Cytokine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Inflammatory profile analysis reveals differences in cytokine expression between smokers, moist snuff users, and dual users compared to non-tobacco consumers

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ARTICLE INFO

Keywords:
Biomarkers
Inflammation
Cytokines
Tobacco use

ABSTRACT

Objective and design: The aim of this study is to investigate the inflammatory alterations due to the use of smokeless tobacco and dual use of smokeless tobacco and cigarettes, relative to smoking.
Subjects: Plasma and saliva samples were collected from healthy smokers (SMK-100 subjects), moist snuff users (MSC-89 subjects), the dual users (DUSMK-49 subjects), and non-tobacco consumers (NTC-99 subjects) from two cross-sectional studies.
Methods: Luminex Human InflammationMAP® 1.0 panel, a multiplex immunoassay.
Results: SMK and DUSMK exhibited larger number of alterations in the expression of inflammatory analytes compared to NTC. Eight analytes were significantly elevated ($p \leq .05$) within plasma samples of SMK compared to NTC, while one 1 analyte was elevated between the MSC and NTC groups. DUSMK exhibited different levels of 11 analytes, relative to NTC.

MSC displayed fewer alterations in inflammatory protein expression compared to smoker groups, and the inflammatory profile of MSC resembles NTC. Five analytes (ICAM-1, VEGF, MMP-9, ferritin and fibrinogen) emerged as potential biomarkers distinguishing tobacco consumers ($p < .02$).
Conclusions: We identified a set of five proteins as potential biomarkers that can inform of inflammation status due to tobacco usage. Our findings contribute a better understanding of how the use of different tobacco products contributes to inflammation.

1. Introduction

Cigarette smoke is a complex aerosol that consists of over 8000 chemicals [1], including 93 harmful and potentially harmful constituents as designated by the FDA [2]. Smoking has been causally related to several diseases, primarily those affecting the pulmonary and cardiovascular systems including cancer, coronary heart disease, and chronic obstructive pulmonary disease (COPD) [3]. The effects of cigarette smoking on human health have been extensively investigated at the organ, cellular, and molecular levels. Mechanistically, cigarette smoking has been linked to perturbations in many molecular pathways, including oxidative stress and immune response [4]. Oxidative moieties generated during cigarette combustion lead to lipid and DNA damage, and to the activation of lung epithelial cells and macrophages [5,6]. Activation of immune cells leads to secretion of inflammatory mediators, promotion of systemic inflammation, and elevation in the number of circulating leukocytes in chronic smokers [4]. Changes in the levels of inflammatory mediators, including the cytokines TNF-α and IL-6, and acute-phase proteins (APPs) such as C-reactive protein (CRP) and fibrinogen, have been documented in cigarette smokers [4]. These and other inflammatory markers have been identified as potential biomarkers of tobacco effect (BioEff) [7–10].

While cigarette smoking remains as the most common form of tobacco use in the United States, smokeless tobacco products (STPs), such as moist snuff and snus, are also marketed [11]. In contrast to cigarette smoking, oral STPs do not generate toxicants associated with the combustion process and are associated with reduced risk of adverse health effects compared to smoking [12]. It is now well recognized that

Abbreviations: AAT, alpha 1 antitrypsin; APP, acute-phase proteins; BDNF, brain derived neurotrophic factor; BioEff, biomarkers of tobacco effect; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; DUSMK, dual users of moist snuff and cigarettes; FRTN, ferritin; LLOQ, lower limit of quantitation; MMP-9, matrix metalloproteinase-9; MSC, moist snuff consumers; NK, natural killer cells; NTC, non-tobacco consumers; RJRT, R.J. Reynolds Tobacco Company; SMK, smokers; STPs, smokeless tobacco products; TSNA, tobacco-specific nitrosamines; VEGF, vascular endothelial growth factor

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https://doi.org/10.1016/j.cyto.2017.11.013
Received 28 September 2017; Received in revised form 16 November 2017; Accepted 18 November 2017

a risk continuum is associated with different tobacco product classes. While combustible cigarette smoking is the most harmful and the non-use of any tobacco is the least harmful option, STPs are associated with significantly reduced risk relative to cigarettes and non-combustible tobacco products may present less risk than cigarette smoking [13,14]. Epidemiological data from USA and Sweden indicate that the consumption of moist snuff and snus is associated with reduced risk of cancer, COPD, and cardiovascular diseases [15–19]. Additionally, cross-sectional studies have demonstrated that across several biological pathways, STP users generally experience fewer perturbations than smokers, notwithstanding higher exposure to nicotine and tobacco specific nitrosamines [9]. These and other studies have led to the identification of several potential BioEff, which could aid in predicting the health effects associated with the use of different tobacco product classes [10,20].

Studies on the dual use of cigarettes and smokeless tobacco products are limited, and it is unclear where dual users fall on the risk continuum of tobacco use. It has been suggested that dual use may result in a reduction in smoking-related harm due to generally reduced cigarette consumption with dual users [21,22]. With over 10% of adults in the United States using multiple tobacco products [23], more studies on the health risks and systemic effects associated with the consumption of multiple tobacco products are necessary.

Since cigarette smoking induces a state of chronic inflammation, we investigated inflammatory protein profiles in plasma and saliva samples from smokers (SMK), STP users (moist snuff consumers [MSC]), dual-users of combustible cigarettes and moist snuff (DUSMK), and non-tobacco consumers (NTC) recruited into two cross-sectional studies. The Human InflammationMAP® 1.0 panel consisted of a large number of inflammatory proteins, which have been reported to be altered in smokers. Hence, this panel was chosen for investigating inflammation changes in smokers and STP users. The inflammatory profile data was compared among the different groups of tobacco consumers and NTC. This data will help in understanding the effects of these tobacco products on the inflammatory/immune responses and assist in understanding the comparative risks between combustible cigarettes and non-combustible tobacco products. Our results indicate that inflammatory cytokine levels are higher among combustible tobacco consumers relative to STP users and NTC, and five analytes emerged as potential BioEff.

2. Materials and methods

2.1. Study design

The clinical conduct of the two studies presented herein was approved by Independent Investigational Review Board, Inc. (currently Schulman Associates IRB, Inc., Fort Lauderdale, FL). Study and subject materials (inclusive of protocol, informed consent forms, recruitment materials, subject instructions) were IRB reviewed and approved; the first study on 18 March 2010 and the second study on 03 November 2009. Both studies were conducted in accordance with the International Conference on Harmonisation Good Clinical Practice [24] and the Declaration of Helsinki [25]. In both studies, written informed consent was obtained from subjects prior to any study procedures being performed. Subjects were free to withdraw from the study for any reason and at any time, and were compensated for their time and travel.

The first study, termed “Biomarker Discovery Study,” with Clinical Trials Registry identifier number NCT01923402, was a single-site, cross sectional study of male tobacco-product consumers and a male non-tobacco-consuming control group, and was previously described [9,10]. Briefly, a total of 120 generally healthy male subjects, aged 35–60 years were enrolled into one of three groups (40 subjects per group completed the study): exclusive long-term SMK, exclusive MSC, and NTC.

The second study was a multi-site, cross sectional study of regular users of different types of tobacco products. In this study, there were three groups that were consistent with the tobacco user groups in the first study. Those groups and a fourth group of DUSMK are presented herein. This multi-site study enrolled 220 male and female subjects aged 19–73 years into the following groups: SMK MSC, DUSMK, and NTC (Supplementary Table 1). A total of 217 subjects across the four cohorts completed the study: SMK = 60, MSC = 49, DUSMK = 49, and NTC = 59. The objectives and the design of this multi-site study were described previously at a scientific conference.[1]

For both studies plasma and un-stimulated saliva samples were collected at clinical research units and shipped to a central laboratory per the study protocols. Samples were collected from the study subjects who fasted overnight from food (all groups) and tobacco (all tobacco consumer groups). The samples were shipped to Rules-Based Medicine (RBM, now Myriad RBM; Austin, TX) for cytokine profile measurements.

2.2. Cytokine profiling

Myriad RBM performed cytokine profile measurements using the Human InflammationMAP® 1.0 panel from Luminex Corporation. Briefly, this multiplex microsphere-based assay allows for the interrogation of 45 analytes simultaneously in a capture-sandwich format. In this assay, 5 µL of diluted capture-antibody microspheres with 5 µL blocker and 10 µL of either standard, pre-diluted sample, or control were incubated at room temperature in a hard-bottom microtiter plate for 1 h. Following incubation, 10 µL of biotinylated detection antibody was added to each well, mixed thoroughly and incubated for 1 h. The reaction contents were added to a pre-wet filter-membrane microtiter plate, the supernatant was aspirated, and the contents were washed twice with 100 µL wash buffer. After the final wash, the beads were suspended in 100 µL of wash buffer and the plate was analyzed on the Luminex platform.

2.3. Statistical analysis

Results for the plasma and saliva measures were sent from Myriad RBM to SciWit Inc. (Boulder, CO) for data analysis. For analytes where the reported value fell below the lower assay limit of quantitation (LLOQ) as defined by Myriad RBM, the entry was recorded as “LOW.” For analytes where < 50% of the entries were LOW, the LLOQ for that analyte was divided by 2 and this value replaced the “LOW” entry for the analyte. Analytes where ≥50% of the entries were LOW, were removed from further analysis. Mean and standard deviations of the analytes were calculated for each group and comparisons between cohort groups were completed using data transformed via the column range method. For this method, the transformed final value equals the original value minus the analyte minimum value divided by the analyte range, yielding a value between 0 and 1 for each analyte. Analysis of variance (ANOVA) using the un-pooled variance method was carried out to compare each of the groups to each other using the transformed data. All of the analytes identified at the $p < .05$ level of significance are described herein.

3. Results

3.1. Smokers exhibit markedly higher inflammatory protein expression compared to non-tobacco consumers

The general status of inflammation was assessed in tobacco consumers by profiling the levels of 45 inflammatory analytes in plasma and saliva samples collected from two cross-sectional studies conducted

[1] Caraway JW et al. (2014) Assessment of Exposure Biomarkers in U.S. Consumers of Snus, Moist Snuff, Cigarettes, and Dual Use. Meeting Presentation at 68th Tobacco Science Research Conference, Charlottesville, VA.