Original Article

Differences of Saliva Composition in Relation to Tooth Decay and Gender

(dental caries / DMFT / gender / saliva / proteins)

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Abstract. Most people worldwide suffer from dental caries. Only a small part of the population is caries-resistant and the reason for this resistance in unknown. Only a few studies compared the saliva protein composition of persons with carious teeth and persons with no caries. Our study is the first to relate proteomic analysis of the caries aetiology with gender. In this study, we compared the differences in the abundances of proteins in the saliva between caries-resistant and caries-susceptible females and males by nano-liquid chromatography-tandem mass spectrometry (Label-Free Quantitative Proteomics). Our results demonstrate that the observed differences in the protein levels might have an influence on anti-caries resistance. A total of 19 potential markers of tooth caries were found, for example proteins S100A8 and annexin A1 with higher expression in the caries-susceptible group in comparison with the caries-free group and mucin-5B, lactoferrin, lysozyme C with higher expression in the caries-free group in comparison with the caries-susceptible group. The pre-sented study is the first complex proteomic and gender project where the saliva protein content of caries-free and caries-susceptible persons were compared by label-free MS. The newly detected potential protein markers of dental caries can be a good basis for further research and for possible future therapeutic use.

Introduction

Human saliva is a major body fluid and is very important for oral health (saliva production equals approx. 0.75–1.5 l per day). The physiology of the whole saliva and salivary secretion was reviewed in Proctor (2016).

Saliva includes many markers that can foretell the potential risk of some diseases, for example periodontal diseases, cardiovascular diseases, diabetes mellitus, oncological, psychiatric, viral, gynecological and endocrinological diseases (Podzimek et al., 2016).

As World Health Organization states, "Dental caries is still a major oral health problem in most industrialized countries, affecting 60–90 % of schoolchildren and the vast majority of adults" (http://www.who.int/oral_health/disease_burden/global/en; 26.5.2018). Only a small part of the world's population (ca 10 % with DMFT = 0 (DMFT – decayed, missing, filled tooth) is well protected against the prevalence of dental caries. The social-economic status plays an important role in dental caries, as processed in a cross-sectinal study by Wang et al. (2017). The study shows that people aged 65–74 with a low social-economic status have poor oral health.

Possible saliva biomarkers and their association with dental caries (microorganisms in the saliva, salivary electrolytes, salivary proteins and peptides in reaction to dental caries, immune studies focused on particular individual markers) were reviewed in Gao et al. (2016). Only a few studies compared the protein saliva composition of people with carious teeth and people with no caries, and they were reviewed with different results in

Al-Tarawneh et al. (2011). Comparative proteomic analysis of oral fluids found differences in protein expression based on the gender and age (Fleissig et al., 2010).

The present study was focused on human proteins in the saliva (not on microbiome profiles of saliva samples) and is a continuation of our previous research on protein differences in dental pulp in relation to tooth decay (Jágr et al., 2016). This study, based on label-free mass spectrometry (MS) quantification, was performed to compare differences in the abundances of proteins in the saliva between caries-resistant and caries-susceptible persons and also to perform a gender comparison. No such complex proteomic comparison of the saliva composition has been performed to date.

Material and Methods

Preparation of samples for label-free quantitative analysis

Saliva samples of the whole saliva (100 µl) were collected from healthy female volunteers aged between 20 and 35 years, caries-resistant (N = 14, DMFT ranging from 0 to 1) and caries-susceptible (N = 16, DMFT ranging from 5 to 14) females, and from healthy male volunteers aged between 23 and 47 years, caries-resistant (N = 12, DMFT ranging from 0 to 1) and caries-susceptible (N = 18, DMFT ranging from 5 to 12) males. All procedures performed in the studies involving human participants were in accordance with the Ethical Standards and with the World Medical Association Declaration of Helsinki (version 2008). All of the volunteers were requested not to eat, drink, or brush and wash their teeth for 1–2.5 h prior to the trial. Samples were kept on ice and protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN)) were added to inhibit protease activity. Unstimulated whole-saliva samples were frozen at –80 °C until futher analysis. Saliva collection was performed once in sterile bottles of each volunteer over a period of three months. The criteria for group inclusion were overall health of the volunteeer, age, sex, and number of teeth treated. Seventy % of participants had completed high school education. DMFT was assesesed based on clinical examination by one experienced dentist. Furthermore, panoramic X-ray was evaluated in each patient.

Samples were centrifuged at 13,000 g for 30 min at 4 °C. The supernatant (A) of each sample was collected. Proteins were precipitated using trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 % (w/v) and dithiotreitol (0.12 % w/v) (Sigma-Aldrich). After vortexing and incubation at 25 °C for 15 min, the precipitated proteins were centrifuged (13,000 g, 15 min, 4 °C). Protein pellets (AI) from the collected supernatants (A) were washed three times with ice-cold 100% acetone and lyophilized. All the samples were then digested in a solution containing NH_4HCO_3 (0.05 mol/l) and trypsin (0.2 mg/ml) (1/50 w/w trypsin/sample) at 37 °C for 16 h. Peptides were extracted using Stage Tips-aided (Rappsilber, 2007) purification of samples for nano liquid chromatography (nLC) MS/MS. The extracted solutions were lyophilized and dissolved in 20 µl of 2% formic acid (v/v).

The protein concentration was determined using a Nanodrop ND-1000 spectrometer (ThermoFisher, Wilmington, DE) (average sample concentration was 0.33 mg/ml). Finally, 0.8 µg of the peptide mixture was loaded to the column.

Analysis of tryptic digests with LC-MS/MS

A nano liquid chromatography (nLC) apparatus Proxeon Easy-nLC (Proxeon, Odense, Denmark) was used for analysing the protein digests, similar to our previous work, coupled to a MaXis quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) (Ošťádal et al., 2015). Auto MS/MS with active exclusion (after one spectrum and release after 0.3 min) was used for MS/MS analyses.

Database searches were performed as described (Eckhardt et al., 2014; Ošťádal et al., 2015) with the taxonomy restricted to *Homo sapiens* to remove protein identification redundancy. Only significant hits (MASCOT score ≥ 80 for proteins; ≥ 20 for peptides, http://www.matrixscience.com) were accepted.

Label-free quantification

Label-free quantification is a method for determination of the relative amount of proteins in two (or more) biological samples, i.e., without using stable isotopes. In our case it was based on the comparison of signal intensities (at MS) of individual particular peptides at different sets of samples. Profile Analysis software (version 2.1, Bruker Daltonics GmbH) was used to evaluate differences in the protein composition of the caries-susceptible and caries-resistant persons (females and males) by means of label-free quantification (Student's t-test; $P < 0.05$). The peptides under consideration had to be found in at least 50 % of all the samples, regardless of the group, and they had to be found in at least one of the two groups (group of susceptible persons and/or group of resistant persons (females and/or males)) as well as in at least 50 % of the group. For correct evaluation of ions with similar m/z values and similar retention times, the Time Alignment option was enabled.

Results

Comparison of caries-susceptible and caries-free saliva samples

In our complex proteomic study, we found protein differences between caries-susceptible and caries-free groups. We detected nine proteins with higher expression in the caries-susceptible male group and seven proteins with higher expression in the caries-free male group (Table 1). Our comparison of female saliva samples showed four statistically significantly higher values in caries-susceptible females (Table 1). We observed

Table 1. List of over-expressed proteins in the saliva of caries-susceptible and caries-free persons

Accession Number	Protein	Total number of peptides	Molecular function	P	Fold change (caries-susceptible : caries-free)
	Up-regulated in caries-susceptible males				
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	35	glycosidase/hydrolase	3.12E-03	1.8
P04083	ANXA1 Annexin A1	17	phosholipase A2 inhibitor	5.19E-05	1.8
P06702	S100A9 Protein S100-A9	11	antimicrobial	3.77E-03	2.4
P60709	ACTB Actin	9	ATB-binging	4.56E-03	1.6
Q08188	TGM3 Protein-glutamine γ-glutamyltransferase E	10	acyltransferase/transferase	1.27E-02	1.8
P05109	S100A8 Protein S100-A8	7	antimicrobial	6.46E-03	2.7
P07355	ANXA2 Annexin A2	7	RNA-binding	3.64E-03	1.9
P29508	SERPINB3 Serpin B3	6	protease inhibitor	3.03E-02	1.3
P62937	PPIA Peptidyl-prolyl *cis-trans* isomerase A	3	isomerase	1.46E-02	1.3
	Up-regulated in caries-free males				
P02814	SMR3B Submaxillary gland androgen-regulated protein 3B	16		5.84E-04	0.6
Q6P5S2	C6orf58 C6orf58	11	developmetal protein	6.51E-04	0.6
P01833	PIGR Polymeric immunoglobulin receptor	8	polymeric immunoglobulin receptor activity	4.03E-02	0.8
P01037	CST1 Cystatin-SN	6	protease inhibitor	3.09E-02	0.8
Q9HC84	MUC5B Mucin-5B	4		2.84E-02	0.6
P61626	LYZ Lysozyme C	3	antimicrobial	1.34E-02	0.5
P31025	LCN1 Lipocalin 1	3	chloride and zinc binding	5.29E-03	0.5
	Up-regulated in caries-susceptible females				
P04083	ANXA1 Annexin A1	9	phosholipase A2 inhibitor	1.08E-02	1.7
P01876	IGHA1 Immunoglobulin heavy constant α 1	8	antigen binding	4.02E-02	1.2
P06702	S100A9 Protein S100-A9	9	antimicrobial	4.95E-03	3.5
P25311	AZGP1 Zinc-α-2-glycoprotein	9	transmembrane activity	1.20E-02	1.8
	Up-regulated in caries-susceptible persons				
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	25	glycosidase/hydrolase	2.38E-02	1.6
P04083	ANXA1 Annexin A1	15	phosholipase A2 inhibitor	4.00E-04	1.7
P05109	S100A8 Protein S100-A8	7	antimicrobial	9.99E-03	2.5
P04406	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	5	oxidoreductase/transferase	2.69E-02	1.4
P60174	TPI1 Triosephosphate isomerase	4	isomerase	4.42E-03	1.4

Accession Number (Uniprot); P – significance; the molecular functions were categorized according to the classification system used in the public database available at http://www.uniprot.org.

five differences in the entire comparison (without gender specification) (Table 1). Most of these protein differences were observed for the first time. We observed 111 proteins in total. The list of identified proteins is attached in Table 3.

The Venn diagram describes the relationships amongst proteins that were found to be differently produced in DMFT comparisons (Fig. 1). The distribution of the biological functions of proteins that were found with different expression in human saliva is shown in Fig. 2.

Gender differences in the saliva

We compared saliva proteins on a gender basis. Most of these gender protein differences were observed for the first time. We identified 18 up-regulated proteins by MS label-free quantification in the group of caries-susceptible males. One protein was up-regulated in the saliva of females (caries-susceptible females) (Table 2). We found six proteins over-expressed in males when we compared samples obtained from caries-free persons (Table 2). We also compared saliva samples obtained from all males and all females (both combined caries-susceptible and caries-free). We identified 14 proteins up-regulated in the group of males compared to females

(Table 2). The Venn diagram describes the relationships of proteins that were found to be differently produced in gender comparisons (Fig. 3).

Discussion

The present study provides the most complex proteomic comparison of the saliva to date (in both fields: caries protection and gender differences). It was observed that the quality of the protein composition did not differ in all the compared groups. However, significant differences were observed in the quantitative contents of some proteins (most of them for the first time). In the present project, we found 21 differences in protein expression between caries-susceptible and caries-free persons (Table 1) (Fig. 2) and 23 gender-related differences (Table 2) (Fig. 3). Proteins AMY1A, ANXA1 and S100A9 represent the most abundant proteins in human saliva (based on the study by Grassl et al., 2016).

The incidence of lipoprotein PPIA limits *Streptococcus mutans* phagocytosis, as described in the report by Mukouhara et al. (2011). We found this protein with significantly higher expression in the caries-susceptible male group (Table 1), and it could therefore be a poten-

Table 2. List of over-expressed proteins in the saliva of males and females

Accession Number	Protein	Total number of peptides	P	Fold change (female : male)
	Caries-susceptible persons			
	Up-regulated in saliva of males			
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	39	2.95E-03	0.9
P04083	ANXA1 Annexin A1	17	4.61E-05	0.8
P60709	ACTB Actin	13	1.32E-02	0.7
Q08188	TGM3 Protein-glutamine γ-glutamyltransferase E	7	1.56E-04	0.6
P05109	S100A8 Protein S100-A8	8	3.48E-03	0.7
Q8N4F0	BPIFB2 Bactericidal/permeability-increasing protein-like 1	5	9.89E-04	0.8
P25311	AZGP1 Zinc-α-2-glycoprotein	7	2.20E-03	0.7
P01037	CST1 Cystatin-SN	7	2.00E-03	0.7
A8K2U0	A2ML1 α-2-Macroglobulin-like protein 1	3	3.46E-02	0.8
P07355	ANXA2 Annexin A2	5	2.61E-02	0.8
P01834	IGKC Immunoglobulin κ constant	8	3.60E-02	0.8
P01704	IGLV2-14 Immunoglobulin λ variable 2-14	6	8.41E-03	0.7
P29508	SERPINB3 Serpin B3	5	1.02E-02	0.7
P30740	SERPINB1 Leukocyte elastase inhibitor	4	1.97E-02	0.8
Q9HC84	MUC5B Mucin-5B	4	5.73E-03	0.7
P04080	CSTB Cystatin-B	3	4.02E-02	0.6
P01591	IGJ Immunoglobulin J chain	3	1.65E-02	0.7
P31025	LCN1 Lipocalin 1	3	2.80E-02	0.7
	Up-regulated in saliva of females			
P02814	SMR3B Submaxillary gland androgen-regulated protein 3B	13	1.32E-02	1.3
	Caries-free persons			
	Up-regulated in saliva of males			
P01876	IGHA1 Immunoglobulin heavy constant α 1	11	1.37E-02	0.8
P02788	LTF Lactotransferrin	4	1.85E-02	0.6
P05109	S100A8 Protein S100-A8	6	1.33E-04	0.7
P25311	AZGP1 Zinc-α-2-glycoprotein	9	2.06E-04	0.6
P01704	IGLV2-14 Immunoglobulin λ variable 2-14	6	1.12E-02	0.8
P80188	LCN2 Neutrophil gelatinase-associated lipocalin	5	7.51E-03	0.7
	Up-regulated in saliva of females			
P04745	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	32	7.71E-04	1.4
	Caries-susceptible and caries-free persons			
	Up-regulated in saliva of males			
P04083	ANXA1 Annexin A1	17	5.77E-07	0.7
P01876	IGHA1 Immunoglobulin heavy constant α 1	12	2.07E-02	0.8
P01833	PIGR Polymeric immunoglobulin receptor	7	1.18E-02	0.9
Q08188	TGM3 Protein-glutamine γ-glutamyltransferase E	6	1.19E-03	0.6
P05109	S100A8 Protein S100-A8	8	7.92E-03	0.7
Q8N4F0	BPIFB2 Bactericidal/permeability-increasing protein-like 1	5	1.19E-02	0.8
P25311	AZGP1 Zinc-α-2-glycoprotein	7	9.38E-05	0.5
P69905	HBA2; HBA1 Haemoglobin subunit α	3	4.25E-02	0.4
P01834	IGKC Immunoglobulin κ constant	5	4.54E-02	0.8
P29508	SERPINB3 Serpin B3	4	2.74E-02	0.7
P30740	SERPINB1 Leukocyte elastase inhibitor	5	1.39E-02	0.7
P04080	CSTB Cystatin-B	4	3.15E-02	0.6
P80188	LCN2 Neutrophil gelatinase-associated lipocalin	4	3.00E-02	0.6
P31025	LCN1 Lipocalin-1	3	4.47E-02	0.7

Accession Number (Uniprot); P – significance; the molecular functions were categorized according to the classification system used in the public database available at http://www.uniprot.org.

tial risk biomarker for dental caries in the saliva. In an earlier study, Vitorino's group also detected a high number of PPIA peptide fragments in the caries-susceptible group, which suggests high proteolytic activity (Vitorino et al., 2005).

We detected six proteins (α-amylase, transglutaminase E, S100A9, S 100A8, annexin A1, annexin A2) with calcium-binding properties at higher concentrations in the caries-susceptible groups (Table 1). As concerns α-amylase and transglutaminase E (Tgase E), the key reason for their occurrence in the dental caries-susceptible group cound be binding of α-amylase to bacteria (Scannapieco et al., 1993). Tgase E is a calcium-dependent acyl-transfer enzyme catalysing cross-links between proteins or peptides (Ahvazi et al., 2004). Calcium- and zinc-binding proteins such as S100A9 and S100-A8 play a role in the regulation of inflammatory processes and immune response. S100A8 a S100A9 are highly expressed in neutrophils and monocytes. These proteins were detected at elevated levels at extracellular

Table 3. List of identified proteins in saliva samples

Row	Accession Number	Protein	MW [kDa]	pI	Mascot Scores
1	IPI00300786	AMY1C; AMY1A; AMY1B; AMY2A α-Amylase 1	57.7	6.5	5857.3 (M:5857.3)
2	IPI00218918	ANXA1 Annexin A1	38.7	6.7	2138.7 (M:2138.7)
3	IPI00386879	IGHA1 Immunoglobulin heavy constant α 1	53.1	6.5	2057.3 (M:2057.3)
4	IPI00784950	IGHA2 Immunoglobulin heavy constant α 2	51.6	5.4	1957.0 (M:1957.0)
5	IPI00023011	SMR3B Submaxillary gland androgen-regulated protein 3B	8.2	10.2	1910.8 (M:1910.8)
6	IPI00374315	C6orf58 UPF0762 protein C6orf58	37.9	5.7	1866.2 (M:1866.2)
7	IPI00426060	IGHA1 Putative uncharacterized protein DKFZp686J11235 (Fragment)	54.4	6.3	1806.1 (M:1806.1)
8	IPI00785067	IGHA2 IGH@ protein	52.0	5.9	1744.8 (M:1744.8)
9	IPI00647704	IGHA1 cDNA FLJ41552	53.3	6.1	1722.6 (M:1722.6)
10	IPI00925547	LTF Lactotransferrin	77.9	9.6	1475.7 (M:1475.7)
11	IPI00027462	S100A9 Protein S100-A9	13.2	5.7	1353.1 (M:1353.1)
12	IPI00654755	HBB Haemoglobin subunit β	16.0	6.9	1291.1 (M:1291.1)
13	IPI00021439	ACTB Actin, cytoplasmic 1	41.7	5.2	1287.6 (M:1287.6)
14	IPI00004573	PIGR Polymeric immunoglobulin receptor	83.2	5.5	1267.9 (M:1267.9)
15	IPI00300376	TGM3 Protein-glutamine glutamyltransferase E	76.6	5.5	1222.5 (M:1222.5)
16	IPI00645363	IGHV4-31 Immunoglobulin heavy variable 4-31	51.7	9.0	1157.4 (M:1157.4)
17	IPI00007047	S100A8 Protein S100-A8	10.8	6.6	1031.4 (M:1031.4)
18	IPI00465248	ENO1 α-Enolase	47.1	7.7	978.0 (M:978.0)
19	IPI00473011	HBD Haemoglobin subunit δ	16.0	9.1	928.1 (M:928.1)
20	IPI00922693	ACTB Actin, α skeletal muscle	38.6	5.1	921.1 (M:921.1)
21	IPI00745872	ALB Serum albumin	69.3	5.9	875.6 (M:875.6)
22	IPI00296654	BPIFB2 Bactericidal/permeability-increasing protein-like 1	49.1	9.5	870.1 (M:870.1)
23	IPI00166729	AZGP1 Zinc-α-2-glycoprotein	34.2	5.7	817.5 (M:817.5)
24	IPI00305477	CST1 Cystatin-SN	16.4	7.6	767.7 (M:767.7)
25	IPI00022463	TF Serotransferrin	77.0	7.0	752.3 (M:752.3)
26	IPI01010670	A2ML1 α-2-Macroglobulin-like protein 1	161.0	5.4	749.7 (M:749.7)
27	IPI00455315	ANXA2 Annexin A2	38.6	8.5	712.5 (M:712.5)
28	IPI00410714	HBA2; HBA1 Haemoglobin subunit α	15.2	9.4	711.1 (M:711.1)
29	IPI00969456	IGKC Putative uncharacterized protein	25.8	9.2	699.5 (M:699.5)
30	IPI00154742	IGLV2-14 Immunoglobulin λ variable 2-14	24.8	5.9	692.6 (M:692.6)
31	IPI00887169	IGLV1-44 Immunoglobulin λ variable 1-44	25.0	8.8	692.0 (M:692.0)
32	IPI00979250	IGKC Ig κ chain C region	25.6	8.8	629.6 (M:629.6)
33	IPI00550731	- Putative uncharacterized protein	26.2	9.2	629.0 (M:629.0)
34	IPI00022204	SERPINB3 Serpin B3	44.5	6.4	622.0 (M:622.0)
35	IPI00784865	IGK@ IGK@ protein	25.8	5.9	620.1 (M:620.1)
36	IPI00853045	IGKC Immunoglobulin κ constant	25.7	9.5	581.6 (M:581.6)
37	IPI00219757	GSTP1 Glutathione S-transferase P	23.3	5.3	558.4 (M:558.4)
38	IPI00013382	CST2 Cystatin-SA	16.4	4.7	533.6 (M:533.6)
39	IPI00219018	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	36.0	9.3	527.7 (M:527.7)
40	IPI00032294	CST4 Cystatin-S	16.2	4.8	515.4 (M:515.4)
41	IPI01014238	SERPINB1 Leukocyte elastase inhibitor	38.7	6.2	512.5 (M:512.5)
42	IPI00411765	SFN 14-3-3 protein ς	24.3	4.6	498.8 (M:498.8)
43	IPI00896380	IGHM Ig μ chain C region	51.8	5.8	488.8 (M:488.8)
44	IPI00060800	ZG16B Zymogen granule protein 16 homologue B	22.7	7.6	460.9 (M:460.9)
45	IPI00021263	YWHAZ 14-3-3 protein ζ/δ	27.7	4.6	448.2 (M:448.2)
46	IPI00021841	APOA1 Apolipoprotein A-I	30.8	5.5	441.3 (M:441.3)
47	IPI00295105	CA6 Carbonic anhydrase 6	35.8	6.6	441.2 (M:441.2)
48	IPI00936444	MUC5B Mucin-5B	590.4	6.2	421.9 (M:421.9)
49	IPI00985211	- Similar to VH-3 family (VH26)D/J protein	18.8	7.2	415.6 (M:415.6)
50	IPI00553177	SERPINA1 α-1-Antitrypsin	46.7	5.3	412.6 (M:412.6)
51	IPI00021828	CSTB Cystatin-B	11.1	7.9	391.1 (M:391.1)
52	IPI01012311	DMBT1 Uncharacterized protein	124.4	5.1	373.6 (M:373.6)
53	IPI00304557	BPIFA2 BPI fold-containing family A member 2	27.0	5.2	372.3 (M:372.3)
54	IPI00022974	PIP Prolactin-inducible protein	16.6	9.3	365.8 (M:365.8)
55	IPI01013763	LCN2 Neutrophil gelatinase-associated lipocalin	22.4	9.8	360.2 (M:360.2)
56	IPI00002851	CST5 Cystatin-D	16.1	7.6	357.2 (M:357.2)
57	IPI00916434	- Anti-(ED-B) scFV (Fragment)	25.1	9.2	350.3 (M:350.3)
58	IPI00908881	GPI Glucose-6-phosphate isomerase	60.0	9.4	342.6 (M:342.6)
59	IPI00216691	PFN1 Profilin-1	15.0	9.4	337.4 (M:337.4)
60	IPI00783810	LPO Lactoperoxidase isoform 3 preproprotein	70.9	8.9	325.2 (M:325.2)
61	IPI00940673	TKT Transketolase	58.9	8.6	313.1 (M:313.1)
62	IPI00642247	SPRR3 Small proline-rich protein 3	17.0	9.5	312.2 (M:312.2)
63	IPI00797270	TPI1; TPI1P1 Triosephosphate isomerase	26.7	6.5	299.4 (M:299.4)
64	IPI00218131	S100A12 Protein S100-A12	10.6	5.8	296.7 (M:296.7)
65	IPI00646773	GSN Gelsolin	80.6	5.5	294.7 (M:294.7)
66	IPI01020720	HSPA1B Heat shock 70 kDa protein 1B	67.5	5.2	294.1 (M:294.1)

67	IPI00019038	LYZ Lysozyme C	16.5	10.6	285.1 (M:285.1)
68	IPI00947235	IGJ Uncharacterized protein	8.2	10.0	257.4 (M:257.4)
69	IPI00478003	A2M α-2-Macroglobulin	163.2	6.0	247.3 (M:247.3)
70	IPI00916818	PGK1 Phosphoglycerate kinase	35.0	9.3	222.7 (M:222.7)
71	IPI00032325	CSTA Cystatin-A	11.0	5.3	221.2 (M:221.2)
72	IPI00009650	LCN1 Lipocalin-1	19.2	5.3	219.8 (M:219.8)
73	IPI00377025	PRH1; PRH2 Proline-rich protein HaeIII subfamily 1	17.0	4.6	213.0 (M:213.0)
74	IPI00100630	MLLT1 Protein ENL	62.0	9.5	212.7 (M:212.7)
75	IPI00297056	CRNN Cornulin	53.5	5.7	212.0 (M:212.0)
76	IPI01013441	PRTN3 Myeloblastin	23.6	10.2	191.2 (M:191.2)
77	IPI00640006	GDI2 rab GDP dissociation inhibitor β isoform 2	45.6	5.9	190.2 (M:190.2)
78	IPI00419920	CES2 Cocaine esterase isoform 2	67.0	6.1	187.4 (M:187.4)
79	IPI00007797	FABP5 Fatty acid-binding protein, epidermal	15.2	7.5	182.2 (M:182.2)
80	IPI01022836	ATP5B ATP synthase subunit β, mitochondrial	55.3	5.1	179.7 (M:179.7)
81	IPI00010471	LCP1 Plastin-2	70.2	5.2	170.0 (M:170.0)
82	IPI00387116	- Ig κ chain V-III region NG9 (Fragment)	10.7	7.1	159.5 (M:159.5)
83	IPI01014727	- cDNA FLJ51983, highly similar to Phosphoglycerate mutase 1	27.1	9.2	153.8 (M:153.8)
84	IPI00220494	SERPINB13 Serpin B13	38.4	5.5	150.2 (M:150.2)
85	IPI00925411	PPIA Peptidyl-prolyl cis-trans isomerase AU	13.0	6.4	149.2 (M:149.2)
86	IPI00872684	EZR Ezrin	65.5	5.6	148.3 (M:148.3)
87	IPI00554798	HIST1H2BM Histone H2B type 1-M	14.0	10.8	146.1 (M:146.1)
88	IPI00465439	ALDOA Fructose-bisphosphate aldolase A	39.4	9.2	144.9 (M:144.9)
89	IPI00216298	TXN Thioredoxin	11.7	4.7	143.6 (M:143.6)
90	IPI00974112	CRISP3 22 kDa protein	21.6	9.2	141.8 (M:141.8)
91	IPI00220146	DSC2 Desmocollin-2	93.7	5.2	136.4 (M:136.4)
92	IPI01009918	PRSS1 Uncharacterized protein	25.4	8.8	135.9 (M:135.9)
93	IPI00431645	HPR 31 kDa protein	31.4	9.3	134.6 (M:134.6)
94	IPI00022488	HPX Haemopexin	51.6	6.6	131.0 (M:131.0)
95	IPI00081836	HIST1H2AH Histone H2A type 1-H	13.9	11.3	127.8 (M:127.8)
96	IPI00908762	LGALS3BP Galectin-3-binding protein	46.4	5.0	127.0 (M:127.0)
97	IPI00952922	KRT13 Keratin, type I cytoskeletal 13	38.5	4.8	125.1 (M:125.1)
98	IPI00022432	TTR Transthyretin	15.9	5.4	124.2 (M:124.2)
99	IPI00009792	IGHV1OR15-1 Ig heavy chain V-I region V35	13.0	10.1	123.4 (M:123.4)
100	IPI00013895	S100A11 Protein S100-A11	11.7	7.5	120.5 (M:120.5)
101	IPI00242956	FCGBP IgGFc-binding protein	571.6	5.0	111.9 (M:111.9)
102	IPI00965713	FGB Fibrinogen β chain isoform 2 preproprotein	49.9	9.0	109.2 (M:109.2)
103	IPI01024806	ACTN1 α-Actinin 1	24.4	5.3	107.4 (M:107.4)
104	IPI00023038	PRB1 Basic salivary proline-rich protein 1	38.5	11.7	99.2 (M:99.2)
105	IPI00641244	PRDX1 11 kDa protein	10.7	9.6	96.9 (M:96.9)
106	IPI00982472	TALDO1 Transaldolase	35.3	9.7	95.1 (M:95.1)
107	IPI00978296	CA1 Uncharacterized protein	9.4	10.0	93.3 (M:93.3)
108	IPI01010447	UBC Uncharacterized protein	10.0	10.3	90.3 (M:90.3)
109	IPI00644531	TAGLN2 21 kDa protein	21.1	9.0	88.8 (M:88.8)
110	IPI00964070	ANXA3 Annexin A3	32.1	5.6	87.5 (M:87.5)
111	IPI00386755	ERO1L ERO1-like protein α	54.4	5.4	82.7 (M:82.7)

locations during inflammatory processes (Ryckman et al., 2003).

Anti-inflammatory mediator annexin A1 can affect migration and cellular responses of the innate immune system (Weyd, 2016). Annexin A1 is a membrane-localized and Ca^{2+}-dependent phospholipid-binding protein. Annexin A2 has an important role in the regulation of the coagulation cascade (Iaccarino et al., 2011). We assume that the reason for higher concentrations of these immune proteins in caries-susceptible groups cound be a consequence of the prior experience with dental caries. Salivary mucins are well recognized as an important factor in conservation of the health of the oral cavity (Frenkel and Ribbeck, 2015), which is in agreement with our measurements, showing mucin-5B in significantly higher concentrations in the caries-free saliva (Table 1). An antimicrobial protein such as lysozyme C could take part in protecting teeth against tooth decay, which is also in agreement with our measurements. We assume that all three of the above-mentioned proteins (mucin-5B, lactoferrin, and lysozyme C) could play specific roles in oral protection and could thus be promising "anti-caries" biomarkers.

Preza et al. (2009) compared parotid gland secretion from two groups of elderly persons with and without root caries. Some protein differences unique to the subjects (α-1-acid glycoprotein 1; cathepsin D; collagen α-1 (VI) chain; collagen α-2 (VI) chain; cytokeratin-17; glucose-regulated protein-78 kDa; glutathione S-transferase P; Ig κ chain V–IV region LEN; SPARC-like protein 1) were observed in this comparison. Similar changes were found in patients with Sjogren's syndrome, a condition associated with dental decay (Preza et al., 2009). In our results described here, we did not found any changes in these proteins.

A study by Vitorino et al. (2006) was focused on salivary protein composition in cases of *in vitro* dental pellicle formation and its possible correlation with dental
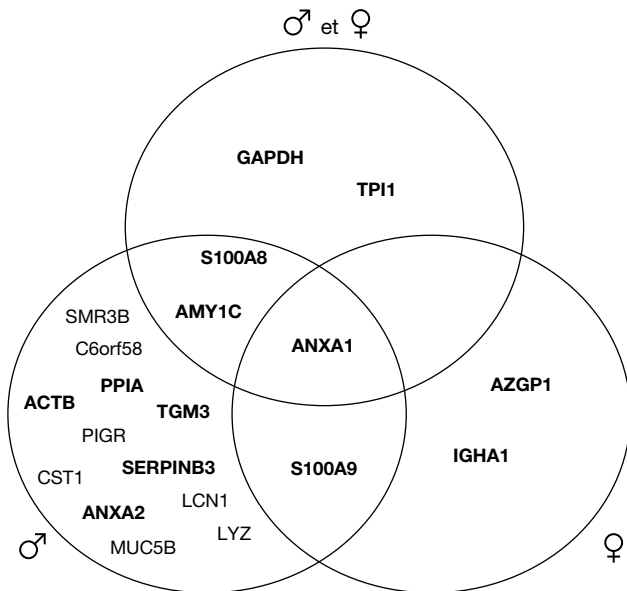
Fig. 1. Comparison of caries-susceptible and caries-free individuals. Proteins found in significantly higher concentrations in the caries-susceptible group (bold) and in the caries-free group (plain) in the individual genders
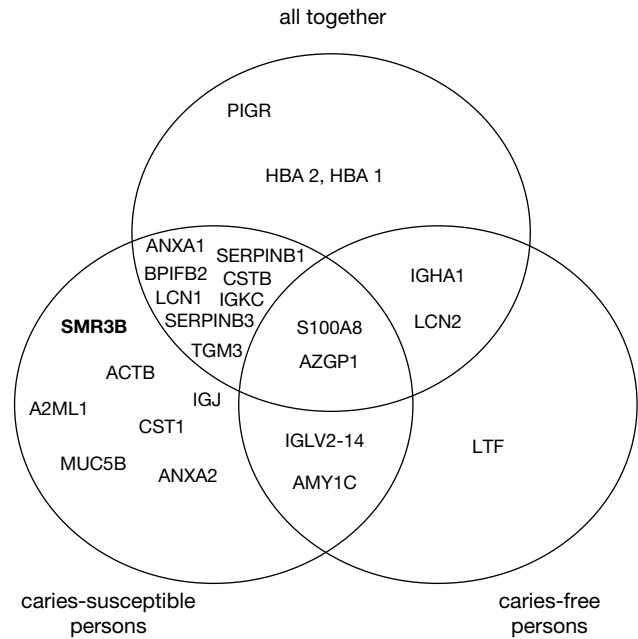

Fig. 3. Gender differences in saliva proteomes. Proteins with significantly higher concentrations in female saliva are shown in bold, and in males in plain fonts
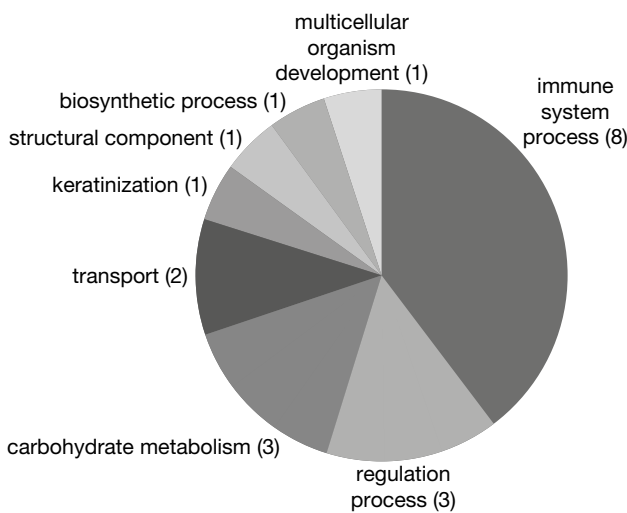

Fig. 2. Distribution of biological processes of proteins found with different expression in human saliva. The protein functions in biological processes categorized according to http://www.uniprot.org.

caries. Analysis of the salivary protein composition showed significantly more abundant concentrations of acidic proline-rich proteins (PRPs), lipocalin, cystatin SN and cystatin S in samples from the caries-free group. In our study we found significantly higher expression of lipocalin 1 and cystatin-SN in the caries-free group and α-amylase in the caries-susceptible group of males, which is in agreement with the results reported by Vitorino et al. (2006), and we assume that these proteins are potential targets for further research of oral health caries.

The present study brings the most detailed gender proteomic comparison of saliva to date. This is the first time that MS quantification was used for this purpose. We discovered 23 gender-related differences (Table 2) (Fig. 3) and assume that these differences could play important roles in the saliva physiology in both genders.

Only a few comparisons of the saliva proteome based on the gender have been performed to date. Lukacs et al. (2011) described sexually-determined proteome differences in the dental caries prevalence. Females were found to exhibit higher prevalence rates than males. The reasons are explained by three factors: earlier eruption of teeth in girls, longer exposure of girls' teeth to a cariogenic oral environment, and pregnancy. The higher incidence of dental caries related to pregnancy is known. There are many factors that are involved in this process, such as higher amount and frequency of consumption of cariogenic diet, reduction of pH of the oral cavity caused by frequent vomiting and decreased attention to maintaining oral hygiene (Christensen et al., 1998; Laine, 2002).

Fleissing et al. (2010) found that gender differences revealed six proteins with significantly higher expression in females. In our results, leukocyte elastase inhibitor (SERPINB1) was observed with significantly higher expression in the caries-susceptible male group and in the group of all males. A second protein, calgranulin A (S100-A8), was found to be up-regulated in the saliva of males in all the gender comparisons. Our results do not agree with those of Fleissing et al. (2010), possibly because of different experimental methods, patient ages, etc.

A cross-sectional study was presented to evaluate inter-individual biochemical variation in unstimulated whole

saliva (18–30 years of age) (Prodan et al., 2015). Females displayed reduced levels of salivary pH, and the protein contents of MUC5B were lower in female subjects compared with male subjects. This is in conformity with our results, showing significantly higher expression of protein MUC5B in the caries-susceptible male group in comparison with the caries-susceptible females (Table 2).

In the study by Li-Hui et al. (2016), where the salivary flow rate was compared, no evident change in the salivary α-amylase was observed. In our work, we found a change in the α-amylase expression, with higher expression in males.

We observed many differences between genders. The saliva protein composition was observed in only one time-point interval, and the results could be influenced by the presence of false-positive identification of differences. The conditions of collection were strictly respected because we wanted to prevent pre-analytical errors. We performed validation of the individual steps of preparation and analyses. In our future work, we would like to extend the number of individuals in the compared groups and present an expanded questionnaire to the volunteers (interindividual and intraindividual variability) with several time-point intervals of sample collection to map the possible effects.

Some correlations were found across the comparisons (DMFT and gender comparisons). We can see, for example, that the protein annexin A1 was found with significantly higher expression in the group of caries-susceptible persons, and this protein was also differently expressed in gender comparisons (annexin A1 was found with significantly higher expression in the male group (comparison of all males vs. all females)).

Our study presents a complex proteomic project, where the saliva protein contents of caries-free and caries-susceptible persons were compared by label-free MS. These results revealed 21 protein differences between caries-susceptible and caries-free persons. These proteins (e.g., with immune and Ca^{2+}-binding functions) could play an important role in oral protection and are promising biomarkers for dental caries and/or for oral health in general. The present study also detected 23 gender-related differences. The specificity of these proteins could play unique roles in the saliva physiology of both genders. Further research including larger groups of the caries-free and caries-susceptible patients is needed.

To date, no similar work has been published describing such a complex proteomic project that included comparisons of the human saliva proteome on the basis of both gender and DMFT by label-free MS.

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