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Impact of Smoking on Salivary Lipid Profile and Oxidative Stress in Young Adults: A Comparative Analysis between Traditional Cigarettes, E-Cigarettes, and Heat-Not-Burn Products

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 Sara Zięba
ABCD 2 Agnieszka Błachnio-Zabielska
ABCDE 3 Mateusz Maciejczyk
BCD 2 Karolina Pogodzińska
ABDF 4 Mariusz Szuta
ACD 5 Giuseppe Lo Giudice
BCD 6 Roberto Lo Giudice
ABCDEFG 1,7 Anna Zalewska

1 Independent Laboratory of Experimental Dentistry, Medical University of Białystok, Białystok, Poland
2 Department of Hygiene, Epidemiology and Metabolic Disorders, Medical University of Białystok, Białystok, Poland
3 Department of Hygiene, Epidemiology and Ergonomics, Medical University of Białystok, Białystok, Poland
4 Department of Oral Surgery, Jagiellonian University Medical College, Cracow, Poland
5 Department of Biomedical and Dental Sciences and Morphofunctional Imaging, Messina University, Messina, Italy
6 Department of Human Pathology of the Adult and Evolutive Age. G. Barresi, Messina University, Messina, Italy
7 Department of Restorative Dentistry, Medical University of Białystok, Białystok, Poland

Corresponding Author: Sara Zięba, e-mail: sarap.zieba@gmail.com
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Background: Smoking nicotine is considered to be one of the most harmful addictions, leading to the development of a number of health complications, including many pathologies in the oral cavity. The aim of this study was to examine the effect of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on profiles of salivary lipids and lipid peroxidation products in the unstimulated and stimulated saliva of healthy young adults with a smoking habit of up to 3 years.

Material/Methods: We enrolled 3 groups of 25 smoking patients each and a control group matched for age, gender, and oral status. In saliva collected from patients from the study groups and participants from the control group, the concentrations of sphingolipids: sphingosine, sphinganine, sphingosine-1-phosphate, ceramides, and salivary lipid peroxidation products – malondialdehyde (MDA) and 4-hydroxynonenal (HNE) – were measured. The normality of distribution was assessed using the Shapiro-Wilk test. For comparison of the results, one-way analysis of variance (ANOVA) followed by post hoc Tukey test was used.

Results: We demonstrated that each type of smoking causes a decrease in the concentration of salivary lipids, and there was an increased concentration of salivary MDA and 4-HNE.

Conclusions: Smoking in the initial period of addiction leads to an increase in the concentration of lipid peroxidation products through increased oxidative stress, leading to disturbance of the lipid balance of the oral cavity (eg, due to damage to cell membranes).

Keywords: Cigarette Smoking • Electronic Nicotine Delivery Systems • Lipid Peroxidation • Lipids • Oxidative Stress • Salivary Glands

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Background

The negative effects of smoking tobacco and e-cigarette vaping are primarily evaluated in terms of causes of development of cardiovascular diseases, lung cancer, respiratory chronic inflammatory diseases, or disorders of the gastrointestinal microbiota [1-3]. However, other tissues also succumb to toxic effects of nicotine contained in commonly available nicotine carriers, including the oral cavity and upper respiratory tract [4,5].

The oral cavity is the place of first contact with cigarette smoke in the human body. Evidence has shown that smoking is a risk factor in the development and progression of periodontal diseases, cancer, and precancerous conditions of the oral cavity area, as well as salivary gland dysfunction and disorders of saliva composition [6-9].

It is well known that smoking traditional cigarettes leads to redox imbalance. We can observe increased production of free radicals, which can cause damage to cell membranes or DNA. It has been demonstrated that long-term smoking leads to a decrease in the activity of endogenous salivary enzymatic antioxidants such as SOD, CAT, and Px, and significantly reduces the efficiency of non-enzymatic endo- and exo-antioxidant systems: GSH, UA, and vitamin C [6,10,11]. Similarly, e-cigarettes can induce oxidative stress and increase the expression of advanced glycation end products (AGEs) and their cellular receptors (RAGEs) in gingival and periodontal tissues within just 1 year of starting smoking [12-14]. Furthermore, in an in vitro study, Ganapathy et al [13] showed that a 14-day exposure of cells to e-cigarette aerosol extracts increases DNA damage in oral epithelial cells, which is expressed by increased concentrations of 8-oxo-dG levels. Long-term smoking of traditional cigarettes and e-cigarettes reduces the content of salivary components of specific and non-specific immunity, such as sIgA, peroxidase, lactoferrin, and lysozyme [6,15,16].

Moreover, saliva contains a wide variety of lipids, including cholesterol and its esters, fatty acids, triglycerides, wax esters, and polar lipids such as phosphatidylcholine, phosphatidylethanolamine, sulfides, and glycolipids, including ceramides [15,16]. Ceramides are composed of sphingosine linked by an amide bond to any fatty acid. The most common ceramides are C14: 0-Cer, C16: 0-Cer, C18: 1-Cer, C18: 0-Cer, C20: 0-Cer, C22: 0-Cer, C24: 1-Cer, and C24: 0-Cer. These lipids form cell membranes and are also precursors of more complex sphingolipids, such as sphingomyelin, ceramide-1 phosphate, and glycerosphingolipids. In addition to their structural function, ceramides determine the process of cell differentiation, proliferation, and apoptosis, and regulate the process of protein phosphorylation, which is essential in signal transduction [16,17]. Sphingolipids, on the other hand, show antimicrobial and antiviral activity in a dose-dependent manner, and induce cellular damage. Pretreatment of cells with sphingosine prevents the viral spike protein of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) from interacting with host cell receptors and inhibits the propagation of herpes simplex virus type 1 (HSV-1) in macrophages [18]. Cigarette smoke strongly activates inflammatory pathways in lungs and in myocardial and skeletal muscle cells, which increases biosynthesis of ceramide and its derivatives in these tissues [3,19,20]. High concentration of this group of lipids in response to exposure to cigarette smoke has been linked to endothelial barrier dysfunction, emphysema, inflammation, and altered myocardial mitochondrial function [21,22]. Lipidomic profiling of sputum samples showed increased levels of 28 ceramides in long-term smokers with COPD (chronic obstructive pulmonary disease) compared to long-term smokers without COPD. Differences between smokers without COPD and people who have never smoked cigarettes revealed significant changes only in the level of salivary glycosphingolipids. Interestingly, disorders in plasma sphingolipid composition were observed only in smokers of traditional cigarettes, while subjects using e-cigarettes only showed dysregulation of tricarboxylic acid cycle-related metabolites [22].

Lipids perform many important functions in the oral cavity, from structural to functional. In addition to their key role in maintaining the integrity and function of cells, they affect the processes of digestion, protection, and communication, as well as maintaining the internal balance in the oral cavity [17]. Lipids contained in saliva help to moisturize and protect the mucous membranes, facilitating eating, speaking, and other functions of the oral cavity. In the oral cavity, lipids can form a thin protective layer on the surface of the teeth and mucous membranes, which helps protect against the effects of irritants and infectious substances and prevents excessive evaporation of water from tissue surfaces [17,23].

Considering the role of saliva and its lipids in maintaining oral homeostasis, we decided to evaluate the effect of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on the concentration of selected sphingolipids (eg, sphingosine, sphinganine, and sphingosine-1-phosphate), ceramides, and the lipid peroxidation products 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) in unstimulated and stimulated saliva from healthy young adults who had been smoking for 1-3 years.

Material and Methods

Approval for the study was obtained from the Bioethics Committee in Białystok (permission number: APK.002.343.2020). Each patient signed a written consent to participate in the study, and could ask questions or withdraw any time during the experiment.

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Subjects

A group of 75 smokers was enrolled in the study group. Smokers were divided into 4 subgroups according to the type of the smoking: Group 1 was traditional cigarette smokers (n=25), Group 2 was e-cigarette smokers (n=25), and Group 3 was heated tobacco device smokers (n=25). Each patient in the study group had been smoking for 1-3 years and used only 1 of the 3 methods of delivering nicotine to the body. Participants smoked on average about 10 cigarettes a day. The control group consisted of non-smokers (n=25) matched by age and gender to the subjects from the study group. The study participants were under continuous care of the Department of Restorative Dentistry at the Medical University of Białystok, reporting regularly for follow-up visits. The number of subjects was determined according to our previous study, assuming power of the test=0.8 (α=0.05) using Fisher's formula [24]. All study subjects were young adults, under the age of 30 years, in generally good health (no chronic diseases of any kind), without any oral inflammatory lesions, with a normal BMI (within the range of 18.5-25), drinking alcohol only occasionally, and not taking psychoactive drugs. At that time, participants in the study were not using fixed orthodontic appliances or retainers, Invisalign splints, did not have removable dentures, fixed restorations, implants, or titanium implants. The subjects had not taken medicines, vitamins, or other dietary supplements within 6 months before the study. Their diet was typical, consisting of 70% carbohydrates, 20% proteins, and 10% fats.

Saliva Collection and Dental Examination

Saliva was collected by an experienced person (S. Z.) at a prior dental examination, including assessment of DMFT (decayed, missing, and filled teeth), GI (gingival index), and PPD (periodontal pocket depth). The examination was performed under artificial lighting, using a mirror, an explorer, and a periodontal probe (WHO, 621). The examiner was previously calibrated, and 20 patients were randomly examined by another dentist (A. Z.). Interrater agreement for DMFT was r=1.0, for GI: r=0.96, for PPD: r=0.9. The tested material consisted of unstimulated and stimulated saliva, collected via the spitting method between 8 and 10 a.m. Before collection of the diagnostic material, patients were instructed not to smoke or consume food or beverages other than water and not to perform any oral hygiene procedures at least 2 hours before the visit. To avoid patients' embarrassment, saliva was collected in a separate room, in a sitting position, with the head slightly inclined downwards, with minimal movement of the face and lips. Before spitting unstimulated saliva into a plastic centrifuge tube, patients rinsed their mouths 3 times with room-temperature water. Saliva collected within the first minute was discarded. Unstimulated saliva was then collected for 15 minutes into a calibrated tube. Stimulated saliva was gathered in a similar manner for 5 minutes, during which 20 µl of citric acid was spotted on the dorsal surface of the patient's tongue every 30 seconds. Prior to centrifugation, the volume of the spat secretion was measured (with a calibrated pipette) and the rate of saliva secretion was determined by dividing the volume of saliva in the tube by the time required to obtain it. The saliva was centrifuged for 20 minutes at 4°C, 10000×g, then the fluid was collected from above the sediment, frozen at -84°C, and stored until assays were performed, but no longer than 4 months.

Lipids Analysis

The concentration of sphingolipids (sphingosine (Sph), sphinganine (SPA), sphingosine-1-phosphate (S1P) and ceramides (C14: 0-Cer, C16: 0-Cer, C18: 1-Cer, C18: 0-Cer, C20: 0-Cer, C22: 0-Cer, C24: 1-Cer, C24: 0-Cer) in saliva was measured according to the method described by Blachnio-Zabielska et al via ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS), with minor modification [21]. Briefly, an internal standard mixture (Sph-d7, SPA-d7, S1P-d7, C15: 0-d7-Cer, C16: 0-d7-Cer, C18: 1-d7-Cer, C18: 0-d7-Cer, 17C20: 0-Cer, C24: 1-d7-Cer and C24-d7-Cer) (Avanti Polar Lipids, Alabaster, Al, USA) and an extraction mixture (isopropanol: ethyl acetate, 15: 85; v/v) (Merck, Saint Louis, MO, USA) were added to each sample (100 µL of saliva). Samples were then vortexed, sonicated, and centrifuged (5 minutes at 3000 g, 4°C). The supernatants were transferred to new vials and the pellets were re-extracted. Both supernatants were combined and evaporated under a nitrogen stream and reconstituted in solvent B (2 mM ammonium formate (Sigma-Aldrich, Saint Louis, MO, USA), 0.1% formic acid (Honeywell Fluka, Morris Township, NJ, USA) in methanol (Merck, Saint Louis, MO, USA)). Sphingolipids were analyzed with a Sciex QTRAP 6500 + triple quadrupole mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany) using a positive ion electrospray ionization (ESI) source (except for S1P, which was analyzed in the negative mode) with multiple reaction monitoring (MRM) against standard curves constructed for each compound. The chromatographic separation was performed on a reverse-phase Zorbax SB-C8 column 2.1×150 mm, 1.8 µm (Agilent Technologies, Santa Clara, CA, USA) in binary gradient using 1 mM ammonium formate (Sigma-Aldrich, Saint Louis, MO, USA), 0.1% formic acid (Honeywell Fluka, Morris Township, NJ, USA) in water (Merck, Saint Louis, MO, USA) as solvent A, 2 mM ammonium formate (Sigma-Aldrich, Saint Louis, MO, USA) and 0.1% formic acid (Honeywell Fluka, Morris Township, NJ, USA) in methanol (Merck, Saint Louis, MO, USA) as solvent B at the flow rate of 0.4 mL/min. To acquire and process the data, we used Analyst (Software version 1.7., AB Sciex Germany GmbH, Darmstadt, Germany) and Sciex OS-Q (AB Sciex Germany GmbH, Darmstadt, Germany).

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Table 1. Clinical and stomatological characteristics of patients and control group participants.

	Non-smokers, *n*=25	Traditional smokers, *n*=25	E-cigarette smokers, *n*=25	Heat-not-burn products smokers, *n*=25	p
Age (years)	24.7±2.4	25.3±3.1	23.4±3.2	23.7±1.9	NS
BMI (kg/m^2)	20.6±1.7	21.9±1.8	21.2±1.2	20.8±1.9	NS
Duration of addiction (years)	–	2.1±0.3	2.3±0.4	2.2±0.3	NS
US (mL/min)	0.68±0.1	0.62±0.1	0.65±0.1	0.69±0.1	NS
SWS (mL/min)	0.91±0.02	0.93±0.01	0.91±0.01	0.9±0.02	NS
DMFT	17±0.23	18±0.32	17±0.31	18±0.28	NS
API	24.56±0.36	21.54±0.31	21.54±0.27	21.54±0.31	NS
PBI	0.36±0.1	0.35±0.12	0.34±0.1	0.34±0.1	NS
PPD (mm)	2.0±0.5	2.0±0.5	2.0±0.5	2.0±0.5	NS

BMI – body mass index; UWS – unstimulated whole saliva; DMFT – Decayed, Missing, Filled Teeth; API – approximal plaque index; PBI – papilla bleeding index; PPD – periodontal pocket depth; NS – statistically insignificant; SWS – stimulated whole saliva.

Oxidative Damage Assays

MDA concentration was assayed colorimetrically using the thiobarbituric acid reactive substances (TBARS) method with 1,3,3,3-tetraethoxypropane (Sigma-Aldrich, Saint Louis, MO, USA) as a standard [25]. The absorbance was measured at 535 nm with microplate reader ELx800 and Gen5 2.01 software (BioTek Instruments, Winooski, VT, USA).

4-HNE concentrations was measured using a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cell Biolabs, Inc., San Diego, CA, USA, and USCN Life Science). The absorbance was measured at 405 nm with microplate reader ELx800 and Gen5 2.01 software (BioTek Instruments, Winooski, VT, USA).

Statistical Analyses

GraphPad Prism 8.3.0 for MacOS (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Normality of distribution was assessed using the Shapiro-Wilk test. For comparison of the quantitative variables, one-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used. The statistical significance level was established at $P<0.05$

Results

Clinical and Stomatological Findings

There were no significant differences in age, BMI, duration of addiction, unstimulated and stimulated saliva flow rate, DMFT, API, PBI, and PPD among the 3 study groups and among the study groups vs the control group. Clinical and stomatological characteristics of participants are presented in **Table 1**.

Unstimulated (US) and Stimulated (S) Saliva

SpH concentration was significantly lower in US and S in all nicotine users (IQOS, e-cigarette users, CS) compared to the controls (US: $P<0.0001$, $P<0.0001$, $P<0.0001$, respectively; S: $P<0.0001$, $P<0.0001$, $P<0.0001$, respectively). In the group of IQOS users, SpH concentration was significantly lower compared to e-cigarette group, both in US ($P<0.01$) and S ($P=0.03$).

SPA concentration was significantly lower in US and S of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls (US: $P<0.0001$, $P<0.0001$, $P<0.0001$, respectively; S: $P<0.0001$, $P=0.006$, $P<0.0001$, respectively). In IQOS users, SPA concentration in US was considerably lower compared to e-cigarette smokers ($P=0.002$), SPA concentration in stimulated saliva of IQOS subjects was significantly lower compared to e-cigarette smokers ($P<0.0001$) and CS ($P=0.002$).

The concentration of S1P was significantly lower in US of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls ($P<0.0001$, $P<0.0001$, $P<0.0001$, respectively). S1P concentration in S did not differ significantly between the study groups.

Similarly, ceramide C14 content was considerably lower in unstimulated saliva of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls ($P<0.0001$, $P<0.0001$, $P<0.003$, respectively). In IQOS users, the concentration of the parameter

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in question was significantly lower compared to the CS group (*P*=0.006). The concentration of ceramide C14 in S in the IQOS group was considerably higher compared to the e-cigarette (*P*=0.02) and CS (*P*=0.008) groups.

The concentrations of ceramides C16 and C24 were significantly lower in US and S of all nicotine users (IQOS, e-cigarette users, CS) compared to the control group (C16, US: *P*<0.001, *P*<0.001, *P*<0.001, respectively, C16, S: *P*=0.04, *P*=0.003, *P*=0.04, respectively; C24, US: *P*<0.001, *P*<0.001, *P*<0.001, respectively; C24, S: *P*<0.0001, *P*<0.0001, *P*<0.0001, respectively).

The level of ceramide C18 was considerably lower in US and S of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls (US: *P*<0.0001, *P*<0.0001, *P*<0.0001, respectively; S: *P*<0.0001, *P*<0.0001, *P*<0.0001, respectively). In US and S of the CS group, the concentration of the discussed parameter was significantly lower in both the IQOS and e-cig groups (US: *P*<0.0001, *P*<0.0001, respectively, S: *P*<0.0001, *P*<0.0001, respectively).

Ceramide C18 concentration was clearly lower in US of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls (*P*<0.0001, *P*<0.0001, *P*<0.0001, respectively). In the US of the IQOS group, the concentration of the parameter in question was significantly higher compared to both the e-cig and CS groups (*P*=0.005, *P*=0.004, respectively). The content of ceramide C18 was considerably lower in S of e-cigarette and CS groups compared to the controls (*P*<0.0001, *P*<0.0001, respectively). In S of the IQOS group, the concentration of the discussed parameter was significantly higher compared to the CS group (*P*=0.04).

Ceramide C20 concentration was significantly lower in US and S of all nicotine users (IQOS, e-cigarette users, CS) compared to the control group (US: *P*=0.001, *P*<0.0001, *P*<0.0001, respectively; S: *P*=0.03, *P*<0.0001, *P*=0.0004, respectively). In the US and S of the CS group, the concentration of the parameter analyzed was significantly lower compared to both the IQOS and e-cig groups (US: *P*<0.0001, *P*<0.0001, respectively; S: *P*<0.0001, *P*<0.0001, respectively).

Ceramide C22 concentration was significantly lower in US of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls (*P*<0.0001, *P*<0.0001, *P*<0.0001, respectively). The content of C22 in US of CS subjects was significantly lower compared to IQOS users (*P*=0.02) as well as the CS group (*P*=0.0006). Ceramide C22 concentration in the S of CS-group participants was significantly lower compared to the control group (*P*=0.0001) as well as the e-cigarette group (*P*=0.004).

The concentration of ceramide C24 1 and total Cer was notedly lower in US and S of all nicotine users (IQOS, e-cigarette users, CS) compared to the controls (US, C 24.1: *P*<0.0001, *P*<0.0001, *P*<0. 0001, respectively, US, total Cer: *P*<0.0001, *P*<0.0001, *P*<0.0001, respectively; S, C24 1 *P*<0.0001, p<0.0001, p<0.0001, respectively, S, total Cer: *P*<0.0001, *P*<0.0001, *P*<0.0001, respectively). The concentration of C24 1 in the US of e-cigarette group subjects was significantly higher compared to the CS group (*P*=0.005), while total Cer concentration in US of IQOS users was significantly higher compared to the CS group (*P*=0.003). Ceramide C24 1 concentration in S of e-cigarette group was significantly higher compared to the IQOS group (*P*=0.008) and CS group (*P*<0.0001).

The content of ceramide C24 1 in the S of the e-cig group was significantly elevated compared to the groups: IQOS (*P*=0.008) and CS (*P*<0.0001).

The concentration of 4-HNE and MDA in US was significantly higher in the group of traditional cigarette smokers compared to the controls (4-HNE *P*=0.0022; MDA *P*=0.0008), whereas in stimulated saliva we demonstrated considerably higher 4-HNE concentration in the group of traditional cigarette smokers vs non-smokers (*P*<0.0001). Moreover, 4-HNE levels in stimulated saliva of IQOS and e-cigarette users were significantly lower compared to the traditional cigarette smoking group (*P*=0.0002, *P*<0.0001, respectively). The content of MDA in the stimulated saliva of traditional cigarette smokers was significantly higher compared to the non-smoking group as well as the e-cigarette smoking group (*P*=0.0030; *P*=0.0078, respectively).

Graphical presentation of the results is presented in **Figures 1-4**.

Discussion

Saliva is the secretion of the even-numbered salivary glands: parotid, submandibular, sublingual as well as numerous smaller glands scattered in the oral mucosa [17]. It contains a number of proteins, glycoproteins, and lipids that determine its function and properties. The secretion of the salivary glands provides, inter alia, lubrication for the surface of the teeth and mucosa, is responsible for the initial stage of food digestion, and conditions protection of oral tissues from irritating stimuli, which certainly include cigarette smoke [26-28].

Cigarette smoke contains over 4000 toxic components responsible for a number of irregularities in the body [29]. It has been proven that in long-term compulsive smokers, cigarette smoke can lead to changes in the amount of saliva secreted, as well as trigger qualitative changes in the salivary gland secretion (decreased buffering capacity, altered bacterial microflora, increased concentration of oxygen free radicals, and changes in the local immune system) [30-35]. The solution to the harmful effect of traditional smoking was supposed to

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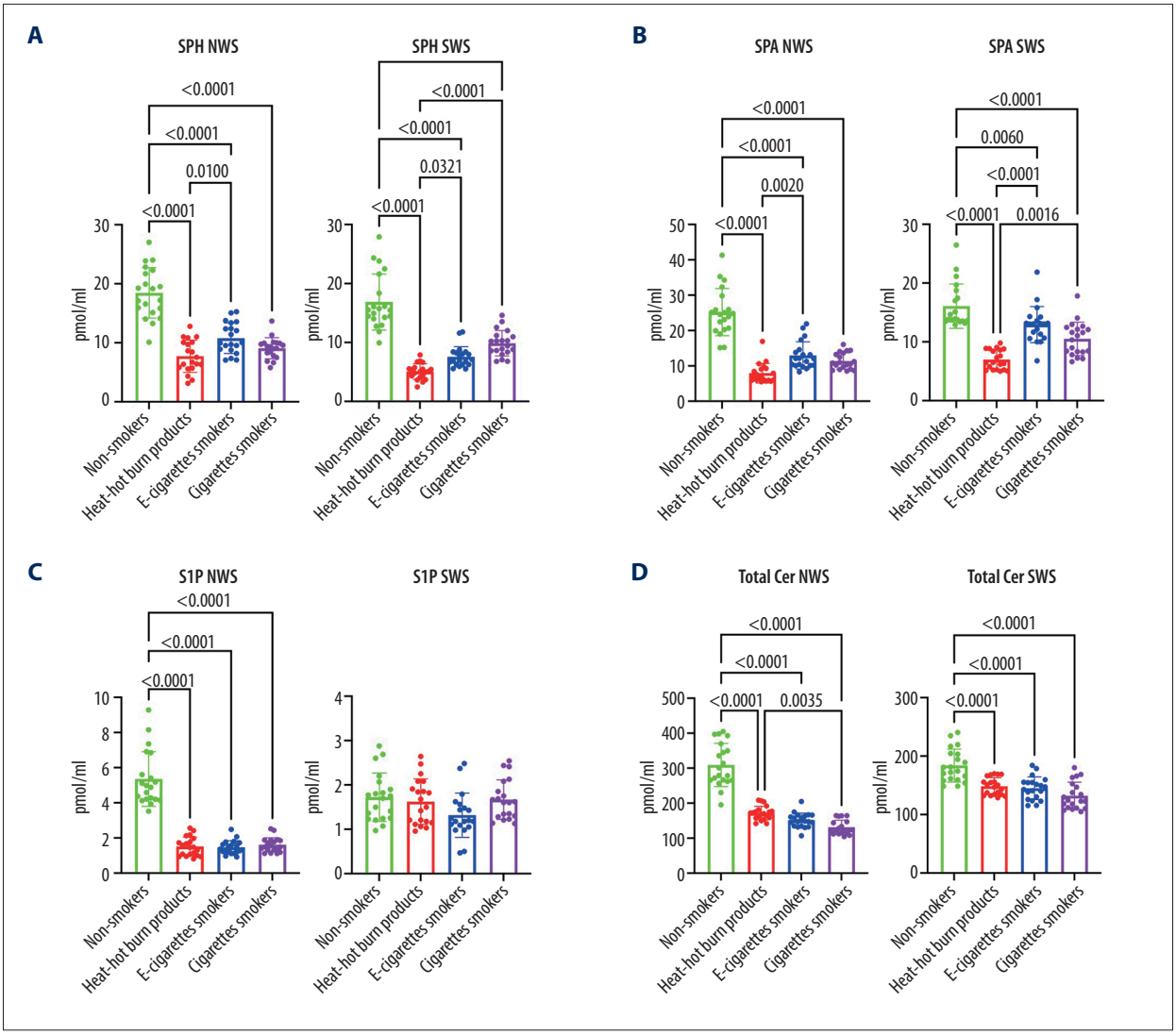

Figure 1. (**A-D**)Influence of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on the concentration of lipids in unstimulated and stimulated saliva. NWS – non-stimulated whole saliva; SPH – sphingosine; SPA – sphinganine; S1P – sphingosine-1-phosphate; SWS – stimulated whole saliva; Total Cer – total ceramide; p<0.0001. *The figure was created in GraphPad Prism.*

be new equipment that delivers nicotine to the body – e-cigarettes and heat-not-burn products.

E-cigarettes are mechanical devices that heat special solutions for inhalation, giving the user a sensation similar to ordinary smoking [36,37]. In addition to propylene glycol, flavors, and nicotine, e-cigarette liquids contain carcinogenic formaldehyde and numerous heavy metals [38,39]. Heat-not-burn products, on the other hand, can be described as a 'hybrid' of the 2 above-mentioned smoking methods. This system is based on tobacco cartridges (similar to traditional cigarettes) and an electronic device designed to heat the tobacco [40]. Heating, rather than burning, is intended to lead to less intense production and supply of harmful substances to the body. According to the literature on the subject, these devices cause adverse health effects, particularly with regard to respiratory organ complications [41,42]. Despite this fact, many people believe that the use of these devices is a "healthy" alternative to traditional cigarettes [39]. Due to their relatively short time on the consumer market, their exact mechanism on both the body and oral health has not been thoroughly evaluated, so any discoveries related to this topic are of great interest in the scientific community. Knowledge regarding the effect of smoking e-cigarettes on saliva composition is scarce, and there are no publications related to the effect of using heat-not-burn products. It is established that e-cigarettes can decrease saliva secretion, change composition of the oral microbiome, and affect the local immune response system [43-45].

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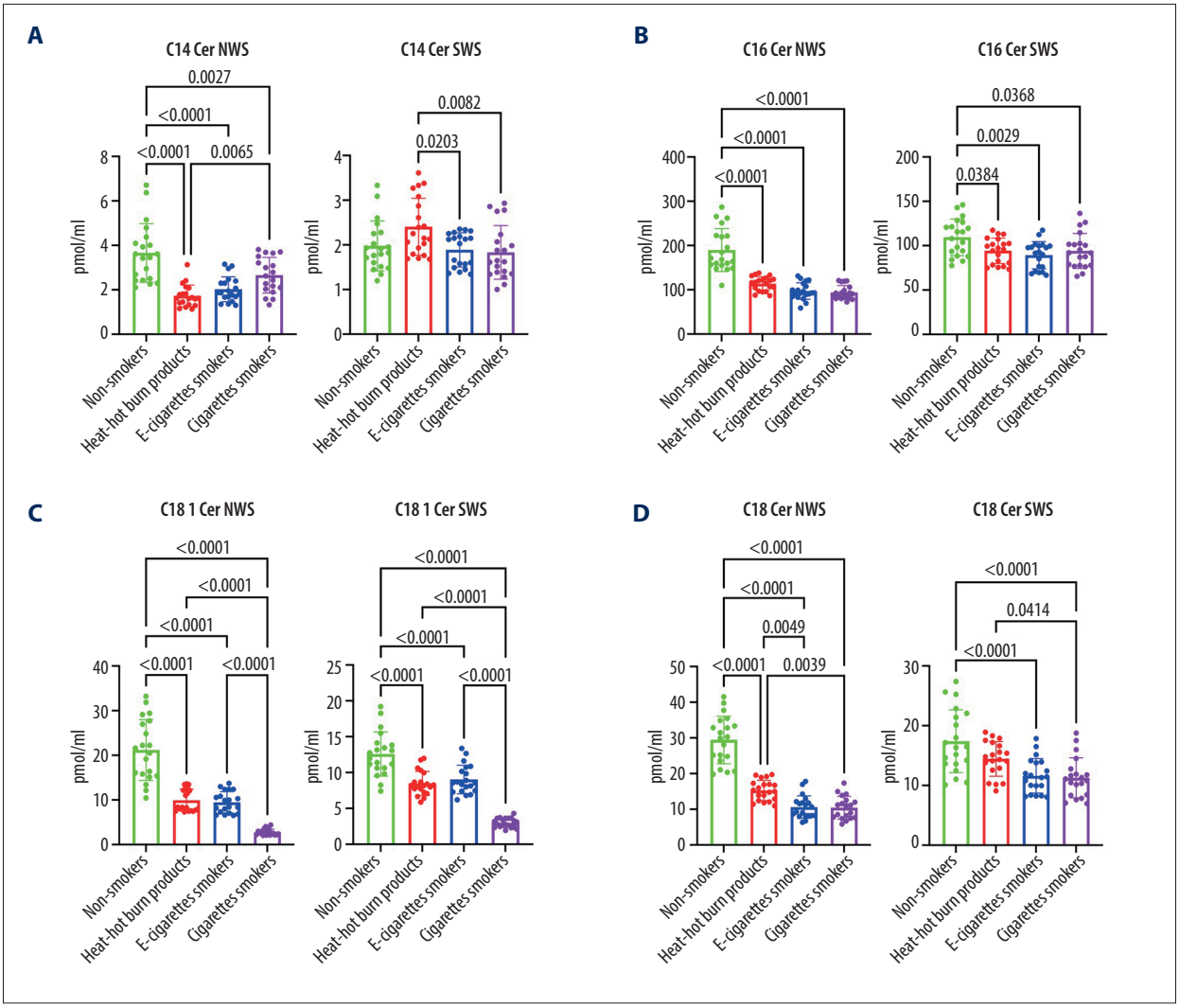

Figure 2. (**A-D** Influence of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on the concentration of lipids in unstimulated and stimulated saliva. Cer – ceramides (C14: 0-Cer, C16: 0-Cer, C18: 1-Cer, C18: 0-Cer); NWS – non-stimulated whole; SWS – stimulated whole saliva; *P*<0.0001. *The figure was created in GraphPad Prism.*

Salivary lipids are considered a very important component of saliva, as their qualitative and quantitative composition can be altered in the course of many pathological conditions [17]. It has been proven that disorders of lipid homeostasis in saliva are associated with the occurrence of periodontal diseases, and may occur in the course of a number of systemic diseases (including Sjögren's syndrome, cystic fibrosis, and Alzheimer's disease) [17,46,47].

There has been no research to evaluate the effect of using different nicotine delivery methods on the concentration of sphingolipids and ceramides in unstimulated and stimulated saliva. In addition, there have been no studies examining the processes of salivary lipid peroxidation in smokers of e-cigarettes and heat-not-burn products.

The purpose of our study was to evaluate the effect of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on the concentration of sphingolipids and lipid peroxidation products (4-HNE, MDA) in unstimulated and stimulated saliva. The participants in the study were generally healthy young adults whose had been smoking for 1-3 years. Furthermore, participants could only use 1 method of delivering nicotine to the body. The fact that young people were enrolled in the study group and the predetermined duration of the addiction are not coincidental. First, the new nicotine delivery devices have been mainly popularized among the "younger generation." Older smokers, due to the length of their addiction and the associated habit, are far less likely to decide to change their nicotine delivery method. Moreover, harmful agents accumulate in the oral cavity with age and prolonged smoking, as has already been proven.

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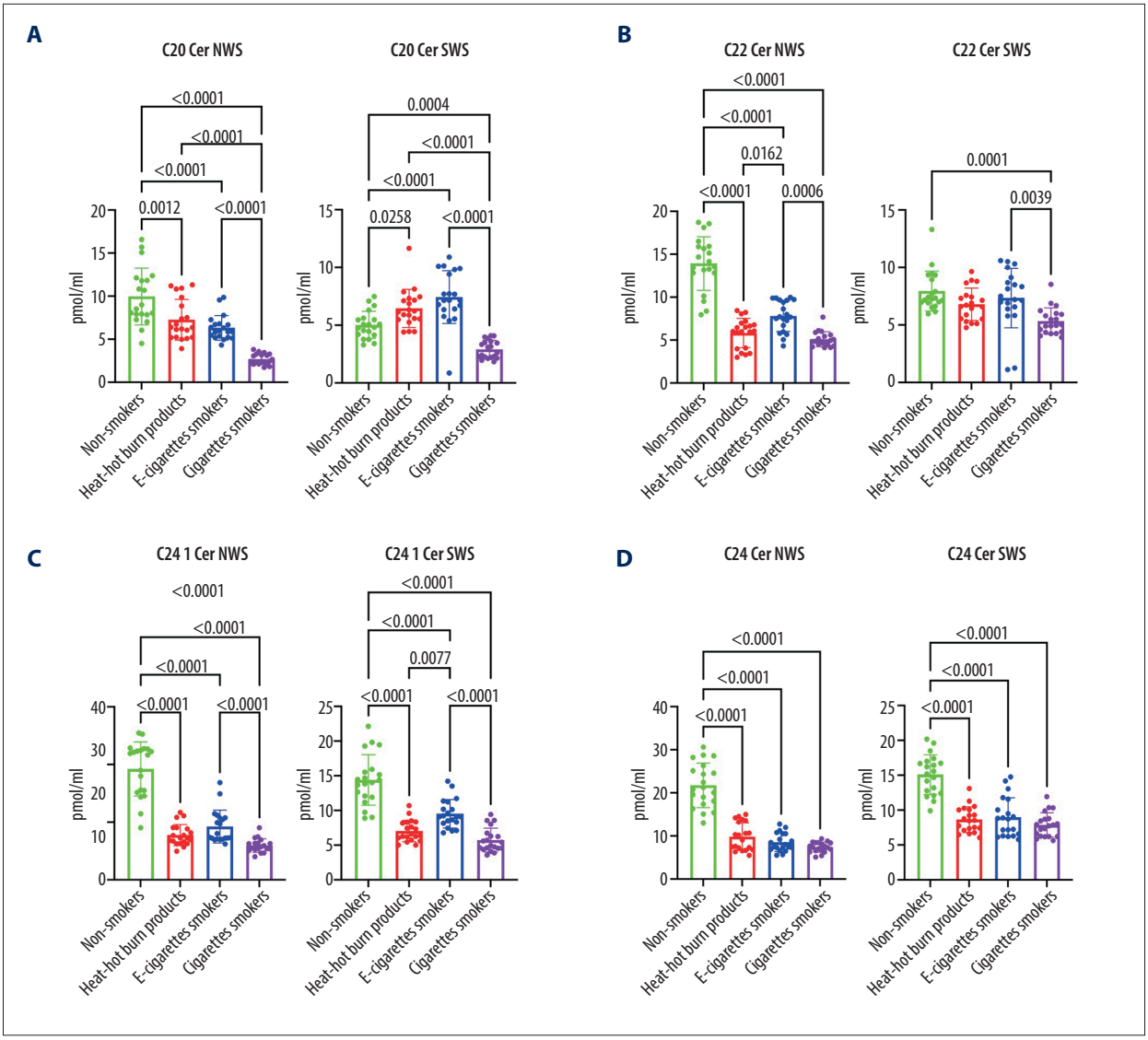

Figure 3. (**A-D**) Influence of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on the concentration of lipids in unstimulated and stimulated saliva. Cer – ceramides (C20: 0-Cer, C22: 0-Cer, C24: 1-Cer, C24: 0-Cer); NWS – non-stimulated whole; SWS – stimulated whole saliva; *P*<0.0001. *The figure was created in GraphPad Prism.*

According to the results of our study, smoking traditional cigarettes, e-cigarettes, or heat-not-burn products reduces the concentrations of all sphingolipids examined by us in the unstimulated and stimulated saliva.

To understand the likely cause of the reduction in sphingolipid levels in the unstimulated and stimulated saliva of smokers, it is necessary to briefly characterize and explain the functions and metabolic processes occurring within this group of compounds.

Sphingolipids and their derivatives constitute a numerous group of bioactive lipid compounds located in the outer layer of eukaryotic cell membranes, thus determining its shape. This group of compounds includes sphingosine, sphinganine, sphingosine-1-phosphate, and ceramides. In addition to their structural functions, sphingolipids act as activators of signaling pathways and secondary signal transducers [48]. The central molecule of the sphingolipid structure is the aliphatic amino alcohol – sphingosine. The combination of sphingosine and fatty acid residue results in the formation of ceramide [48-51]. Ceramides play a primary role in sphingolipid metabolism and are involved in the regulation of such cellular processes as proliferation and differentiation, growth, aging, and cell death [50]. They also provide the basis for the synthesis of other sphingolipids. They can be transformed, with participation of ceramidases, into sphingosine, which is phosphorylated by sphingosine kinases type 1 and 2 to sphingosine-1-phosphate [48,50].

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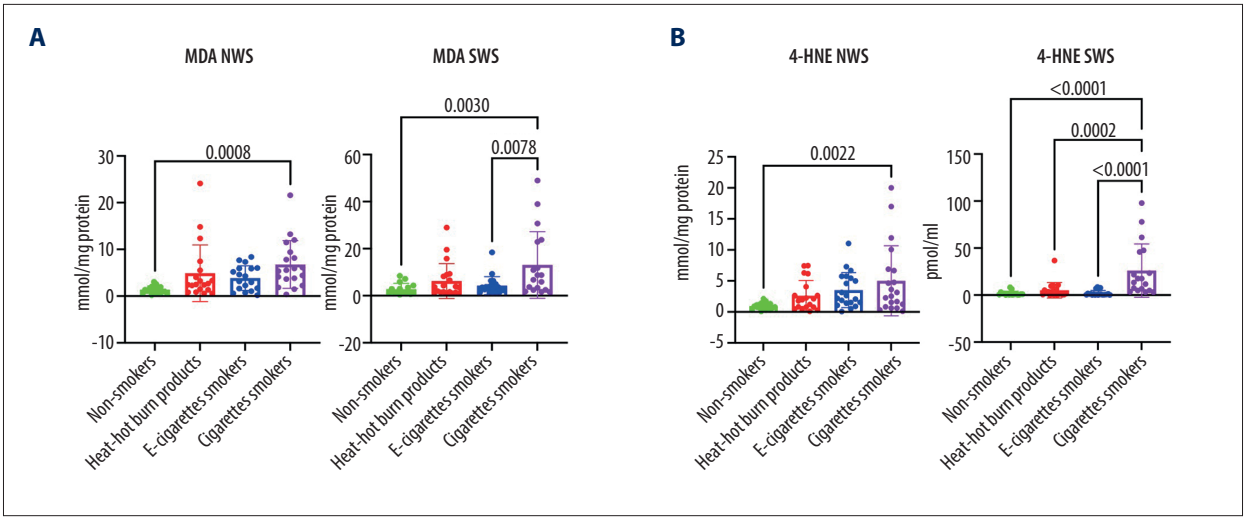

Figure 4. (**A, B**) Influence of smoking traditional cigarettes, e-cigarettes, and heat-not-burn products on the concentration of lipid peroxidation products in unstimulated and stimulated saliva. 4-HNE – 4-hydrokxynonenal; MDA – malondialdehyde; NWS – non-stimulated whole; SWS – stimulated whole saliva; *P*<0.0001. *The figure was created in GraphPad Prism.*

The signaling pathway of sphingosine-1-phosphate is one of the key regulators of cell survival, proliferation, and differentiation. Thus, sphingosine kinases are important enzymes involved in maintaining the balance among bioactive sphingolipids such as S1P, ceramide, and sphingosine [48,49].

The results of our study may seem surprising, since most studies assessing the effect of smoking on sphingolipid concentration within tissues and plasma demonstrated increased sphingolipid levels [52-54]. Although saliva predominantly constitutes the ultra-filtrate of plasma (which might appear controversial in the context of the results we obtained), the vast majority (nearly 98%) of lipids found in saliva are synthesized directly in salivary gland cells [17,47]. This suggests that the significant changes in sphingolipid content in unstimulated and stimulated saliva of smokers compared to the control group directly reflect the ongoing pathology within the salivary glands.

Toxins in cigarette smoke are responsible for DNA damage within the cells of secretory glands. In turn, sphingomyelin, ceramide – synthesized de novo in cells – and sphingosine are characterized by antiproliferative and pro-apoptotic properties. It is likely that cigarette smoke "activates" these functions of sphingolipids to lead to programmed cell death. Ceramides present in the cell are not stored but rather are transported directly to the Golgi apparatus, where they are transformed into derivative compounds. It is possible that chronic irritation of the oral cavity with tobacco smoke toxins decreases the concentration of the compounds we studied as a result of depletion of reserves of sphingolipids, which attempts to mitigate the adverse effects of cigarette toxins on secretory cells of salivary glands.

Reduced ceramide concentrations were observed in gastrointestinal cancer. Changes in the activity of enzymes responsible for the metabolism of this compound trigger an increase in glycosylation of ceramide, resulting in reduced concentration of this compound. In addition, elevated levels of S1P have been observed in many types of cancer, including colorectal cancer cells. This sphingolipid – S1P – unlike ceramide, is anti-apoptotic, enhances proliferation of tumor cells, and stimulates their angiogenesis. This contradicts the results of our study in which S1P concentrations were also reduced in the studied material. Nevertheless, cancer is an advanced form of complications of organs connected with smoking. Further studies are necessary to better understand the effects of smoking on salivary sphingolipid concentration.

Moreover, in the groups of all smokers, regardless of the source of nicotine delivery to the body, we observed an increase in 4-HNE and MDA concentrations in unstimulated and stimulated saliva compared to the controls; however, only in the group of smokers of traditional cigarettes vs the controls was this result statistically significant, suggesting that smoking traditional cigarettes is the most harmful in terms of oxidative modification of salivary lipids, whereas it is possible that a longer period of using modern nicotine delivery devices may give similar results. This is particularly important considering that lipids perform many important functions in the oral cavity, from structural to functional. They affect the processes of digestion, protection, and communication, as well as maintaining the internal balance in the oral cavity [17].

4-HNE is one of the byproducts of lipid peroxidation that the body under oxidative stress. It is mainly formed by oxidation of linoleic acid, one of the unsaturated fatty acids. 4-HNE is a

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highly reactive aldehyde that affects various physiological processes in the body; it can lead to cell membrane damage, protein degradation, enzyme inactivation, and inflammatory reactions. In addition, 4-HNE can affect mitochondrial function, introducing disorders in the process of cellular energy production. MDA, on the other hand, is a byproduct of lipid peroxidation and can damage body cells and tissues through its toxic and pro-inflammatory effects. It can also lead to the formation of free radicals, which cause further damage to cells and can contribute to development of numerous diseases, including heart disease, respiratory diseases, and cancer.

The increased concentrations of these compounds in unstimulated and stimulated saliva obtained in our study clearly indicate a redox imbalance in the salivary glands of smokers. Increased levels of free radicals lead to oxidative modification of lipids and thus damage to cell membranes. Similar results were obtained by Celec et al, who did not demonstrate any correlation between the concentrations of lipid peroxidation products in saliva and plasma, which confirms the glandular origin of lipids in saliva. Additionally, some recently discovered compounds have a significant influence on the oral environment; lysates and postbiotics can modify clinical and microbiological parameters, so these products should be considered in future trials as they could also influence saliva composition and balance [55-57].

Lipids are one of the main components of cell membranes; therefore, changes in the lipid composition of saliva can reflect changes in the composition of salivary gland cell membranes. Salivary lipids perform many functions, the disruption of which can lead to disintegration throughout the oral cavity [17]. They participate in the signal for saliva secretion, which can consequently lead to impaired salivary secretion. They also have a protective function towards the oral mucosa. Both reduced saliva secretion and weakened protective function may consequently lead to a number of abnormalities, including dry mouth, difficulties in forming a bolus of food, increased susceptibility to mechanical injuries in the oral mucosa (including during consumption or use of prosthetic devices), and an increased risk of bacterial, fungal and viral infections of the gums and oral mucosa (including increased susceptibility to HSV-1 infection and lichen planus) [17,58]. As a consequence, discomfort increases and the patient's overall well-being deteriorates. Of course, in our study, considering the age of the recruited patients and the duration of their addiction, we did not observe the above phenomena, but it cannot be ruled out that in the long-term, smokers may develop these pathologies.

Limitations

Our study has several limitations. Due to the small group size, this should be regarded as a pilot study. The people qualified for the study and control groups were considered matched, with deficiencies in systemic diseases and other factors that may directly affect the increase in oxidative stress in the body. Therefore, fewer patients were included in the study.

Because of the young age of our participants and relatively short smoking histories (up to 3 years), our findings have limited generalizability to long-term or older smokers who may experience different oral health effects. The results may not capture the full spectrum of oral health effects associated with smoking, particularly in older individuals with longer smoking durations.

Moreover, only some salivary lipids were included in our study, so the results do not reflect the overall effect of smoking on the salivary lipid profile. This study also did not compare heavy vs light smokers.

Conclusions

The results of our research clearly show that:

- Decrease the concentration of all sphingolipids in unstimulated and stimulated saliva is not dependent on the mode of delivery of nicotine.
- Significant changes in the content of sphingolipids in the unstimulated and stimulated saliva of smokers in comparison to the control group are a direct reflection of ongoing pathology within the salivary glands.
- The increased concentration of the 4-HNE and MDA in unstimulated and stimulated saliva indicates an imbalance in the redox balance in the salivary glands of smokers.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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