Salivary Exoglycosidases as Markers of Alcohol Dependence

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Abstract — Background: Some salivary markers of alcohol abuse/dependence have been proposed so far: aminotransferases, gamma-glutamyltransferase, ethanol, ethyl glucuronide, ethyl sulfate, sialic acid, β-hexosaminidase A, oral peroxidase, methanol, diethylene/ethylene glycol, α-amylase, clusterin, haptoglobin, heavy/light chains of immunoglobulins and transferrin. Aim: To investigate the effect of chronic alcohol drinking and smoking on the activity (pKat/ml) and output (pKat/min) of salivary lysosomal exoglycosidases: α-fucosidase (FUC), α-mannosidase (MAN), β-galactosidase (GAL), and β-glucuronidase (GLU), and their applicability as markers of alcohol dependence. Methods: The activity of FUC, MAN, GAL and GLU was measured colorimetrically in the saliva of healthy social drinkers, alcohol-dependent non-smokers and alcohol-dependent smokers. Results: We observed an increased salivary activity of FUC, GAL, GLU and MAN, as well as an increased output of GAL and GLU, in comparison with controls. The highest increase in the activity/output was found in salivary GLU and MAN (GLU, even 7- to 18-fold), and the least in GAL. We found an excellent sensitivity and specificity and a high accuracy (measured by the area under the ROC curve) for salivary FUC, GAL and MAN activities. The salivary GLU activity positively correlated with the number of days of last alcohol intoxication. Salivary activity of FUC, GAL and MAN, but not GLU, positively correlated with the periodontal parameters such as gingival index and papilla bleeding index. Conclusions: Although we found an excellent sensitivity and specificity as well as a high accuracy for the salivary activity of FUC, GAL and MAN, the GLU activity seems to be mostly applicable as a marker of chronic alcohol drinking (alcohol dependence).

INTRODUCTION

Epidemiological studies estimate the prevalence rate of 12-month alcohol dependence in 2 to 4% of the population (Waszkiewicz et al., 2010a, 2013a). Approximately 80% of alcoholics smoke cigarettes (Romberger and Grant, 2004).

There is increasing number of new diagnostic biomarkers of alcohol abuse/dependence in the literature. The most commonly used new blood or urinary biomarkers are 5-hydroxytryptophol, fatty acid ethyl esters, ethyl glucuronide (EtG), phosphatidyl ethanol, ethyl sulfate (EiS), mitochondrial aspartate aminotransferase, carbohydrate-deficient transferrin (CDT), acetaldehyde adducts, β-hexosaminidase (HEX) and sialic acid. There are also other markers associated with alcohol abuse such as acetaldehyde, acetate, methanol, alpha-amino-n-butyrice acid, dolichol and some proteins, e.g. immunoglobulin A. The sensitivity and specificity of new markers are generally higher than those of traditional biomarkers such as gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) or the mean corpuscular volume (MCV) (Waszkiewicz et al., 2010a,b).

The use of saliva for the diagnosis of substance abuse/dependence and monitoring of substance levels, including alcohol and tobacco, has received increasing attention in the recent years (Soo-Quee Koh and Choon-Huat Koh, 2007; Waszkiewicz et al., 2013b,c). There is nothing unusual in that, as saliva contains a wide array of constituents that are very sensitive to toxic substances, and reflects real-time level of biomarkers. Worse real-time urinary biomarker levels compared with salivary biomarker levels are generally attributable to the urine storage in the bladder after alcohol consumption. There are also some advantages of using saliva instead of blood in clinical practice, as non-invasive saliva collection is devoid of stress, with no risk of needlestick injuries and easy to perform, and self-collection of saliva sample is possible after simple instructions (Høiseth et al., 2010; Waszkiewicz et al., 2013b,c).

For the detection of alcohol abuse/dependence, determination of salivary aminotransferases and GGT, ethanol, EtG, EtS, sialic acid, β-hexosaminidase A (HEX A) and oral peroxidase (OPO), as well as indirect methods of detecting ethanol poisoning, including congeners such as methanol and diethylene and ethylene glycol, has been proposed (Tu et al., 1992; Pönniö et al., 1999; Shin et al., 2008; Heberlein et al., 2010; Høiseth et al., 2010; Shivashankara et al., 2011;Waszkiewicz et al., 2012a, 2013b). Some of the salivary glycoproteins, such as α-amylase, clusterin, haptoglobin, heavy and light chains of immunoglobulins and transferrin, also seem to be worthy of detailed glycosylation analysis in the detection of ethyl alcohol dependence (Kratz et al., 2013). For the detection of exposure to tobacco smoke, measurements of nicotine and its metabolites, cotinine, or thiocyanate, in the saliva have been proposed (Galanti, 1997; Tricker, 2006; Soo-Quee Koh and Choon-Huat Koh, 2007; Waszkiewicz et al., 2013b,c).

Alpha-mannosidase, FUC, GLU and GAL are lysosomal exoglycosidases involved in the degradation of the oligosaccharide chains of glycoconjugates, such as glycoproteins, glycolipids and proteoglycans, through the release of monosaccharides from non-reducing ends of their oligosaccharide chains (Waszkiewicz et al., 2009). As our earlier study (Waszkiewicz et al., 2013b) has shown that salivary β-hexosaminidase (its isoenzyme HEX A), the most active of exoglycosidases, may be a valuable marker of chronic alcohol drinking in alcoholics (smoking and non-smoking), the aim of this study was to investigate the effect of chronic alcohol drinking on the activity of salivary α-fucosidase, α-mannosidase, β-galactosidase, and β-glucuronidase, and their applicability as an alcohol consumption as well as alcohol dependence markers.
METHODS

Participants
Sixty males were recruited to the study: 25 healthy social drinkers (Group C) aged 33–55 years [mean (SD): 42 (7)] without a history of alcohol abuse or smoking, 10 alcohol-dependent non-smoking (ANS) individuals aged 33–57 years [46 (5)], and 25 alcohol-dependent smoking (AS) individuals aged 29–61 years [44 (11)]. ANS and AS group participants were recruited from the Unit of Treatment of Alcohol Withdrawal Symptoms. The chronic alcohol-drinking period before hospital admission ranged from 3 to 10 days [7 (2)] in ANS and from 3 to 65 days [19 (19)] in AS group. During intoxication, ANS subjects drank 100–200 g of alcohol per day [167 (37)], whereas AS subjects drank 150–650 g of alcohol per day [216 (122)]. Alcohol-dependent persons met the criteria for the alcohol and nicotine (AS) or only for alcohol dependence (ANS), according to the ICD-10 and DSM-IV criteria. The time of alcohol dependence ranged from 2 to 20 years [11 (7)] for ANS and from 2 to 30 [13 (8)] for AS group. The time of nicotine dependence ranged from 8 to 40 years [21 (8)] for AS subjects, and they smoked 4–35 [18 (7)] cigarettes per day.

Procedures
Ethical issues
The study was approved by the local Bioethical Committee (R-I-003/289/2005) and conducted in accordance with the Helsinki Declaration. Informed written consent was obtained from all the participants after explanation of the nature, purpose and potential risks of the study.

Data and sample collection
Material in the Unit of Treatment of Alcohol Withdrawal Symptoms was collected at the second day of the alcohol abstinence period. A checkup of the oral cavity was done by one qualified dentist in artificial light, by using a dental mirror and a probe. Following the WHO criteria, the dental state was qualified dentist in artificial light, by using a dental mirror and a probe. Following the WHO criteria, the dental state was

RESULTS

Descriptive characteristics of smoking and non-smoking alcoholics
There were no significant differences in age between the groups. Smoking alcohol-dependent persons had a slight tendency to longer-time drinking (days of last drinking period) of alcohol than non-smoking alcohol-dependent subjects ($P = 0.188$).
There were no significant differences in the amounts of alcohol (g) consumed and the duration of alcohol dependence between ANS and AS groups (respectively, $P = 0.226$ and $P = 0.387$).

**Oral cavity parameters**

We found significant decrease in the SF in alcoholic (ANS and AS) groups in comparison with control C, and no difference in the SF between ANS and AS groups (Table 1). There were no significant differences between C, ANS and AS groups in the salivary protein content (Sp).

We also found a slight tendency to worsening dental state and significantly worse dental state in ANS and AS groups, respectively, when compared with controls. The periodontal state (reflected by GI and PBI indexes) was significantly worse in ANS and AS groups than in controls. There were no significant differences between oral parameters in smoking and non-smoking alcohol-dependent subjects (Table 1).

In the ANS group, we found negative correlations of DMFT and PBI as well as positive correlations of GI and the time period of denture wearing (TPDW), with the activity of salivary MAN ($r = -0.811$, $P = 0.049$ for DMFT; $r = -0.948$, $P = 0.013$ for PBI; $r = 0.904$, $P = 0.012$ for GI; $r = 0.908$, $P = 0.014$ for TPDW, respectively). The correlations between salivary protein (Sp) and age ($r = 0.745$, $P = 0.033$), DMFT ($r = 0.824$, $P = 0.043$) and number of days of last alcohol intoxication ($r = -0.810$, $P = 0.014$) were also found. The SF values negatively correlated with salivary GAL activity ($r = -0.981$, $P < 0.001$) as well as salivary FUC activity ($r = -0.763$, $P = 0.027$), and had a tendency to negative correlation with Sp ($r = -0.625$, $P = 0.096$). The DMFT index negatively correlated with salivary FUC and GLU output ($r = -0.811$, $P = 0.049$ for FUC, and $r = -0.828$, $P = 0.043$ for GLU, respectively).

In the AS group, we found a positive correlation of DMFT with salivary GAL activity ($r = 0.441$, $P = 0.045$) as well as between GI and TPDW ($r = 0.713$, $P = 0.006$), salivary GAL activity ($r = 0.502$, $P = 0.040$) and output ($r = 0.520$, $P = 0.046$), and a tendency to correlate with the salivary FUC output ($r = 0.490$, $P = 0.063$).

**Salivary FUC, GAL, GLU and MAN activity**

The salivary activity of FUC, GAL, GLU and MAN was significantly higher in ANS and AS groups than in controls (Fig. 1). There were no significant differences in activity of exoglycosidases between ANS and AS groups. The output of salivary GAL and GLU was significantly higher in ANS and AS groups than in controls, whereas no significant differences in output of salivary FUC and MAN activity were noted between alcoholics and controls. There were no significant differences in the output of salivary exoglycosidases between ANS and AS groups.

The median value of activity of salivary FUC was higher by 142% in ANS and by 94% in AS, in comparison with Group C. In comparison with Group C, the median of salivary GAL activity was higher by 35% in ANS and by 26% in AS, salivary GLU activity was 18 times higher in ANS and 19 times higher in AS (by 1800% and 1900%), and salivary MAN activity was higher by 300% in ANS and by 280% in AS.

The median value of output of salivary FUC was higher by 67% in ANS and by 12% in AS, when compared with Group C. The median output of salivary GAL was lower by 43% in ANS and by 68% in AS, GLU was 11 times higher in ANS and more than 7 times higher in AS (1148% and 748%, respectively), and salivary MAN output was higher by 100% in ANS and by 17% in AS, in comparison with Group C.

In the ANS group, the number of days of last alcohol intoxication positively correlated with salivary GLU activity ($r = 0.801$, $P = 0.016$), and the amount of consumed alcohol per day (g) positively correlated with salivary GAL output ($r = 0.677$, $P = 0.05$). The duration of abstinence syndrome (in hours) negatively correlated with the salivary output of FUC, GAL, GLU and MAN ($r = -0.788$, $P = 0.020$ for FUC; $r = -0.793$, $P = 0.018$ for GAL; $r = -0.783$, $P = 0.017$ for GLU and $r = -0.768$, $P = 0.018$ for MAN). We did not find any significant correlations between duration and amount of cigarettes smoking and activity or output of any of the salivary exoglycosidase.

In the AS group, we did not find any significant correlations between the amount/duration time of alcohol drinking or smoking as well as the time of dependence and the activity or output of any of the investigated salivary exoglycosidase.

**Serum AST and ALT**

The mean serum AST activity (U/l) was 55 (11–153) in ANS and AS groups, respectively. The mean serum ALT activity (U/l) was 46 (9–118) and 56 (11–153) in ANS and AS groups, respectively. The activity of serum AST had strong tendency to be higher in ANS than in AS group. The activity of serum ALT had slight tendency to be higher in AS than in ANS group (Mann–Whitney U-test). In patients with biochemical evidence of liver dysfunction (serum AST and ALT over the normal range), we did not find any correlations between serum AST or ALT markers and any of the investigated salivary exoglycosidase (Spearman’s rank correlation).
Fig. 1. The activity (a, c, e and g), and output (b, d, f and h) of salivary α-fucosidase (FUC), α-mannosidase (MAN), β-galactosidase (GAL), β-glucuronidase (GLU) in controls (C), alcohol-dependent non-smokers (ANS) and alcohol-dependent smokers (AS); $P < 0.05$ is statistically significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

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Diagnostic accuracy of the salivary FUC, GAL, GLU and MAN activity in smoking and non-smoking alcohol-dependent persons

In ANS group, salivary FUC, GLU and MAN activity had excellent sensitivity and specificity, whereas salivary GAL activity had poor sensitivity and specificity. In AS group, only the salivary MAN activity had excellent sensitivity and specificity; salivary FUC and GLU activities had excellent sensitivity and only good specificity, whereas salivary GAL activity had good sensitivity and failed specificity (Table 2).

The diagnostic test accuracy in ANS group, measured by the area under the ROC curve (AUC), was excellent for the activity of salivary FUC, MAN and GLU, and fair for salivary GAL activity. In the AS group, salivary FUC, MAN and GLU activity had the excellent accuracy, whereas accuracy of salivary GAL activity was poor. There were no differences between accuracy of salivary FUC and MAN activity concentrations in both ANS and AS groups, and these tests had higher accuracy than salivary GLU activity in both the alcoholic groups (Table 3; Fig. 2).

**DISCUSSION**

Earlier, our studies found that the activity of salivary exoglycosidases, including FUC, GAL, GLU and HEX, increases after single occasional drinking session (binge drinking), whereas MAN remained within the preconsumption level (Waszkiewicz et al., 2008, 2009). Binge drinking increased also the serum/plasma activity of GAL, HEX and MAN, and the urinary activity of FUC and HEX (Waszkiewicz et al., 2008, 2013d). In chronic alcohol drinkers (alcoholics),

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### Table 2. Diagnostic accuracy and cutoff values for salivary activity of exoglycosidases (FUC, GAL, GLU and MAN) in alcohol-dependent non-smoking (ANS) and alcohol-dependent smoking (AS) persons

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cutoff value&lt;sup&gt;a&lt;/sup&gt; activity</th>
<th>ANS</th>
<th>AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUC</td>
<td>40.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GAL</td>
<td>46.1</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>GLU</td>
<td>52.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MAN</td>
<td>52.8</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cutoff values for salivary FUC, GAL, GLU and MAN activities (pKat/ml) were obtained for alcoholic individuals by ROC analysis.

### Table 3. The area under the ROC curve (AUC) and comparison of AUC between salivary activity of FUC, GAL, GLU and MAN, in alcohol-dependent non-smoking (ANS) and alcohol-dependent smoking (AS) persons

<table>
<thead>
<tr>
<th>Group</th>
<th>AUC (SE)</th>
<th>FUC</th>
<th>GAL</th>
<th>GLU</th>
<th>MAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS Activity</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>GAL</td>
<td>0.793 (0.07)</td>
<td>0.689 (0.06)</td>
<td>0.940 (0.02)</td>
<td>0.808 (0.01)</td>
<td>0.808 (0.01)</td>
</tr>
<tr>
<td>GLU</td>
<td>0.974 (0.02)</td>
<td>0.808 (0.01)</td>
<td>0.808 (0.01)</td>
<td>0.808 (0.01)</td>
<td>0.808 (0.01)</td>
</tr>
<tr>
<td>MAN</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0 (0.0)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SE; P-value of < 0.05 is considered statistically significant; *P < 0.05, **P < 0.01, ***P < 0.001; ns, non-statistically significant.

**Diagnostic accuracy of the salivary FUC, GAL, GLU and MAN activity in smoking and non-smoking alcohol-dependent persons**

In ANS group, salivary FUC, GLU and MAN activity had excellent sensitivity and specificity, whereas salivary GAL activity had poor sensitivity and specificity. In AS group, only the salivary MAN activity had excellent sensitivity and specificity; salivary FUC and GLU activities had excellent sensitivity and only good specificity, whereas salivary GAL activity had good sensitivity and failed specificity (Table 2).

The diagnostic test accuracy in ANS group, measured by the area under the ROC curve (AUC), was excellent for the activity of salivary FUC, MAN and GLU, and fair for salivary GAL activity. In the AS group, salivary FUC, MAN and GLU activity had the excellent accuracy, whereas accuracy of salivary GAL activity was poor. There were no differences between accuracy of salivary FUC and MAN activity concentrations in both ANS and AS groups, and these tests had higher accuracy than salivary GLU activity in both the alcoholic groups (Table 3; Fig. 2).

Fig. 2. Area under the curve for the activity of salivary α-fucosidase (FUC), α-mannosidase (MAN), β-galactosidase (GAL), β-glucuronidase (GLU) in alcohol-dependent non-smokers (a) and alcohol-dependent smokers (b).
increased activity of salivary HEX A isoenzyme; serum/plasma activity of FUC, GLU, HEX (and HEX-7 isofroms), MAN and GAL (animal study) as well as urinary HEX activity were reported. Some of exoglycosidases such as salivary, serum and urinary HEX, and serum MAN, as well as urinary product of FUC activity, α-fucosidase, were promoted as markers of chronic alcohol drinking (alcoholism) (Geokas and Rinderknecht, 1973; Wehr et al., 1991; Yamauchi et al., 1993; Stowell et al., 1997; Waszkiewicz et al., 2010b, 2011a, 2013b, d; Maenhout et al., 2013). When serum and urinary exoglycosidases were proposed as markers of alcohol dependence, liver damage was described as a reason of their activity increase in the body fluids (Waszkiewicz et al., 2010b, 2012a).

As it was found earlier that the salivary activity of isoenzyme HEX A may be a possible marker of alcohol dependence, even in smokers (Waszkiewicz et al., 2013b), we checked other exoglycosidases in the saliva of alcohol-dependent smokers and non-smokers. In the present study, we found increased salivary activity of FUC, GAL, GLU and MAN, as well as the salivary output of GLU and GAL, in the saliva of smoking and non-smoking alcohol-dependent persons, in comparison with the controls (Fig. 1). The glycosylation changes are known in heavy alcohol drinkers. Desialylation due to the inhibition of sialyltransferase and induction of plasma sialidase resulted in increased levels of an excellent alcohol abuse marker: CDT (Waszkiewicz et al., 2012b). The hyposialylated forms of transferrin with terminal galactose residues were found to be less eliminated by the hepatocytes compared with normosialylated transferrin, and became senescent glycoproteins (Flahaut et al., 2003). On the other hand, ethanol can inhibit the activity of mannosidase II, resulting in the synthesis of cell surface glycoproteins with high mannose structures (Waszkiewicz et al., 2013d). In addition, we have confirmed in an earlier study that the changes in fucose amount and location seem to be regarded equally important glycochanges in the saliva of alcoholics, and may help in the detection of chronic alcohol drinkers (Kratz et al., 2013). It is observed, in our present study, that the highest increase in salivary GLU and MAN activity (18 and 3 times, respectively) may indirectly suggest degradation of the oligosaccharide chains of glycoconjugates through the release of glucuronic acid from glycosaminoglycans and mannose residues from N-linked glycoproteins, to be the most important glycochanges in the saliva of alcohol-dependent subjects. The release of galactose from oligosaccharide chains of salivary glycoconjugates seems to play a minimal role in glycoconjugate changes of the alcoholic’s saliva, which is consistent with other alcohol studies that stated that there was an increase in the high terminal galactose residues (Flahaut et al. 2003; Waszkiewicz et al., 2013d). Although it is known that ethanol exposure results in the synthesis of glycoproteins with high mannose residues (Tomás et al., 2002; Waszkiewicz et al., 2012b), a higher salivary activity of MAN in alcoholics than in controls, in our study, may suggest that the balance between the action of salivary glycosyltransferases and glycohydrolases (between glycoconjugate synthesis and degradation) may be shifted toward inhibited glycosylation and accelerated degradation processes of high mannose glycoconjugates. It might be attributable to the much higher levels of ethanol and its metabolites in the saliva (and/or tissues neighboring to oral cavity) of alcoholics. It was shown that salivary acetaldehyde exceeds the blood level 10 to 100 times, and has a potency to inhibit or stimulate some of the salivary enzymes (Waszkiewicz et al., 2013b,c).

Chronic alcohol drinking affects oral mucosa (epithelial atrophy or dysplasia with hyperregeneration), salivary glands (fat accumulation, acinar cell swelling, atrophy, diffuse immune infiltration), saliva (reduced: SF rate, sodium, bicarbonate and chloride concentrations, as well as some of salivary proteins and glycoproteins levels, e.g. amylase) (Riedel et al., 2003; Waszkiewicz et al., 2011b, 2013b,c). Chronic cigarette smoking and tobacco use induce pigmentation and thickening of the oral mucosa epithelium as well as inflammation, swollen secretory cells of the salivary glands and decreased secretion of salivary proteins (including peroxidase, lysozyme, lactofernin), or increased levels of immunoglobulin A (Winn, 2001; Waszkiewicz et al., 2012b,c,d,e, 2013c). On the other hand, there is a significantly higher stimulated secretion of the saliva due to the irritating effect of tobacco smoke on the oral mucosa (de Almeida Pdel et al., 2008). The reduced SF in alcohol abusers may lead to inflammatory states of the oral cavity, periodontal diseases or infections (Waszkiewicz et al., 2013b). It is also known that dental and oral hygiene is often neglected by persons addicted to alcohol and that cigarette smoking deteriorates oral hygiene and it is a strong modifiable risk factor for periodontal disease (Johnson and Guthmiller, 2007; Waszkiewicz et al., 2013b,c). In our present study, we confirmed our earlier reports (Waszkiewicz et al., 2012a,c,d,e, 2013b,c) that alcoholics have worse dental (reflected by higher DMFT) and periodontal state (reflected by higher GI and PBI) as well as lower SF, than the controls.

The increased activities of lysosomal exoglycosidases in the saliva of smoking alcoholics are attributable to the ethanol itself as well as alcohol and smoke metabolites, and may occur via many mechanisms: increased lysosomal membrane permeability, delayed removal of the enzymes from the saliva, impaired trafficking of lysosomal hydrolases to organelles, enhanced synthesis of the enzyme by activated leucocytes or leakage from the damaged cells (Waszkiewicz et al., 2012b, 2013b). Therefore, in alcoholics, the lysosomal and cellular membranes of the oral cavity tissues (including oral mucosa and salivary glands) may be damaged, and proteases together with glycosidases (including FUC, GAL, GLU and MAN) may be translocated into the cytosol, the extracellular matrix, and to the saliva (Waszkiewicz et al., 2012a). Smoking may, additively to ethanol, damage tissues of the oral cavity and potentiate enzyme release to the saliva (Knas et al., 2006; Waszkiewicz et al., 2013b). Enzymes in the saliva (including exoglycosidases) may come from salivary gland cells, oral microorganisms, polymorphonuclear leukocytes (PMNs) and macrophages entering the gingival sulcus, as well as from epithelial cells, plasma and dietary constituents. In particular, gingival crevicular fluid outflow to the oral cavity (it reflects the constituents of serum) may be an important contributor to the enzyme pool in the whole saliva (Waszkiewicz et al., 2013c). As smoking and alcohol drinking induce bacterial load/repopulation (Seitz and Stickel, 2007), increased salivary activity of FUC, GAL, GLU and MAN may not only come from serum and damaged cells of the oral tissues, but also from an increased oral bacterial load/repopulation or activated inflammatory cells.

We found that salivary activity of FUC, GLU and MAN activity had excellent sensitivity and specificity in both smoking and non-smoking alcohol-dependent persons, whereas
salivary GAL had poor sensitivity and specificity values (Table 2). The diagnostic test accuracy, measured by the area under the ROC curve (AUC), was excellent for salivary FUC, MAN and GLU activity, but not for salivary GAL (Table 3). We also noted that the activity of salivary exoglycosidasises inversely correlated with the SF. Therefore, after salivary output counting (pKat/min), only the salivary GAL and GLU output values increased in fact in alcohol-dependent persons. Hence after GAL exclusion due to poor marker statistics, the GLU activity mostly seems to be applicable to be used as a marker of the chronic alcohol drinking (alcoholism). Moreover, we noted the highest increase for salivary GLU activity and output (7–19 times), and only the salivary GLU values correlated with the alcohol parameters (number of days of last alcohol intoxication). As GLU output counting may complicate the diagnostic procedure because of the necessary volume and time period of SF recording, a simple GLU activity determination seems to be more applicable for alcohol drinking detection than output of GLU.

It was reported earlier that the activities of AST and ALT in the saliva as well as in the serum were significantly higher in the alcoholics when compared with the healthy controls (Shivashankara et al., 2011). As salivary and serum aminotransferases strongly correlate with each other, and gingival fluid that is a serum transudate (and/or inflammatory exudate) to the oral cavity and contributes in a small part to the whole saliva content (Shivashankara et al., 2011; Waszkiewicz et al., 2013b,c), activities of investigated exoglycosidasises in our study might potentially correlate with the serum aminotransferases. However, we did not find any correlation between any of the investigated salivary exoglycosidase and serum aminotransferases. The lack of correlation is not surprising, since there is mostly a different origin of serum and salivary enzymes (Waszkiewicz et al., 2013c). In addition, the mean activities of salivary exoglycosidasises increased even 18-fold, whereas mean activities of serum aminotransferases increased 2-fold above the normal ranges at best.

Despite some limitations of our present study, such as small C and AS groups as well as very small ANS group, and no group of social drinking smokers, our study clearly shows that alcohol-dependent persons have bad dental and periodontal state. We showed an increased salivary activity of all assayed exoglycosidasises (FUC, GAL, GLU and MAN), and increased output of salivary GAL and GLU. Although we found excellent sensitivity and specificity as well as high accuracy for salivary FUC, GLU and MAN, the GLU activity seems to be mostly applicable as a marker of chronic alcohol drinking (alcoholism).

In conclusion, we confirmed previous reports that alcohol-dependent persons have bad dental and periodontal state. We showed an increased salivary activity of all assayed exoglycosidasises (FUC, GAL, GLU and MAN), and increased output of salivary GAL and GLU. Although we found excellent sensitivity and specificity as well as high accuracy for salivary FUC, GLU and MAN, the GLU activity seems to be mostly applicable as a marker of chronic alcohol drinking (alcoholism).

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**Conflict of interest statement.** None declared.

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