

ORIGINAL ARTICLE

The effect of a non-tobacco-based nicotine pouch on mucosal lesions caused by Swedish smokeless tobacco (snus)

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Abstract

Oral mucosal lesions are commonly found in Swedish smokeless tobacco (snus) users where the pouch is placed. These lesions are reversible, that is, clinical and histological tissue changes return to normal following cessation. However, the exact mechanisms behind these changes are unknown. The main aim of this study was to investigate how snus-like non-tobacco-based nicotine pouches affect the oral mucosa and the severity of pre-existing lesions. Sixty regular users of Swedish smokeless tobacco were encouraged to substitute their snus with non-tobacco-based nicotine pouch products during a 6-week period. Meanwhile, oral mucosal lesions were assessed using a four-degree scale. Over time, a reduction of pre-existing mucosal lesions was observed between baseline and the final visit. In a second part, the effect of exposure to regular snus on the production of 48 different cytokines in peripheral blood mononuclear cells was compared in vitro with that resulting from exposure to the non-tobacco-based nicotine products. Results showed significantly increased production of proinflammatory cytokines in cells exposed to regular snus compared to untreated or cells exposed to the non-tobacco-based nicotine products. This may be related to the improved clinical appearance of the oral mucosa in the participants that used the non-tobacco-based nicotine test pouches.

KEYWORDS

cytokines, inflammation, nicotine pouch, oral mucosa, smokeless tobacco

INTRODUCTION

Smokers frequently exhibit oral mucosal lesions which can be located anywhere in the oral mucosa, such as leukoedema, smoker's palate, smoker's melanosis, lingua villosa/nigra, leukoplakia and erythroplakia [1–3]. It is generally assumed that the main reason for these lesions is the smoker's exposure to the combustion products in tobacco smoke, most of

which are found in the tar particles in the smoke [3–5]. Although similar findings have not been associated with smokeless tobacco use, regular Swedish smokeless tobacco (further referred to as snus) users may particularly develop mucosal lesions in the sulcus at the site at which the snus pinch/pouch is most frequently placed [6, 7].

The biology of these lesions is clearly different from that of most of the mucosal lesions associated with smoking: they are

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strictly localised to the site of exposure in the sulcus, they are reversible within weeks after the cessation of exposure or if the snus user changes the location of the snus pouch and they do not appear to be pre-malignant [8]. The exact mechanisms behind these lesions remain unclear. It has been suggested that the high pH of snus could result in a localised, chemical irritation of the mucosa. However, the observation that snus lesions are much less prevalent among users of pouched snus compared with loose snus suggests that physical irritation by the tobacco particles in snus may also play a role. The nicotine in snus may be another significant factor. For instance, mucosal lesions have been observed with lozenges used during pharmaceutical nicotine replacement therapy. A recent study assessed the oral safety of a sublingual tablet containing 2 mg nicotine with regard to lesions at the site of application [9]. In a prospective follow-up of smokers using sublingual nicotine tablets for 3–6 months, eight of 30 participants displayed lesions in the floor of the mouth during the 6-month medication period, all of which appeared in the first 1–6 weeks. By the 6-month visit, all of these lesions had resolved [9].

Since our knowledge of the side-effects of tobacco today is higher than ever before, there is also an increased desire to refrain from use due to the reported adverse health effects. Instead, tobacco-free nicotine pouches have become a new trend. One of the new non-tobacco-based nicotine products (ZYN pouches) are an alternative form of orally delivered nicotine. The physical properties of these products in terms of pH are the same as those of regular tobacco-based snus and the product is used in the same way, that is, it is placed in the sulcus for 30–60 min. However, the matrix for the nicotine in these products is different from that of regular snus. Maltitol and cellulose are used as fillers instead of ground tobacco leaves. Because of their different properties, it is unclear if, or to what extent the non-tobacco-based nicotine product usage causes mucosal lesions similar to those produced by regular, tobacco-based snus. The comparable pH and the nicotine delivery may indicate similar potential, but the absence of tobacco particles and the difference in size may result in less physical irritation.

In an older study by Axéll et al. [7], biopsies from mucosal lesions caused by snus were examined and a mild clinical inflammatory response and an increased number of various leucocytes were reported. Inflammation is induced to remove harmful stimuli, including damaged cells, irritants or pathogens, and to start the healing process. When foreign compounds come in contact with white blood cells, it may result in the production of various cytokines with different effects on the surrounding tissues. Cytokines have specific effects on the interactions and communications between cells. Some cytokines have the ability to trigger inflammation, while others have the opposite effect and act to dampen the inflammatory response [10]. Previous studies have been

conducted on other products (electronic cigarettes) as an alternative to conventional combustible cigarette smoke and their effect on cytokine production [11]. However, little is known about the cytokine profile in human cells after exposure to smokeless tobacco. A better understanding of this could perhaps explain the mechanisms behind oral lesions caused by snus.

The main aim of this study was to investigate how the oral mucosa was affected if users substituted their regular Swedish snus with non-tobacco-based nicotine products (investigational products). Specifically, we were interested to see if some of the pre-existing oral lesions might improve or resolve during a 6-week observation period. The second aim was to compare the effect of the non-tobacco-based nicotine products to the effect of regular tobacco-containing snus products on the production of proinflammatory cytokines in mononuclear leucocytes *in vitro*.

As previously mentioned, the physical properties of the investigational non-tobacco-based nicotine products in terms of pH and nicotine delivery are the same as those of regular, tobacco-based snus and, in the same way, this product is placed in the upper sulcus for approximately 1 h. Because of these circumstances, the extent to which the use of the investigational non-tobacco-based nicotine pouch may cause mucosal lesions similar to those produced by regular, tobacco-based snus is unclear. Our hypothesis is that the comparable pH and the nicotine delivery may indicate similar potential, but the absence of tobacco particles and the smaller size of the pouch may result in less physical irritation in the oral mucosa and the lower production of proinflammatory cytokines from human mononuclear leucocytes *in vitro*.

MATERIAL AND METHODS

This paper is part of a larger study in which the effect on biofilm properties (unpublished findings) has also been evaluated and it is divided into a clinical part and an *in vitro* part.

Clinical part

Volunteers and informed consent

Healthy adults ($n = 60$) were recruited by an advertisement (looking for healthy regular Swedish snus users) in a local newspaper. Following a telephone interview to evaluate eligibility, potential participants were invited to a screening visit. Potential participants then submitted a health declaration, which was checked by the responsible investigator. In order to be included in the study, participants had to be snus users with a minimum weekly consumption of three

or more snus cans (brands with nicotine content <1%) or two or more cans (brands with nicotine content <1%) or two or more cans (brands with nicotine content >1%) for more than 1 year, be healthy and aged ≥ 19 years, have a negative pregnancy test, and a normal stimulated salivary secretion rate (>0.7 ml/min). Reasons for exclusion were a history or presence of diagnosed hypertension or any cardiovascular disease, previous surgery within 6 months of the screening visit that, in the opinion of the investigator (PL), could negatively impact the subject's participation in the clinical study, any medical condition, which, in the judgement of the investigator (PL), might interfere with the absorption, distribution, metabolism or excretion of nicotine, pregnancy, allergy to composite materials and antibiotic use during or within the last 4 weeks prior to study start. Eligible participants were given written and verbal information about the study's objectives, and signed informed consent was obtained from each participant.

Demographics and other baseline characteristics

A total of 60 participants, 21 females and 39 males, were included in the study. The participants had a mean age of 31 years (SD 10 years) and a normal stimulated salivary secretion rate (mean 2.3 [SD 1.0] ml/min) at the screening. All female participants had a negative pregnancy test at screening.

Restrictions during the study

The participants had to refrain from approximal tooth cleaning for 72 h prior to each visit and toothbrushing during the last 48 h prior to the visit. They did not eat or drink anything during the last 2 h prior to the visit. Study participants were not allowed to participate in any other clinical study during the study period.

Treatments

The subjects were recommended to replace as many as possible of their regular snus products (ideally all) with the investigational non-tobacco-based nicotine pouch (ZYN; Swedish Match) during the 6 weeks and to use unlimited amounts of this product. It was entirely at the discretion of the participant when he/she used the pouch during the day and how many pouches a day that were used. At each clinical visit the participants received enough products for 14-day use (until the next visit) and the number of products and regular snus used by the subjects was recorded in a paper diary. Participants were able to choose among 3 or 6 mg pouches of three different flavour variants, smooth, peppermint or cinnamon.

Clinical visits were scheduled at baseline (screening), after 2 weeks (visit 1), after 4 weeks (visit 2) and 6 weeks (visit 3) and on each visit the participants were given new products for use.

Examination of the oral cavity

A clinical examination of the oral cavity was performed at baseline and on the follow-up visits. The oral mucosa was inspected, and any clinical changes were recorded and classified. Tooth status (DMFT) and the presence or absence of gingival retractions was recorded at the start of the study (screening/baseline).

Lesions in the mucosa at the site of placement of the pouch were scored according to a four-grade clinical scale. The clinical examination of the oral mucosa at the site of pouch placement was performed according to Axéll et al. [7], and scored in one of the following categories: degree 1 – superficial lesion with a colour similar to the surrounding mucosa and with slight wrinkling but no visible mucosal thickening; degree 2 – superficial, whitish or yellowish lesion with wrinkling but no visible thickening; degree 3 – whitish-yellowish to brown, wrinkled lesion with intervening furrows of normal mucosal colours and obvious thickening and degree 4 – marked white-yellowish to brown and heavily wrinkled lesion with intervening deep reddened furrows and/or heavy thickening. Lesions clinically suspected as oral leucoplakias or erythroplakias were followed up according to standard clinical routines including assessment of specialised dentist in oral medicine. The oral mucosa of the site of snus placement was documented on every visit by photography taken by an iPhone. The participants were asked to remove their upper lip with their own fingers.

Local symptoms reported by the subject were recorded on each visit, by using open-ended general questions. Any adverse events and their duration which were reported spontaneously were also recorded.

Data quality assurance

The study was performed in compliance with good clinical practice, applicable regulations and Clinical Trial Consultants Standard Operating Procedures (www.ctc-ab.se).

Before the inclusion of the participants in the study, a study initiation visit was made at the research clinic by a sponsor representative in order to inform and train relevant study staff. The investigator was then responsible for providing appropriate study-related training to new staff and for forwarding any further information of relevance to the performance of this study to the team involved.

Determination of sample size

A 6-week observation period was considered reasonable to assess putative changes in the oral mucosa resulting from the use of the non-tobacco-based nicotine pouches, given that lesions caused due to snus usage among habitual snus users regress within a few weeks after the cessation of exposure [9]. With an estimated dropout rate of 25%, a total of 45 fully evaluable participants were expected with a total inclusion of 60 participants.

Ethical approval and regulatory requirements

The study was performed in accordance with ethical principles that have their origin in the Declaration of Helsinki [12] and are consistent with the International Conference of Harmonization/GCP, European Union Clinical Trials Directive [13] and applicable local regulatory requirements.

The study did not start until approval of the clinical study protocol, the subject information and the informed consent forms had been obtained from the by the Research Ethics Committee of Gothenburg University (Dnr 778-17).

In vitro part

In order to compare the effect of the non-tobacco-based nicotine products to the effect of regular tobacco-containing snus products on the production of proinflammatory cytokines, human mononuclear leucocytes were exposed to the different products in vitro.

Ethical approval

For the in vitro experiments, the peripheral blood cells that were obtained from the Sahlgrenska University Hospital blood bank were de-identified and, according to the Swedish legislation section code 4§ 3 p SFS2003:460, no informed consent was needed.

Isolation of mononuclear cells from human blood

Blood cells from healthy blood donors ($n = 11$) were obtained from Sahlgrenska University Hospital in Gothenburg, Sweden. Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood using a hydrophilic polysaccharide (Ficoll-Paque), followed by centrifugation, which separated blood into layers so that different cell types could be purified by a gradient. The top layer contained plasma,

followed by a layer of PBMCs, while the bottom fraction consisted of neutrophils, eosinophils, and erythrocytes.

The PBMCs were resuspended in phosphate-buffered saline (PBS; Sigma), centrifuged and then resuspended in Dulbecco's Modified Eagle's Medium (D-MEM; Invitrogen) supplemented with 5% heat-inactivated human serum type AB (Sigma–Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cell viability was determined by staining with 0.4% trypan blue (Sigma–Aldrich) and counting the cells using a Bürker chamber.

Supernatants were prepared from the non-tobacco-based nicotine pouches with different flavours (cinnamon, smooth, peppermint 3 or 6 mg), and from four regular tobacco-based, Swedish snus products (Kaliber Plus vit stark, Granit original white, Göteborgs Rapé original large, and Grov portion) that were most commonly used by the participants before the start of the study. The tobacco-free Lyft Ice Cool, that is a similar product to ZYN, was also included. Firstly, the pouches of snus/the tobacco-free products were cut open. Approximately 1 g of every product was weighed and diluted in medium to the desired concentration. Samples were incubated for 2 h at 37°C in the presence of 5% CO₂. After incubation, the samples/supernatants were strained into new tubes using BD Falcon cell strainers and stored at –20°C for later use.

The isolated PBMCs were exposed to supernatants containing 0.025 g/ml of the different products for 24 h at 37°C. Lipopolysaccharides 250 ng/ml (serotype O127:B8; Sigma–Aldrich) was added as a positive control.

Multiplex cytokine assay

The multiplex assay was performed according to the manufacturer's guidelines. In brief, colour-coded beads coupled with antibodies directed at the desired biomarker, for example, IL-6, were used. The sample was added, and the beads reacted with the biomarker of interest that was present in the sample. After a series of treatments with detergents to remove unbound proteins, a biotinylated detection antibody was added in order to create a sandwich complex. The final detection complex was formed when streptavidin–phycoerythrin (fluorescent indicator or reporter) conjugate was added to bind to the biotinylated antibody. The samples were analysed using a BioPlex 200 instrument equipped with BioManager analysis software (BioRad, Hercules).

Expression level assessment

Hierarchical Clustering Explorer software (<http://www.cs.umd.edu/hcil/multi-cluster/>) was used to create a heat map of the expression levels of selected cytokines produced.

The median value was calculated for each cytokine and the values were normalised and transformed using the Hierarchical Clustering Explorer software into colour codes representing higher, intermediate, and lower expression levels of each cytokine.

Statistics

The results from the self-reported usage of the non-tobacco-based products and the DMFT are presented in terms of number (*n*), mean, and standard deviation (SD). The statistical comparisons of paired samples were made using the Wilcoxon matched-pairs signed-rank test for the cytokine production and scoring of oral lesions. GraphPad Prism Software (GraphPad Software) was used to create parts of the artwork and analyses.

RESULTS

Safety results

The administration of nicotine-containing pouches was safe and well tolerated by the healthy participants. The events assessed by the investigator as related to the investigational products (the tested non-tobacco-based nicotine products) were nausea (two events of mild intensity), dry mouth (mild), gingival blisters (moderate), and dizziness (mild). There were no serious adverse events or discontinuations due to adverse events during the study.

Self-reported usage of the non-tobacco-based nicotine products

The number of products and regular snus used by the 57 participants who completed the study was recorded in a paper diary. The self-reported usage of the non-tobacco-based nicotine products is presented as mean (SD) as the ratio the non-tobacco-based nicotine products/total nicotine products.

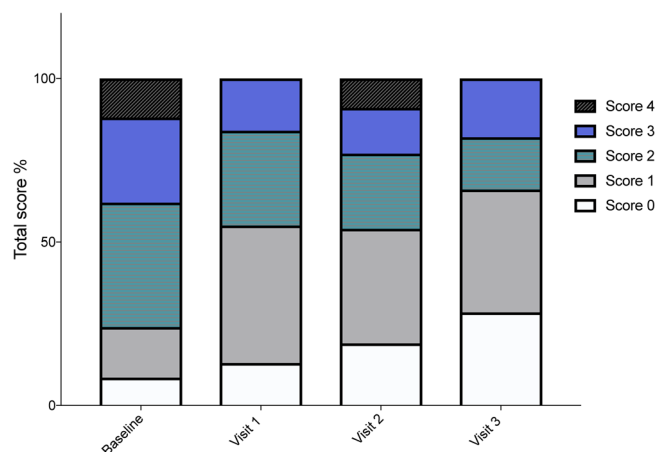


FIGURE 1 The clinical scoring of the lesions presented as percentage for each visit. Score 0 is presented as the white part of each bar, the grey color demonstrates score 1, green demonstrates score 2, blue demonstrates score 3, and black demonstrates score 4

During the time period the ratio from baseline to visit 1 was 0.717 (0.219), from visit 1 to visit 2 was 0.809 (0.178) and from visit 2 to visit 3 was 0.840 (0.165).

DMFT and gingival retraction

DMFT was recorded at the beginning of the study, where the calculated mean (SD) was 3.7 (4.6). The clinical examination of the oral cavity was performed at baseline on visits 1, 2, and 3 and gingival retractions were recorded on each visit. There was no indication of changes in the incidence of gingival retraction during the study; the presence of gingival retraction varied between 54% and 57% during the study.

Snus-induced mucosal lesions

The number of participants with lesions and the clinical scoring of the lesions changed during the study. The percentage of participants with no lesions increased from 9% at baseline to 30% on visit 3. The number of participants with lesions of

TABLE 1 Prevalence and progression of mucosal lesion score at baseline and after 6 weeks

Mucosal lesion score at baseline	Mucosal lesion score at last visit (Week 6)					Total at baseline
	Score 0	Score 1	Score 2	Score 3	Score 4	
Score 0	4	1	0	0	0	5
Score 1	3	4	1	1	0	9
Score 2	8	8	2	3	0	21
Score 3	1	7	5	2	0	15
Score 4	1	1	1	4	0	7
Total at the end of study (week 6)	17	21	9	10	0	57

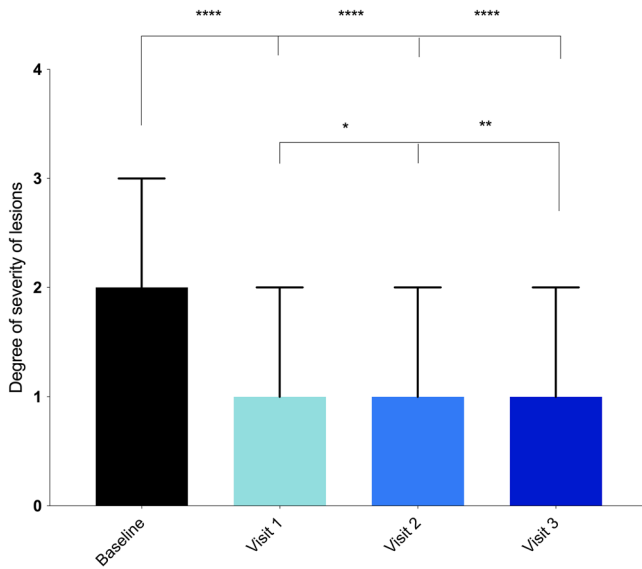


FIGURE 2 Degree of the severity of lesions in the mucosa at the placement of the pouch between visit 1, 2, and 3 compared to baseline/screening was scored according to a four-grade clinical scale by Axéll et al. Statistical analysis was carried out using Wilcoxon matched-pairs signed-rank test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ and **** $p < 0.001$

degrees 3 and 4 decreased, while the number of participants with lesions of degrees 1 and 2 increased (Figure 1). In total, the number of participants with pre-existing mucosal lesions at the placement of the pouch decreased from 90% to 70%. A comparison of the scoring between the degree of lesions at the beginning (baseline) and the end of the study (visit 3) is presented in Table 1.

There was a statistically significant decrease in the scoring of the lesions for the subgroups at the placement of the pouch between visits 1 ($p < 0.0001$), 2 ($p < 0.0001$), and 3 ($p < 0.0001$) compared with baseline (Figure 2).

The scoring of the lesions at visits 1, 2, and 3 in comparison to the usage of non-tobacco-based nicotine pouch are presented in Figure 3. There was a statistically significant correlation between the improvement in pre-existing oral mucosal lesions and the percentage of investigational non-tobacco-based nicotine pouch used for all three visits compared to baseline. The percentage use of the investigational non-tobacco-based nicotine pouch versus standard Swedish snus was calculated from the report of usage at each visit (the percentage use was calculated as the number of test products divided by the total number of pouches used). As seen in Figure 3C, six participants had an enhanced lesion at the last visit (visit 3) compared to baseline. Clinical photographs showing improvement in the oral mucosa for three of the participants ($n = 3$) are presented in Figure 4.

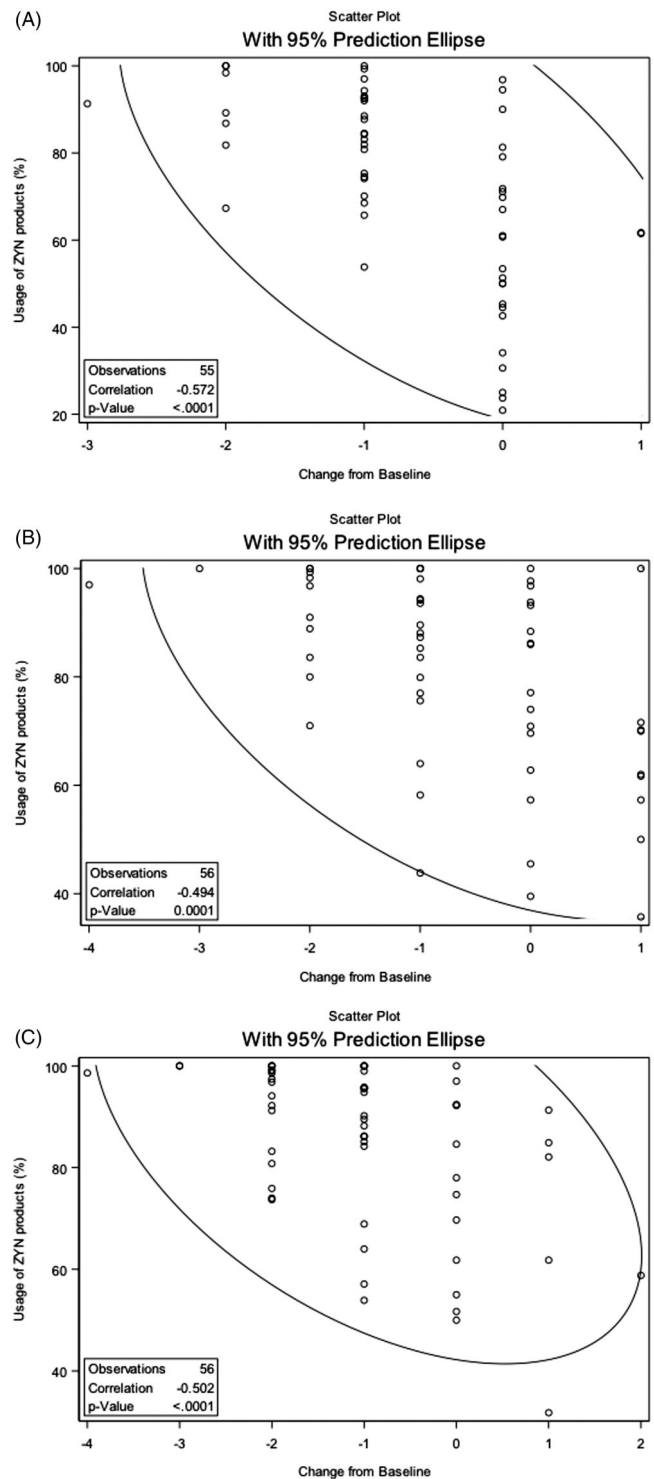


FIGURE 3 The correlation at each visit using the change in oral mucosa and the percent usage of new products vs. ordinary the participants ordinary snus was calculated at each visit: (A) visit 1, (B) visit 2, and (C) visit 3 (the percentage usage was calculated as the number of test products divided by the total number of pouches used) and was compared to baseline



FIGURE 4 Clinical photographs from three participants taken at the start of the study (baseline) and the end of the study (visit 3). The lesions in the oral mucosa at the site of placement of the pouch was scored according to a four-grade clinical scale

In vitro findings

The cytokine concentrations in the human PBMC cultures exposed to the different products are presented in a heat map (Figure 5), where only those with values that were higher than the lowest value of the standard control used for the assay, are shown.

All four tested snus products and the tobacco-free Lyft showed a significant increase in the production of proinflammatory cytokines IL-1 β , IL-6, IL-18, TNF- α , and MIP-1 α from human PBMCs compared with unstimulated cells ($p < 0.05$) (Figure 6). However, the investigational non-tobacco-based nicotine products showed a reduction in the production of the proinflammatory cytokines in comparison to the unstimulated cells and the regular snus. In some cases, the production of the proinflammatory cytokines was slightly increased after exposure to investigational non-tobacco-based nicotine products (flavour Smooth) compared with the other investigational non-tobacco-based nicotine pouch items (Figure 6).

DISCUSSION

The findings of this study demonstrate that the almost complete substitution of snus with a non-tobacco-based nicotine

product resulted in the gradual resolution of pre-existing oral mucosal lesions in healthy snus users over a 6-week period. Furthermore, findings showed that the investigational non-tobacco-based nicotine products do not elicit an inflammatory response in vitro.

The oral mucosa is, in many ways, similar to the skin and acts as a barrier against the external harmful environment. However, since only certain parts of the oral mucosa is covered by keratin, the oral mucosa is more permeable than skin. This is partly the reason why some areas in the mouth are especially sensitive to irritants because it is easy for the irritants to penetrate at these highly vascularised sites [14]. A multitude of different types of interactions are possible between a mixture of chemicals and associated extrinsic factors in the oral mucosa. The interactions are classified as irritative or allergenic in nature and the pathology typically include inflammation in the mucosa [15]. Major factors involved in the potential development of irritation include the amount of exposure, the irritation potential of the agent, the ability of the agent to penetrate the tissue and the reactivity of the subject [15]. Irritation that leads to oral mucosal alterations is a common event that can be caused by different types of exposure to the oral cavity. Most irritation in the oral cavity tends to reverse quickly when the causative agent is removed. Previous research has indicated that, due to its pH, different particles of the product and the content of

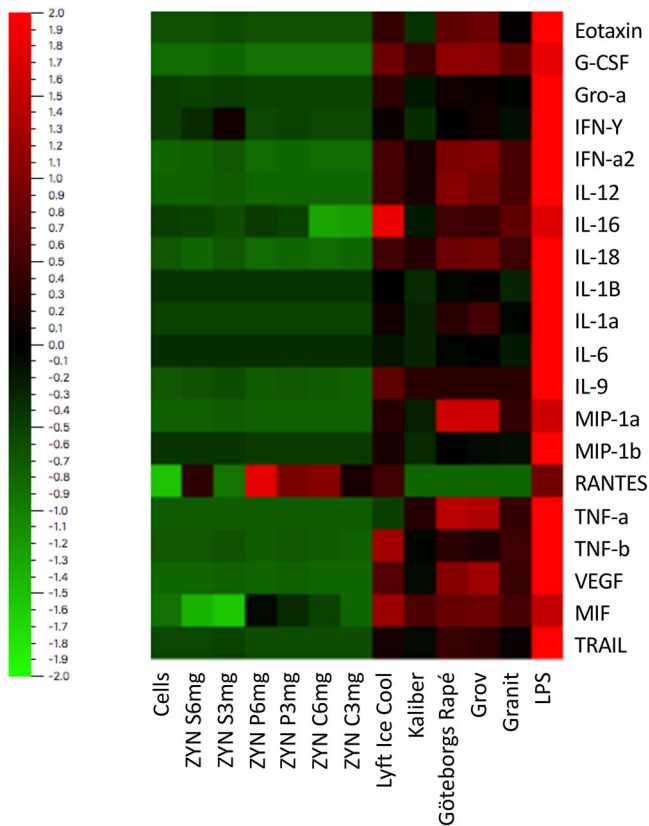


FIGURE 5 Heat map of cytokine levels in culture supernatants of human peripheral blood mononuclear cells (PBMCs) ($n = 11$) exposed to 0.025 g/ml the investigational non-tobacco-based nicotine pouch (ZYN) smooth, peppermint, cinnamon (6 or 3 mg), Lyft Ice Cool, Kaliber, Göteborgs Rapé, Grov, Granit or LPS. The cytokine levels in the culture supernatants were measured with a multiplexed bead-based cytokine immunoassay. The median value was calculated for each cytokine and the values were normalised and transformed using Hierarchical Clustering Explorer into colour codes representing higher (red), intermediate (black), and lower (green) expression of each cytokine

nicotine, the snus may cause irritation to the oral mucosa, thereby causing alterations in the mucosa, that is, oral lesions [16–18].

In this study, a four-point severity scale for subgrouping oral lesions caused by snuff, suggested by Axéll et al. [7], was used. The present results showed an improvement in the oral lesions when the participants replaced their normal snus with the investigational non-tobacco-based nicotine products. There was a statistically significant correlation between the improved change in the pre-existing oral mucosal lesions and the percentage of investigational non-tobacco-based nicotine products used. Six participants displayed an enhancement in the severity of oral lesions at the placement of the pouch at the last visit compared to baseline. However, whether this is due to the increased usage of the investigational non-tobacco-based nicotine products or their own regular snus products is unknown. The participants were able to change to the non-

tobacco-based nicotine pouch as they pleased. It is therefore difficult to identify the amount of product that needs to be used to improve oral lesions.

In a previous study by Andersson et al. [19] a relatively consistent pattern of tissue changes was recorded, indicating that a relationship frequently exists between the clinical grading of the lesions that have been used by Axéll et al. [7] and the histological changes. They were able to demonstrate histological inflammation at the lesion in snus users diagnosed with a clinical degree 4 lesion. When inflammation occurs, different white blood cells are involved. They release various substances that cause inflammation. Since many different cells are present in the oral cavity (in the pulp, gingival cervical fluid, epithelium, etc. [20, 21]), a variety of cells may encounter different substances leaked from the snus thus leading to inflammation studied by Axéll et al. [7].

While there are reports showing a link between the consumption of smokeless tobacco and mucosal inflammation [19, 22], its specific effects on the human immune system have not been determined. Previous in vitro studies performed on macrophages and monocytes exposed to smokeless tobacco have reported elevated levels of the proinflammatory mediators TNF- α , IL-1 β and prostaglandin E2 [22, 23]. Among other reactants, these mediators participate in lymphocyte proliferation, epidermal inflammation and epithelial proliferation [24, 25]. According to a previous study by Seydroudbari and Khan [22], the oral mucosal inflammation elicited by smokeless tobacco that is characterised histologically by leucocyte infiltration may be due to the production of the proinflammatory cytokines IL-1 β and TNF- α . This is in agreement with another study that demonstrates increased levels of IL-1 in smokeless tobacco lesions [26].

In order to acquire a better understanding of the clinical manifestations of the mucosal lesions, more knowledge of the cellular/immunological response to the products in question is required. One approach to measuring the effect on the immune system is to study the effect of cytokines in vitro. In the present study, the production of proinflammatory cytokines produced by mononuclear cells in vitro, after exposure to the investigational non-tobacco-based nicotine products, was compared with exposure to regular tobacco-containing snus products and one tobacco-free nicotine pouch. Regular snus exposure resulted in a significant increase in the production of proinflammatory cytokines in vitro. The cells exposed to the investigational non-tobacco-based nicotine products did not produce cytokines to the same extent as the cells exposed to various types of snus and the tobacco-free nicotine product. This may be related to the improved clinical appearance of the oral mucosa in the participants that used the investigational non-tobacco-based nicotine test articles. The alterations in the inflammatory mediators seen after exposure to the regular snus products and the tobacco-free Lyft could have important implications in the development of oral lesions and

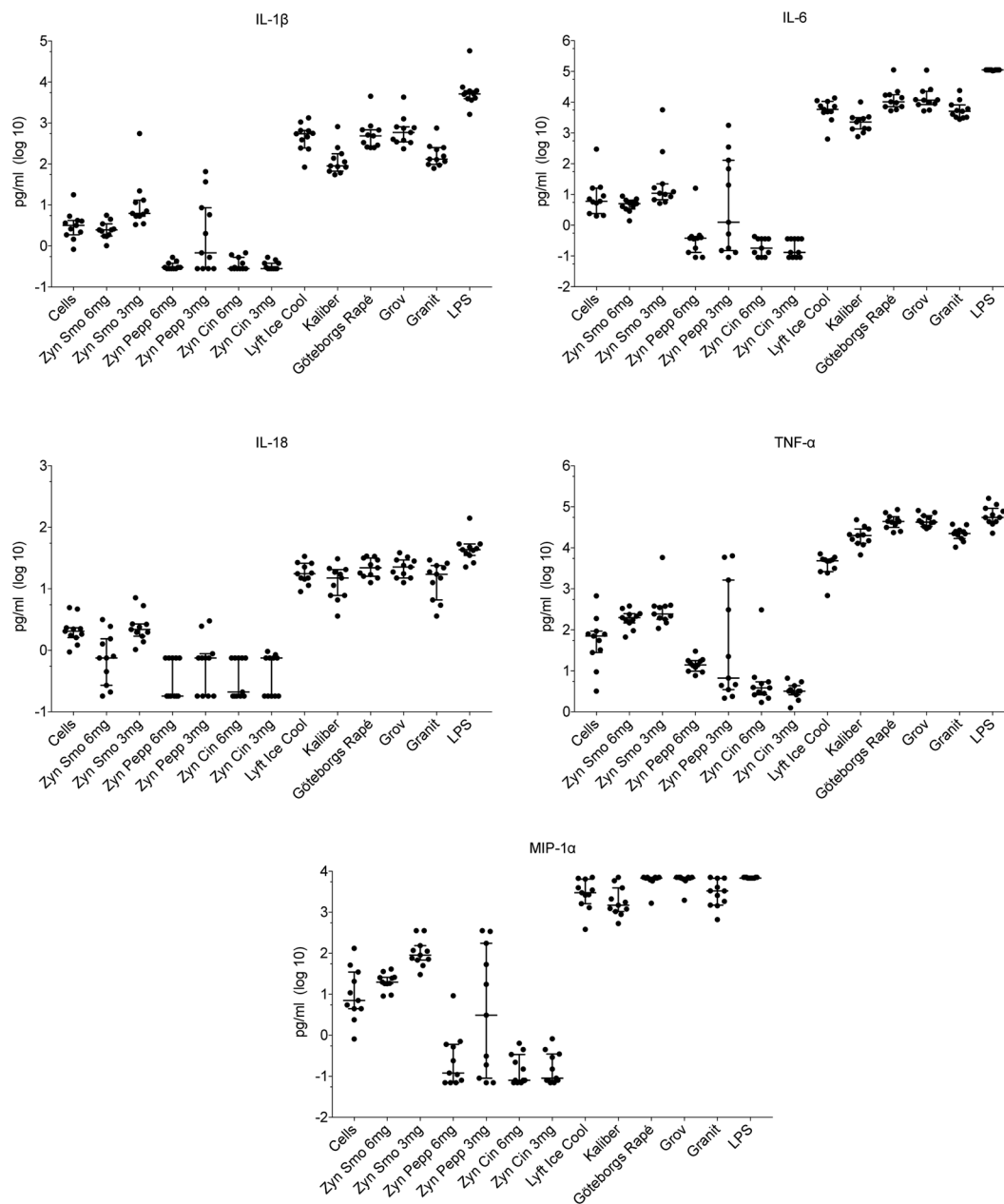


FIGURE 6 Cytokine levels in culture supernatants of human peripheral blood mononuclear cells (PBMCs) ($n = 11$) exposed to 0.025 g/ml investigational non-tobacco-based nicotine pouches (ZYN) smooth, peppermint, cinnamon (6 or 3 mg), Lyft Ice Cool, Kaliber, Göteborgs Rapé, Grov, Granit or LPS. The levels of the proinflammatory cytokines IL-1 β , IL-6, IL-18, TNF- α and MIP-1 α in the culture supernatants were measured using a multiplexed bead-based cytokine immunoassay

also explain the inflammation observed at the lesion site. However, great care should be exercised when extrapolating the conclusions from the *in vitro* part of the study to the clinical part, and further studies, particularly histopathological studies, need to be performed in order to confirm these findings. An interesting aspect of these results is the fact that the size of the pouch/snus was thought to have a significant role in the development of the oral lesions and the observed local inflammatory response in the beginning of the study. However, the results in the *in vitro* study imply that this may not

be the case since the different products (snus and tobacco-free nicotine products) were cut open and the same amount product was used to expose the PBMCs. Regardless of this, there was still a significant difference in the inflammatory effect observed after exposure to regular snus, the tobacco free nicotine product and the investigational non-tobacco-based nicotine products.

The main limitation of the present study is the fact that it is difficult to identify a specific constituent that could have elicited alterations in the cytokine profile, since the chemical

composition of snus is very complex [27, 28]. It would be of interest for future studies to characterise a specific component (for example, flavour) of the products included in the present study. One specially interesting finding in the present study is the fact that, even though Lyft Ice Cool is a tobacco-free product similar to the investigational non-tobacco-based nicotine products, it seemed to have a higher impact on the production of proinflammatory cytokines compared to the investigational non-tobacco-based nicotine products. Whether this is due to its flavour (Ice Cool), or another constituent is not known. Another interesting consideration for the future is to include a dose-response curve for the cytotoxicity of these materials. Further, it would be interesting to perform a similar study in which a biopsy is taken at the beginning of the study and compared with a biopsy taken at the end of the study in order to identify the presence of immune cells and inflammatory markers at the sight of the active lesion.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS


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