due to their far-reaching implications for human health.

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KEY POINTS

1. Exposure to tobacco smoke remains very common, and epidemiologic data indicate that this exposure increases risk for influenza and other respiratory virus infections.
2. Recent experimental data in animal models and in human cell culture suggest that tobacco smoke components can inhibit antiviral pathways and increase susceptibility to influenza, via mechanisms involving oxidant stress.
3. Recent data from studies in human volunteers inoculated with live attenuated influenza virus appear consistent with many of these findings.
4. Additional studies are needed investigating whether antioxidant strategies can enhance innate immunity to influenza and other respiratory viral infections, in populations exposed to air pollutants.

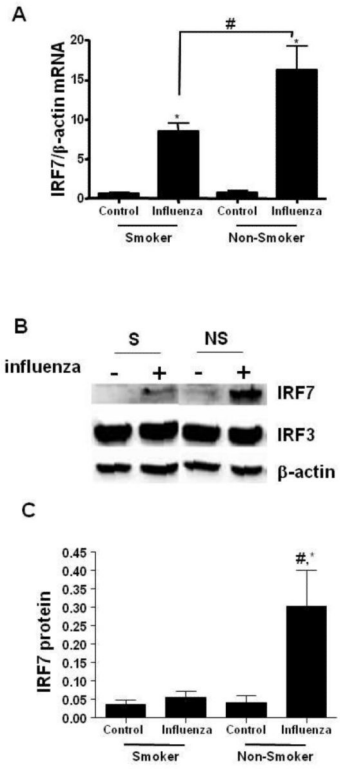

Fig. 1.

Expression of IRF7 in NECs from smokers and nonsmokers. The NECs were infected with influenza A Bangkok/2/79 or left uninfected, and analyzed for IRF7 expression 24 hours after infection. (*A*) Total RNA was analyzed for IRF7 mRNA and normalized to β-actin mRNA concentrations ($n = 16$ smokers; $n = 14$ nonsmokers). (*B*) Whole cell lysates were analyzed for IRF7 and IRF3 protein levels by Western blotting. Membranes were stripped and analyzed for β-actin to assure equal loading. Representative immunoblots are shown. (*C*) Densitometric analysis of IRF7 protein levels ($n = 6$ smokers; $n = 6$ nonsmokers). *Significantly different from noninfected cells ($P < 0.05$). # Significantly different from smokers ($P < 0.05$). Previously published in reference [16].

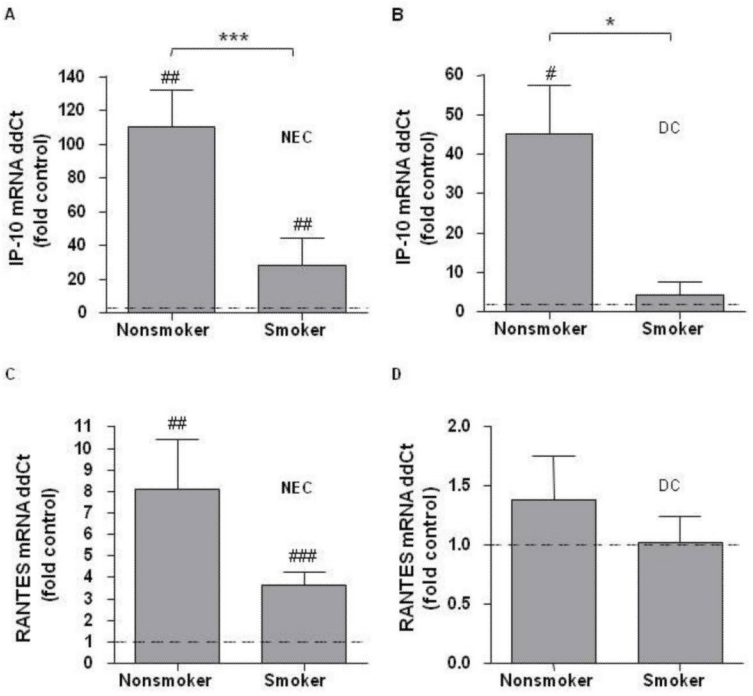

Fig. 2.

Total RNA from nonsmoker and smoker NEC/mono-DC co-cultures was collected 24 h post influenza infection. qRT-PCR (normalized to β-actin and expressed as fold induction over non-infected control) was performed for (A) IP-10 in NEC, (B) IP-10 in mono-DC, (C) RANTES inNEC, and (D) RANTES in mono-DC. Data are expressed as mean ± SEM. Nonsmoker n=5, smoker n=5. #p<0.05, ##p<0.01, ###p<0.001 vs non-infected control, *p<0.05, ***p<0.001 for nonsmoker vs. smoker. Previously published in reference [28].

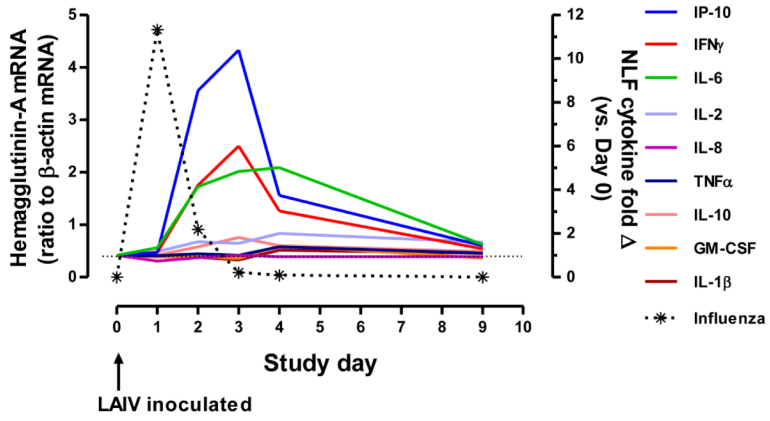

Fig. 3.

Time course of influenza virus measured by qRT-PCR as ratio of influenza type B HA RNA to β-actin mRNA in NLF cells and cytokines in NLF, after inoculation with LAIV in healthy nonsmoking subjects (controls). Cytokine data are shown as fold change (Δ) from day 0 baseline, to illustrate differential responses among the cytokines. All data points are shown as median for study day. IP-10, IL-6, and IFNγ but not other cytokines showed statistically significant increases (days 2-4) compared with day 0. Previously published in reference [35].

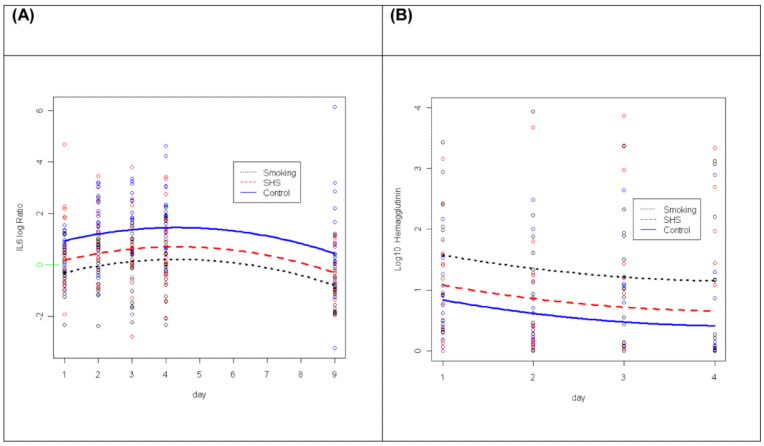

Fig. 4.

(A) Mixed-effects model curves for influenza B virus quantity (hemagglutinin RNA normalized to β-actin RNA) and (B) for IL-6 in NLF from Controls (blue circles and solid line), SHS-exposed nonsmokers (red circles and broken line), and active Smokers (black circles and dotted line). In the linear mixed model fitting, the response variable is the log-transformed fold change from the baseline. Covariates include time, time^2, group and their interactions. Not previously published.

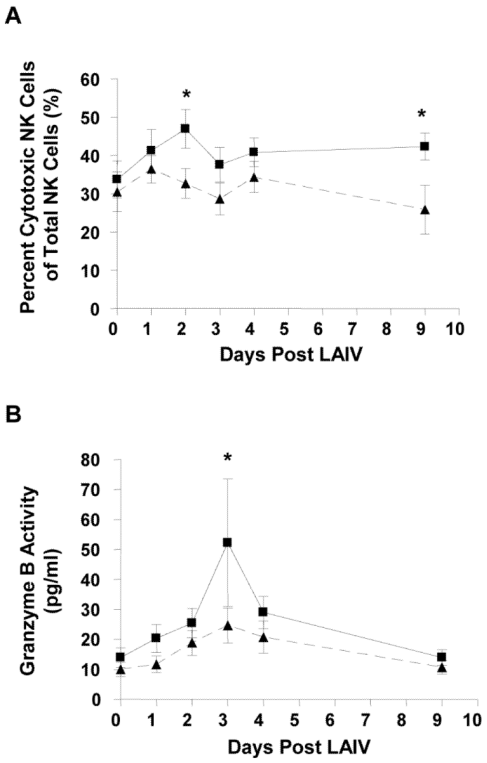

Fig. 5.

Cytotoxic NK cell percentages and activity were analyzed in NLF of nonsmokers and smokers after LAIV inoculation. A) $CD56^{+}CD16^{+}$ cytotoxic NK cell percentages of total NK cells were decreased in the NLF of smokers following LAIV. Kruskal-Wallis p=0.09, *p<0.05 nonsmoker vs smoker posttest. Nonsmokers n=12 (■, solid line), smokers n=9 (▲, dashed line). B) Granzyme B activity was decreased in NLF of smokers following LAIV inoculation. Kruskal-Wallis p<0.01, *p<0.05 nonsmoker vs smoker posttest. Nonsmokers n=13 (■, solid line), smokers n=13 (▲, dashed line). Previously published in reference [38].