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Salivary proteome characterization of alcohol and tobacco dependents

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ABSTRACT

Background: Alcohol and substances found in tobacco may alter salivary flow and amount of saliva proteins. This study aimed to compare salivary proteins between alcohol dependent smokers and controls.
Methods: This is a case-control study with men older than 18 years of age, matched by age. The alcohol-dependent group was composed by heavy smokers and alcohol consumers. Unstimulated whole saliva was collected from all subjects. Analysis of digested peptides was performed in mass spectrometer. Data were processed using ProteinLynx GlobalServer software. Results were obtained by searching the*Homo sapiens* database from the UniProt catalog. The search tool IBI-IMIM was used to identify candidate proteins for biomarkers.
Results: Alcohol-dependent and control groups were composed of nine participants each, with mean age of 36.89 ± 2.57 and 35.78 ± 1.64 years, respectively. 404 salivary proteins were found in both groups; 282 in the alcohol-dependent. Among the 96 proteins presented in both groups, 32 were up-regulated in the alcohol dependents (i.e. “Hemoglobin subunit beta” and “Forkhead box protein P2” were up-regulated at least 10-fold), 23 were down-regulated (i.e. “Statherin” and “RNA-binding protein 25” were down-regulated at least 10-fold), and 41 presented similar expression in both groups. 71 proteins were candidates for biomarkers of disorders 58 presented in alcohol dependents’ saliva. The most common disorders were neoplasms, genetic, cardiovascular, metabolic and glandular diseases.
Conclusions: Salivary protein profile undergoes strong changes in alcohol and tobacco dependents. 34% of salivary proteins present in alcohol and tobacco dependents were present in controls; 14.5% of them were expressed in similar quantity.

1. Introduction

Saliva and its components are the main supporter of oral physiology. Its lubricating function facilitates speech, chewing, swallowing, taste, and maintaining dental health and local homeostasis (Cho et al., 2017; Holmberg and Hoffman, 2014). The organic elements of saliva, especially proteins, are constantly secreted, maintaining acquired dental pellicle formation, and soft and hard tissues lubrication (Holmberg and Hoffman, 2014; Proctor and Carpenter, 2014). The concentration of secreted proteins may vary depending on some stimuli. One stimulus pathway is the release of norepinephrine by sympathetic nerve endings binding to β1-adrenoceptors. Signaling from parasympathetic nerves and cholinergic stimuli can also contribute to the release of proteins in saliva (Proctor and Carpenter, 2014).

Chemical substances, such as alcohol and substances found in tobacco, may alter salivary flow and the amount of saliva proteins. They usually alter the salivary constitution indirectly, by systemic changes in the patient's physiology. Alcohol also acts through stimuli in local nerve

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endings, resulting in decreased salivary flow and local pH changes (Dukić et al., 2013; Enberg et al., 2001; Gelbier and Harris, 1996).

Variations in salivary protein variety and concentration may alter the acquired pellicle formation on enamel surface, resulting in a non-uniform and without multilayer structure with decreased lubrication properties, which may lead to dental demineralization, often present in alcohol users (Zeng et al., 2017). The most common oral conditions are high caries experience, periodontitis and mucosal lesions, besides alcohol being an important risk factor for the development of head and neck carcinomas (Priyanka, 2017; Zygogianni et al., 2011).

To our knowledge, there is no description in the literature of salivary proteomics of alcohol dependent individuals and smokers. Some studies report variations in specific groups of salivary proteins in alcohol dependents, such as proteins related to the immune system (Waszkiewicz et al., 2012, 2008), Epidermal Growth Factor concentration (Benamouzig et al., 1996), TNF-alfa expression (Slomiany et al., 1997), total protein concentration (Enberg et al., 2001), and salivary amylase activity (Enberg et al., 2001).

Describing the salivary protein profile of alcohol dependents associated with smoking may help to understand which mechanisms lead to unfavorable dental and periodontal conditions, and the higher predisposition to benign and malignant oral mucosal lesions. In addition, salivary protein profile may allow the identification of important systemic biomarkers with current methodologies, using different biological matrices than blood for disease screening. Thus, this study aimed to compare the salivary proteins from alcohol dependent smokers and salivary proteins from non-smokers and non-alcohol dependent users.

2. Materials and methods

This is a case-control study about the salivary protein composition between alcohol dependent smokers, and non-smokers and non-alcohol dependent individuals matched by age.

This survey was approved by the Local Institutional Review Board under protocol number 1.825.659.

2.1. Sample collection and processing

Research volunteers were male patients, older than 18 years of age. The alcohol dependent group was composed by individuals recruited from IPTA – Instituto de Pesquisa e Tratamento do Alcoolismo (Campo Largo, Southern Brazil). Inpatients were selected for rehabilitation due to alcohol abuse, who consumed more than 500 mL of ethyl alcohol per week, smokers, with hospitalization time less than 15 days.

The non-alcohol dependent group was composed by patients attended at the Dental Clinic of the Pontifical Catholic University of Paraná (Curitiba, Southern Brazil), matched by age with the alcohol dependent group. All volunteers were informed about the research arrangements and agreed to participate by signing the Free and Informed Consent Form prior to any procedure.

As exclusion criteria, the participants should not have used illicit drugs within 3 months before sample collection, not have systemic diseases, or acute infection symptoms, and not be using antibiotic or anti-inflammatory drugs. For the non-alcohol dependent group, in addition to the exclusion criteria cited above, smokers and ex-smokers were also excluded, as well as alcohol users who consumed more than 99 mL of ethyl alcohol per week.

Nine participants of each group were selected for the present study. The sample size of the groups was based on a previous proteomic study with saliva (Ventura et al., 2018).

All samples were collected between November 2016 and August 2017.

The volunteers were interviewed in relation to socioeconomic data, licit and illicit drugs use profile, systemic health condition, medication use, oral hygiene and xerostomia. According to the tobacco consumption, smokers were classified as mild (less than 10 cigarettes per day), moderate (10–19 cigarettes per day), and heavy (more than 20 cigarettes per day) (Albini et al., 2017; Chiolero et al., 2006). After the interview, extraoral clinical examination (palpation of lymph nodes and salivary glands) and intraoral evaluations (inspection and palpation of oral mucosa and inspection of teeth and gingival tissue) were performed, according to the World Health Organization (WHO, 1997). In cases of the presence of oral mucosa changes, dental caries or gingival alterations, the patients were addressed to the Dental Clinic at Pontifical Catholic University of Paraná.

The collection of saliva was performed according to previous research (Ventura et al., 2018). All samples were collected from 9 to 11am. All participants were instructed to perform oral hygiene and stay for 45 min without ingesting any type of food or liquid, smoking cigarettes, or making use of chewable objects. After this time, all patients underwent oral rinsing with 20 mL of distilled water and remained in rest for 15 min. Unstimulated whole saliva was collected in a 50 mL Falcon tube immersed in ice. The insoluble components in saliva were separated by centrifugation 3000 × *g* for 15 min at 4 °C. The supernatant was stored in cryogenic tube at −80 °C until samples were processed for proteomic analysis (de Jong et al., 2010; Rhodus et al., 2005; Ventura et al., 2018).

2.2. Preparation of the saliva samples

The protocol of proteins extraction was based in a previous study; no IgG depletion columns were used (Ventura et al., 2018). For the extraction, 100 µL of each collected sample was aliquoted in separate tubes. The aliquots were diluted 1:1 with extraction solution, containing 6 M urea, 2 M thiourea and 50 mM NH_4HCO_3, pH 7.8. After dilution, they were agitated for 10 min at 4 °C, then placed in ultrasonic bath for 5 min and then centrifuged at 14,000 x g at the same temperature for 10 min. This step was repeated twice. After extraction, 3 sample pools were formed. Each pool was composed of 3 samples, chosen by order of collection. The samples were concentrated to 150 µL in Falcon Amicon tubes (Merk Millipore®, Darmstadt, Germany). For reduction of proteins disulfide bridges, 5 mM dithiothreitol (DTT) was used during 40 min at 37 °C and subsequently added 10 mM iodoacetamide for 30 min in the dark to prevent cysteine residues from forming new disulfide bonds. The amino acid chains were cleaved with 2% (w/w) trypsin (Promega®, Madison, WI) for 14 h at 37 °C. Then, 10 µL of 5% acid formic were added. The samples were purified and desalted using the C18 Spin columns (Thermo Fisher Scientific®, Waltham, MA) and 1 µL of each sample was used for the protein quantification by the Bradford method (Bio-Rad®, Hercules, CA). The samples were resuspended in the solution containing 3% acetonitrile and 0.1% formic acid and submitted to Nano Liquid Chromatography Electron Spray Ionization Tandem Mass Spectrometry - LC-ESI-MS/MS (Waters, Wilmslow, UK).

2.3. Shotgun label-free quantitative proteomic analysis

The analysis of tryptic peptides was performed in the nanoACQUITY UPLC system (Waters, Milford, MA) coupled to Xevo Q-TOF G2 mass spectrometer (Waters, Milford, MA). The nanoACQUITY UPLC system was equipped with a HSS T3 type column (Acquity UPLC HSS T3 column 75 µm x150 mm; 1.8 µm) (Waters, Milford, MA), previously equilibrated with mobile phase B, 7% (100% Acetonitrile + 0.1% formic acid). Peptides were separated by linear gradient 7–85% mobile phase B for 70 min with 0.35 µL/min flow rate, the column temperature was maintained at 45 °C. The mass spectrometer was operated in positive ion mode, with 75 min of data acquisition time. Data were processed using ProteinLynx GlobalServer software (PLGS) version 3.03 (Waters, Milford, MA). The identification of saliva proteins was performed using the ion count algorithm incorporated into the software. The results were obtained by searching the *Homo sapiens* database from the UniProt catalog (Universal Protein Resource) in August 2017

(http://www.uniprot.org). All pool samples were analyzed in triplicates (Consortium, 2017; Ventura et al., 2018).

For label-free quantitative proteome, three MS raw files from each group were analyzed using the Protein Lynx Global Service (PLGS, v 2.2.5, Waters Co., Manchester, UK) software. All the proteins identified with a score with confidence higher than 95% were included in the quantitative statistical analysis embedded in the PLGS software. Identical peptides from each triplicate by sample were grouped based on mass accuracy (< 10 ppm) and on time of retention tolerance < 0.25 min, using the clustering software embedded in the PLGS. Difference in expression among the groups was calculated using Monte-Carlo algorithm and expressed as $p < 0.05$ for proteins present in lower abundance and $1\text{-}p > 0.95$ for proteins present in higher abundance, when one location was compared to another.

2.4. Protein classification and bioinformatic analysis

Repeated proteins, fragments and reverse proteins were excluded from the initial data by manual review. The salivary proteins presented in each group (alcohol dependent group and non-alcohol dependent group) were compared and classified according to expression and later classified as up-regulated in the tested group (alcohol dependent group), similar expression in both groups or down-regulated in the tested group.

Proteins were classified by molecular function, cellular component, and biological process involved, according to the terms described by the Princeton University generic gene term mapper (Generic Gene Ontology - GO - Term Finder). The search was performed using the GOA database for *Homo sapiens* (Boyle et al., 2004; Princeton University). The search tool IBI-IMIM was used to identify candidate proteins for biomarkers (Database of disease-related biomarkers) (Bravo et al., 2014; Cho et al., 2017).

3. Results

3.1. Salivary protein profile

Alcohol-dependent and non-alcohol dependent groups were composed of nine participants each, all males, with mean age of 36.89 ± 2.57 and 35.78 ± 1.64 years, respectively. The mean alcohol use time in the first group was 14.89 ± 9.25 years and all participants were heavy smokers, with consumption greater than 20 cigarettes per day. The mean smoking time was 18.89 ± 8.54 years until the time of collection.

The amount of total protein in the triplicates after Bradford analysis were 37.11 µg, 40.43 µg and 52.30 µg in the alcohol dependent group and 33.28 µg, 49.62 µg and 62.00 µg in the non-alcohol dependent group. In total, 404 proteins were found in the saliva from both groups, with 282 presented in the alcohol-dependent group. The way the proteins were distributed in each group can be visualized in Fig. 1.

According to the protein salivary concentration (difference in expression), among the 96 proteins presented in both groups, 32 were up-regulated in the alcohol dependent group, 23 were down-regulated, and 41 proteins presented similar expression in both groups according to the Monte Carlo test. In the saliva of alcohol and tobacco dependents, “Hemoglobin subunit beta” and “Forkhead box protein P2” were the two most up-regulated proteins compared to the control group, with ratios of 17.81 and 10.07, respectively, and “Statherin” and “RNA-binding protein 25” were the two most down-regulated salivary proteins, with ratios of 0.03 and 0.10, respectively (Table 1).

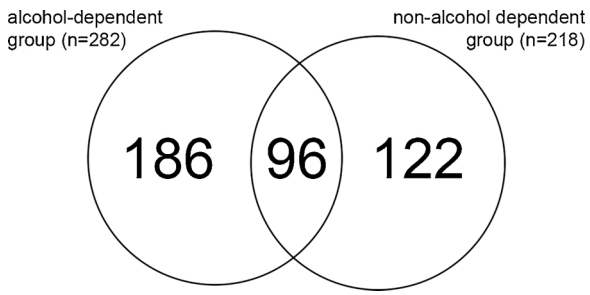

Fig. 1. Venn diagram with the number of proteins detected in saliva of each group.

All identified proteins, including their differences in expression, biological processes, molecular functions and cellular components are described in the supplementary material. The amount of proteins (GO) according to their molecular function, cellular component and biological processes are presented in Fig. 2.

3.2. Salivary biomarkers

The salivary proteins candidates for biomarkers found with IBI-IMIM search tool were divided according to presence and absence in the saliva of each group and by difference in expression (Table 2). Among 404 salivary proteins detected by proteomic analysis, 71 were candidates for biomarkers of local and systemic alterations, with 58 (81.7%) of them presented in the saliva from alcohol dependents individuals.

The most common disorders to which salivary proteins were indicated as candidates for biomarkers are as follows: neoplasms (n = 47), genetic diseases (n = 44), cardiovascular diseases (n = 36), metabolic and glandular diseases (n = 36), hepatobiliary and pancreatic diseases (n = 35) (Fig. 3).

4. Discussion

Chronic and acute alcohol consumption may lead to different changes in body physiology. The amount and variability of proteins presented in saliva may be altered by alcohol abuse, either by local stimuli or systemic physiological changes (Proctor and Carpenter, 2014). Alcohol is responsible for decreasing stimulated and non-stimulated salivary flow (Enberg et al., 2001). The difference of salivary protein composition presented in this study reflects the different habits between alcohol-dependent smokers and non-alcohol dependent groups. Among 404 salivary proteins identified in the two groups, only 96 were present in both of them, of which 41 did not show differences in expression between groups. In addition, the number of salivary proteins candidates for biomarkers from the alcohol-dependent group was higher compared to the salivary proteins from the non-alcohol dependents, especially for neoplastic, cardiovascular, hepatobiliary and pancreatic diseases. However, it is valid to emphasize that the results of this study should be interpreted as changes caused by longtime exposure to alcohol without the acute effects at the time of the analysis.

In this study, proteins presented in both groups represent those typically found in saliva, such as actin, albumin, amylases, hemoglobin chains, immunoglobulin chains, cystatins, histatins, lysozyme, keratins, salivary proline-rich proteins, and statherin. Although the presence of keratin may denote normal differentiation of oral epithelial tissue, some types of keratin may result from contamination by contact between skin and saliva at the time of collection (Baliban et al., 2012; Ventura et al., 2018). The keratins identified in this study were one of type I (keratin 13) and two of type II (keratin 4 and 73). Keratin 13 and 73 are generally found in skin glands and hair follicles, although keratin 13 can also be found in esophageal epithelial and type 4 in the oral mucosa (Consortium, 2017). As mentioned previously, this may occur during the collection procedure, especially in debilitated patients, such as in the present sample.

Molecules involved in the immune response were found with altered concentrations in the saliva from alcohol dependents of the present study. Changes in the immune system of these individuals have been described and reported in other studies (Curtis et al., 2013). Thereafter, exposure to alcohol compromises the immune system action against

Table 1
Proteins identified in saliva of both groups and their differences in expression.

Expression differences	Ratio A/C	Score	Accession Number	Protein name
↑	1.93	1740.62	P68032	Actin alpha cardiac muscle 1
↑	2.12	1753.68	P68133	Actin alpha skeletal muscle
↑	2.16	1740.62	P62736	Actin aortic smooth muscle
↑	2.29	4796.52	P60709	Actin cytoplasmic 1
↑	2.14	4796.52	P63261	Actin cytoplasmic 2
↑	2.46	1740.62	P63267	Actin gamma-enteric smooth muscle
↑	1.34	410.33	P04280	Basic salivary proline-rich protein 1
↑	1.35	4894.05	P01036	Cystatin-S
↑	1.03	3222.1	P01037	Cystatin-SN
↑	17.81	589.65	O15409	Forkhead box protein P2
↑	1.79	238.64	Q86W71	GOLGA4 protein
↑	1.75	268.95	Q13439	Golgin subfamily A member 4
↑	7.61	5534.44	G3V1N2	HCG1745306_ isoform CRA_a
↑	5.05	8580.57	P69905	Hemoglobin subunit alpha
↑	10.07	6003.34	P68871	Hemoglobin subunit beta
↑	4.06	1171.27	P02042	Hemoglobin subunit delta
↑	5.26	387.65	P69891	Hemoglobin subunit gamma-1
↑	4.85	387.65	P69892	Hemoglobin subunit gamma-2
↑	1.52	521.61	O60479	Homeobox protein DLX-3
↑	1.35	432.62	Q86Y46	Keratin_ type II cytoskeletal 73
↑	3.03	443.41	Q6GTX8	Leukocyte-associated immunoglobulin-like receptor 1
↑	2.77	382.08	P31025	Lipocalin-1
↑	1.73	1050.7	P61626	Lysozyme C
↑	2.97	1170.4	Q6S8J3	POTE ankyrin domain family member E
↑	2.89	1170.4	A5A3E0	POTE ankyrin domain family member F
↑	5.53	316.29	Q8IZS8	Voltage-dependent calcium channel subunit alpha-2/delta-3
↑	1.03	1324.57	P09228	Cystatin-SA
↑	4.53	207.1	Q6IQ26	DENN domain-containing protein 5A
↑	1.15	986.28	P12273	Prolactin-inducible protein
↑	2.23	310.65	A6NNI4	Tetraspanin
↑	1.01	27231.79	P04745	Alpha-amylase 1
SE	2.23	305.51	Q12955	Ankyrin-3
SE	1.02	3074.69	P02768	Serum albumin
SE	1.43	485.16	Q75MZ5	Forkhead box P2_ isoform CRA_a
SE	1.04	494.51	Q0PRL4	Forkhead box P2 variant 3
SE	1.03	128.88	Q96QU1	Protocadherin-15
SE	1.12	506.33	O60315	Zinc finger E-box-binding homeobox 2
SE	1.03	411.65	Q9NP78	ATP-binding cassette sub-family B member 9
SE	1.03	255.08	Q9UKM9	RNA-binding protein Raly
SE	1.03	1036.56	P01721	Immunoglobulin lambda variable 6-57
SE	1.04	160.92	Q9BZX4	Ropporin-1B
SE	1.04	274.65	P05161	Ubiquitin-like protein ISG15
SE	1.06	190.4	Q15436	Protein transport protein Sec23A
SE	1.84	387.65	P02100	Hemoglobin subunit epsilon
SE	1.02	333.65	O15021	Microtubule-associated serine/threonine-protein kinase 4
SE	1.08	255.56	Q14232	Translation initiation factor eIF-2B subunit alpha
SE	1.04	202.78	Q96IY1	Kinetochore-associated protein NSL1 homolog
SE	0.97	193.41	A6NDL8	Olfactory receptor 6C68
SE	1.04	237.38	Q15283	Ras GTPase-activating protein 2
SE	1.02	302.1	O14795	Protein unc-13 homolog B
SE	1.03	518.87	Q6ZSG1	E3 ubiquitin-protein ligase RNF165
SE	1.15	196.81	Q5THK1	Protein PRR14L
SE	1.04	278.7	Q8N9H6	Uncharacterized protein C8orf31
SE	1.03	197.98	O00231	26S proteasome non-ATPase regulatory subunit 11
SE	1.03	348.26	Q15582	Transforming growth factor-beta-induced protein ig-h3
SE	1.06	250.34	Q86XF7	Zinc finger protein 575
SE	1.02	284.48	O95780	Zinc finger protein 682
SE	1.02	330.32	Q8WXB4	Zinc finger protein 606
SE	1.03	297.54	B4E159	Zinc finger protein 721
SE	1.01	260.6	Q14002	Carcinoembryonic antigen-related cell adhesion molecule 7
SE	0.99	260.56	A6NEH6	Transmembrane protein 247
SE	0.98	316.62	Q765P7	MTSS1-like protein
SE	1.00	345.48	H0YIY4	Gephyrin (Fragment)
SE	0.98	365.38	P01034	Cystatin-C
SE	1.01	226.42	O15375	Monocarboxylate transporter 6
SE	1.00	301.73	Q5VSP4	Putative lipocalin 1-like protein 1
SE	0.94	525.96	Q96DA0	Zymogen granule protein 16 homolog B
SE	0.99	19911.77	P19961	Alpha-amylase 2B
SE	0.84	302.47	Q6NXT4	Zinc transporter 6
SE	0.84	330.69	Q9BZF1	Oxysterol-binding protein-related protein 8
SE	0.95	763.32	P13646	Keratin type I cytoskeletal 13
SE	0.83	1158.14	Q9P2R6	Arginine-glutamic acid dipeptide repeats protein
SE	0.76	1062.35	P0CG38	POTE ankyrin domain family member I
↓	0.61	545.37	P18615	Negative elongation factor E

(*continued on next page*)

Table 1 (*continued*)

Expression differences	Ratio A/C	Score	Accession Number	Protein name
↓	0.55	181.39	P0CG39	POTE ankyrin domain family member J
↓	0.87	2956.72	P02812	Basic salivary proline-rich protein 2
↓	0.75	736.45	Q562R1	Beta-actin-like protein 2
↓	0.47	240.22	Q9NQY0	Bridging integrator 3
↓	0.40	172.46	P23280	Carbonic anhydrase 6
↓	0.40	1242.75	P15515	Histatin-1
↓	0.36	1349.11	P15516	Histatin-3
↓	0.70	2608.5	P01876	Immunoglobulin heavy constant alpha 1
↓	0.70	2252.35	P01877	Immunoglobulin heavy constant alpha 2
↓	0.54	1650.72	P01591	Immunoglobulin J chain
↓	0.31	349.5	P20592	Interferon-induced GTP-binding protein Mx2
↓	0.87	675.64	P19013	Keratin type II cytoskeletal 4
↓	0.40	688.79	Q9NYZ2	Mitoferrin-1
↓	0.95	18247.75	P04746	Pancreatic alpha-amylase
↓	0.72	592.56	P01833	Polymeric immunoglobulin receptor
↓	0.14	265.2	Q5W0V3	Protein FAM160B1
↓	0.10	328.02	P49756	RNA-binding protein 25
↓	0.28	3979.31	P02810	Salivary acidic proline-rich phosphoprotein 1/2
↓	0.22	190.19	H0YDR5	Single Ig IL-1-related receptor (Fragment)
↓	0.59	251.96	Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1
↓	0.03	1767.57	P02808	Statherin
↓	0.76	6879.29	P02814	Submaxillary gland androgen-regulated protein 3B
↓	0.15	579.79	Q8TAF7	Zinc finger protein 461

Note: SE = similar expression compared to control group; ↑ = up-regulated (1-p > 0.95); ↓ = down-regulated (p < 0.05); Ratio A/C = ratio between alcohol-dependent and control group proteins.

infections, resulting in longer recovery times, longer hospitalization time, and higher morbidity and mortality numbers when compared to non-intoxicated individuals (Curtis et al., 2013; Messingham et al., 2002). In this study, among the main proteins involved with the immune system with lower concentrations in alcohol dependents saliva are the various immunoglobulin chains (apart from Immunoglobulin lambda variable 6–57) and histatins 1 and 3. Conversely, there was an increase of C-lysozyme in the alcohol dependents saliva, which can be explained as an innate immune system compensation, caused by increased phagocytosis by local macrophages, and the non-detection of mucin-7 in the saliva of this group, which show bacterial clearance function in the mouth (Consortium, 2017).

The proteins presented in higher concentration in the alcohol dependents saliva varied, highlighting actin, cystatine, hemoglobin and alpha-amylase. Actin are intracellular cytoplasmic filaments which are part of the composition of the cytoskeleton. They are proteins associated with motility and support the cellular structure. They were also identified as possible biomarkers for the differentiation between premalignant and malignant lesions in the oral mucosa (de Jong et al., 2010). This increased concentration may be associated with alcohol-dependent group habits, since alcohol and tobacco are substances known to be carcinogenic. One type of malignant disease caused by alcohol and tobacco use is oral cancer (Secretan et al., 2009). Further studies are needed to confirm salivary concentrations of actin associated with differentiation between premalignant and malignant lesions in oral mucosa, as inflammatory processes that causes tissue necrosis can release actin filaments in saliva (Blotnick et al., 2017; Geijtenbeek, 2012).

Hemoglobin is a blood protein responsible for oxygen transport to various tissues. The increased presence in alcohol-dependents saliva may be associated with tissue injury and blood hemolysis. In addition, chronic alcohol users may develop macrocytic anemia due to lack of nutrients, especially folate. The ethanol concentrations in bloodstream may destabilize the erythrocyte membrane, leading to intravascular hemolysis (Andersen et al., 2012; Tyulina et al., 2000). Increased salivary hemoglobin may be harmful to oral mucosa, considering its high potential to cause damage to mucosa, which occurs due to the presence of heme group, which is quite reactive. In intravascular space, hemoglobin causes endothelial dysfunction, vascular damage and inflammation (Schaer et al., 2013).

Still involving the participation of red blood cells and related enzymes, mitoferrin 1 appears in low concentration in saliva of alcohol dependents in this study. It is an enzyme responsible for transporting iron through the mitochondrial membranes of erythroblasts. It is usually expressed in large quantities in hematopoietic tissues, and its deficiency makes it difficult to incorporate iron into the heme group, which may result in hypochromic anemia and differentiation delay of immature erythroblasts (Ye and Rouault, 2010).

Proteins that strongly adhere to the enamel pellicle are mucins, statherins, and proline-rich proteins. Acute alcohol consumption causes deformations in film composition (Zeng et al., 2017). In the present study, decreased statherins concentration was detected in alcohol dependents saliva with chronic use. In addition to statherins, several proline-rich proteins were present in decreased concentration or were absent; only 1 (basic salivary proline-rich protein 1) was present in higher concentration in the alcohol-dependents saliva, and mucin 7 was only found in the saliva of non-alcohol dependents (Douglas et al., 1991; Elangovan et al., 2007; Tabak, 1995; Zeng et al., 2017). Decreased statherin is also associated with the presence of dental calculus, possibly due to alterations related to acquired pellicle (Pateel et al., 2017).

Carbonic anhydrase is an enzyme that controls the pH by reversibly catalyzing hydration of carbon dioxide. The type 6 enzyme, described in the supplementary material, is the enzyme commonly found in saliva. It was found with decreased concentrations in the alcohol-dependent group, which may result in pH salivary changes even after alcohol use. It also seems to be involved in taste (Hassan et al., 2013; Lindskog, 1997).

In the present study, the number of proteins candidates for biomarkers in the alcohol dependent group was high, mainly in neoplastic, cardiovascular, hepatobiliary and pancreatic diseases. The fact that the group tested was composed of alcohol-dependent smokers may have contributed to an increased number of salivary biomarkers, since, according to some epidemiological studies (Corrao, 2004; Fagerström, 2002), the number of systemic changes in this population is higher than in healthy individuals. The main diseases related to alcohol abuse are neoplastic lesions in mouth, pharynx, esophagus, larynx, colon, rectum, liver, and breast. The non-neoplastic diseases are hypertension, coronary disease, ischemic and hemorrhagic stroke, gastroduodenal ulcer, liver cirrhosis and chronic pancreatitis, as well as health injury caused

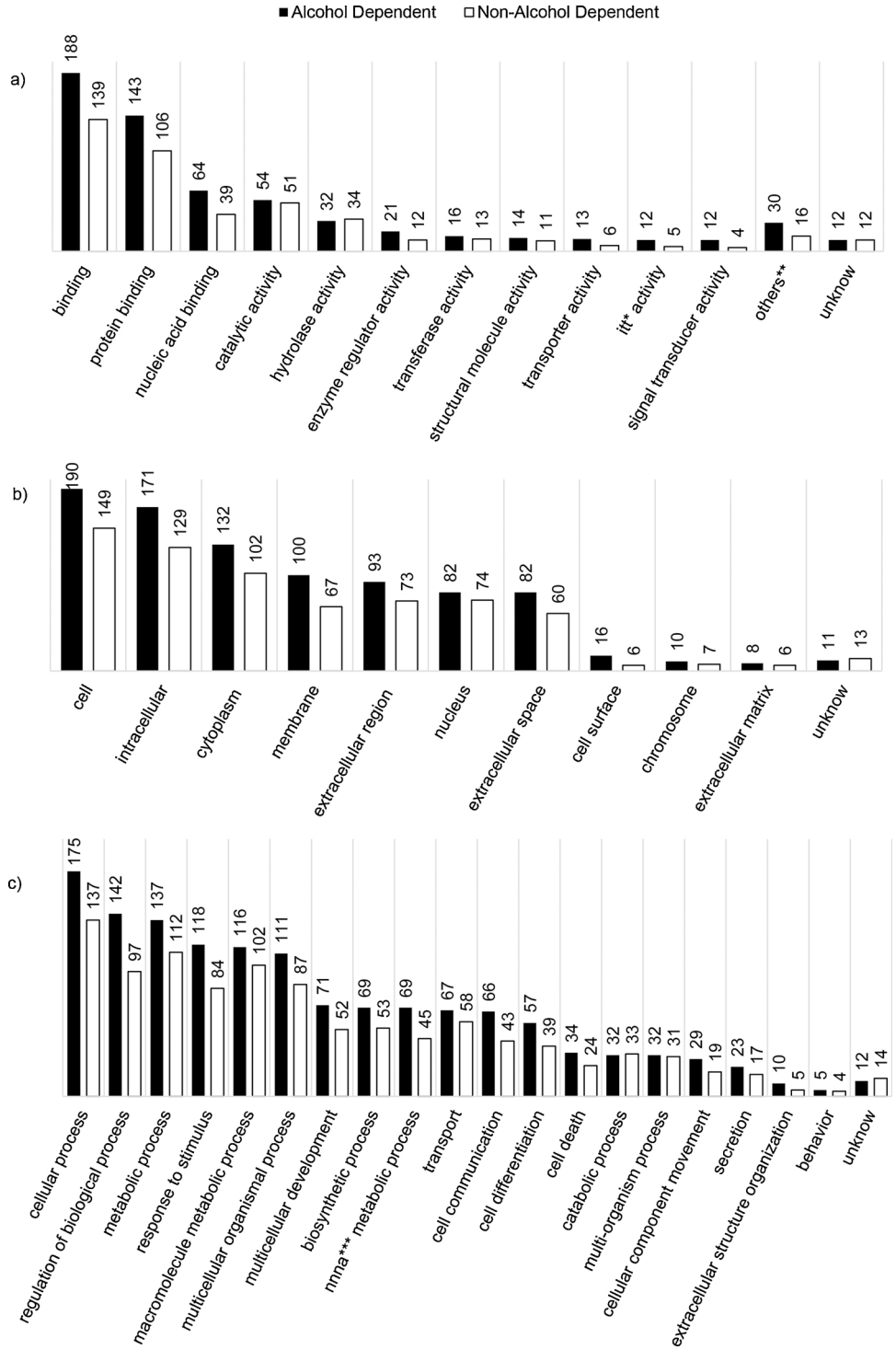

Fig. 2. Number of proteins divided by group and classified by molecular function (a), cellular component (b), and biological processes involved (c).
Note: *itt = ion transmembrane transporter; **others = kinase activity, oxidoreductase activity, channel activity, lyase activity, helicase activity, ligase activity, antioxidant activity, translation regulator activity, electron carrier activity, protein transporter activity, isomerase activity; ***nnna = nucleobase, nucleoside, nucleotide and nucleic acid.

by accidents and violence (Corrao, 2004). Neoplastic lesions for smokers are also common, such as neoplastic disease of the esophagus, lung, bladder, kidney, stomach and pancreas. As well as non-neoplastic diseases, such as chronic obstructive pulmonary disease, coronary disease, stroke, peripheral vascular disease and ulcer (Fagerström, 2002). Despite this, it is important to emphasize that the presence of salivary biomarkers does not indicate the presence of disease, since there are several biomarkers that are proteins found in healthy patients. It will only direct the diagnosis of disease when proteins present changes, for example, high or low concentration, or chain mutations which lead to conformational changes in protein structure (FDA-NIH Biomarker Working Group, 2016). Describing these proteins and their differences in expression between groups is extremely important to serve as basis for new researches that focuses on biological detection and biomarkers description of some diseases.

Biomarkers are biological molecules used as indicators of normal or

Table 2
Proteins candidates for biomarkers per group and divided by difference in expression.

Salivary Protein Expression	Proteins candidate for biomarkers n (%)	Total proteins n
Only in alcohol-dependent group	32 (17.2)	186
Only in non-alcohol dependent group	13 (10.6)	122
Similar expression in both groups	8 (19.5)	41
Up-regulated proteins in alcohol-dependent group in relation to non-alcohol dependent group	14 (43.7)	32
Down-regulated proteins in alcohol-dependent group in relation to non-alcohol dependent group	4 (17.4)	23

pathogenic biological processes or monitoring physiological events on drugs action. There are several types, such as diagnostic, monitoring and response biomarkers, among others (FDA-NIH Biomarker Working Group, 2016; Ghallab, 2018). The search tool of IBI-IMIM group was elaborated from a research data collection tool. There is vast amount of information on biomarkers related to physiological and pathological changes in scientific literature; such tool provides gene identification related to possible biomarkers by means of MeSH terms data mining in MEDLINE publications (Bravo et al., 2014). However, it is important to point out weaknesses in this tool, since it is an automatic mining tool that uses specific algorithms. It does not guarantee that all proteins candidates for biomarkers reported in the scientific research are present in these data, neither all listed proteins will prove to be biomarkers; for example, some studies refer biomarkers to the appearance of secondary diseases not directly related to primary disease (data not shown). Biomarkers pointed by this tool must be manually reviewed before confirmation.

The fact that no female population was assessed is one limitation of the present study. However, based on the fact that the inclusion of female patients could present changes in salivary protein secretion due to hormonal variations (Abrao et al., 2014; Rukmini et al., 2018) and that only male patients over 18 years of age are attended at IPTA, we assumed the inclusion of only male subjects in the present study. Besides this, the individual health condition was obtained by self-report, therefore with possible bias. To reduce the risk of bias, questions regarding the continuous use of medications were also performed, which helped to clarify possible unreported diseases. Meanwhile, we believe that hepatic, renal and cardiovascular damage in discrete degrees may have been present in our sample but are representative of chronic alcohol and tobacco users. Finally, the alcohol-dependent group was composed by heavy smokers, which make it unfeasible to know which proteins were changed due to the alcohol and which due to the smoking habit. In a pilot study, we observed that most of the alcohol-dependent volunteers were smokers classified as heavy users of tobacco. Thus, we decided to assume that tobacco consumption is a characteristic of alcohol dependent population due to its high frequency and amount, and that the changes caused by the synergism of both drugs may be even more important than the changes caused by only one.

Proteomic analysis is a powerful tool for protein description of complex biological matrices such as saliva. Therefore, standardization is required in preanalytical phase (Kwasnik et al., 2016; Ventura et al., 2018). Age-paired groups was an option to reduce bias, as well as the option to perform the study only with male volunteers. Conversely, the protein variety between both groups may represent just part of the changes caused by chronic alcohol consumption. Less evident is the difference demonstrated by ethnic variability present in the population, or daily habits, either hygiene habits and other factors that may interact in individual's physiology. Therefore, protein differences found in each

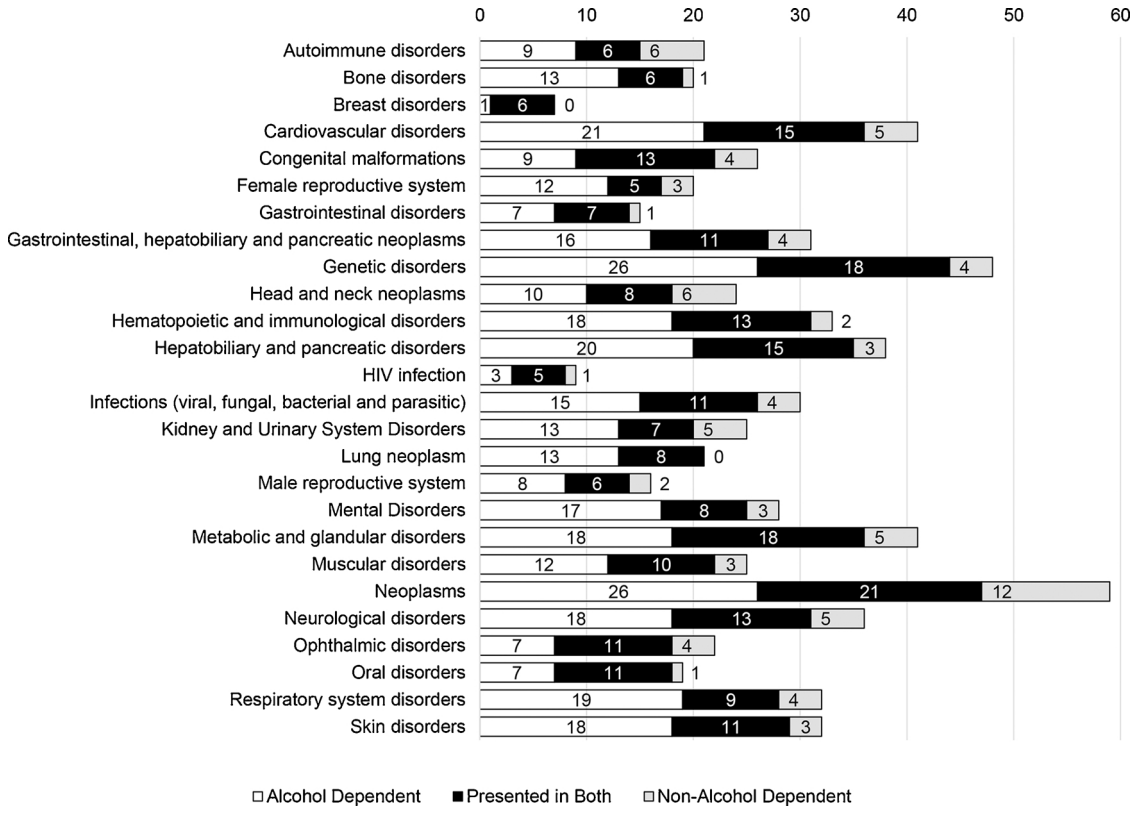

Fig. 3. List of proteins candidates for biomarkers, classified by groups of diseases present in each group.

group may be a limitation presented by this methodology, in the detection of proteins in low concentrations. The use of pools with small numbers of patients in proteomic studies can reduce such variations, avoiding the dilution of proteins present in few individuals (Ventura et al., 2018; Winck et al., 2015).

With more robust studies involving proteomic analysis associated with decreased material costs, this analysis may become more frequent in clinical testing laboratories.

5. Conclusions

The salivary protein constitution undergoes strong changes in alcohol and tobacco dependents. The applied methodology allowed identification of 404 proteins in both alcohol dependent and non-alcohol dependent groups. Only 34% of proteins present in the saliva of alcohol-dependent smokers were present in the saliva of non-dependents, and 14.5% of them were expressed in similar quantity. In the saliva of alcohol and tobacco dependents, “Hemoglobin subunit beta” and “Forkhead box protein P2” were up-regulated at least 10-fold, and “Statherin” and “RNA-binding protein 25” were down-regulated at least 10-fold compared to controls. In addition, the number of candidates for biomarkers in the saliva of alcohol-dependent smokers was higher than that on the saliva of non-alcohol dependents, with a high number of biomarkers for disorders compatible with this behavior, especially neoplasms, cardiovascular disorders, hepatobiliary and pancreatic disorders.

Contributors

The contributions made by each author to the four following conditions: 1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content and (3) final approval of the version to be published, and (4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, are listed below:

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Declaration of Competing Interest

No conflicts were declared.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.drugalcdep.2019.06.013.

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