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**Ethyl glucuronide concentrations in hair: a controlled alcohol dosing study in healthy volunteers<sup>1</sup>**

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**ABSTRACT**

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<sup>1</sup> Parts of the work have been presented at the 20th Meeting of the SoHT

**Background:** Ethyl glucuronide (EtG) is a minor phase II metabolite of alcohol that accumulates in hair. It has been established as a sensitive marker to assess the retrospective consumption of alcohol over the past months using a cut-off of  $\geq 7$  pg/mg hair to assess repeated alcohol consumption.

**Aims:** The primary aim was to assess whether amounts of alcohol consumed correlated with EtG concentrations in hair. Additionally, we investigated whether the current applied cut-off value of 7 pg/mg hair is adequate to assess the regular consumption of low-to-moderate amounts of alcohol.

**Methods:** A prospective controlled alcohol dosing study in 30 healthy individuals matched on age and gender. Individuals were instructed to drink no alcohol (N = 10), 100 g alcohol per week (N = 10) or 150 g alcohol per week (N = 10) for 12 consecutive weeks, before and after which hair was collected. Throughout the study, compliance to daily alcohol consumption was assessed by analyzing urine EtG 3 times weekly.

**Results:** Participants in the non-drinking group had median EtG concentrations of 0.5 pg/mg hair (interquartile range (IQR) 1.7 pg/mg; range < 0.21 – 4.5 pg/mg). Participants consuming 100 g and 150 g alcohol per week showed median EtG concentrations of 5.6 pg/mg hair (IQR 4.7 pg/mg; range 2.0 – 9.8 pg/mg) and 11.3 pg/mg hair (IQR 5.0 pg/mg; range 7.7 – 38.9 pg/mg), respectively. Hair EtG concentrations between the 3 study groups differed significantly from one another ( $p < 0.001$ ).

**Conclusions:** Hair EtG concentrations can be used to differentiate between repeated (low-to-moderate) amounts of alcohol consumed over a long time period. For the assessment of repeated alcohol use, we propose the current cut-off of 7 pg/mg could be re-evaluated.

**Keywords:** ethyl glucuronide; hair; alcohol use; alcohol biomarker; controlled study; cut-off

## Introduction

Ethyl glucuronide (EtG) is a minor phase II metabolite of alcohol that incorporates in hair after alcohol consumption. In contrast to traditional alcohol markers, such as carbohydrate-deficient transferrin (CDT), EtG is a direct marker of alcohol that is exclusively produced upon alcohol consumption [1]. Hair provides an easy and non-invasive matrix for stable accumulation of compounds over extended periods of time. Thus, hair EtG is proposed as a reliable marker to quantify the retrospective consumption of alcohol over a relatively long period of several months [2]. As the concentrations of EtG measured in hair are in the pg/mg range sensitive analytical methods such as liquid chromatography (LC) and gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS), are necessary [3]. The Society of Hair Testing (SoHT) proposes a concentration  $\geq 7$  pg/mg hair to be indicative for repeated alcohol consumption [4].

In humans, using self-report questionnaires and medical records, correlations between hair EtG and alcohol consumption have been reported [5-8]. Though, in participants consuming either 16 g or 32 g daily alcohol, hair EtG concentrations were detected in only 24% of alcohol consumers [9]. These studies show important inter-individual variability in EtG hair concentrations even when using data normalized for body weight [8] and within participants with similar alcohol consumption reports [6]. This variability in hair EtG concentrations may be related to EtG incorporation or accumulation in hair (e.g., cosmetic hair treatments [10]), but could also be related to differences in alcohol consumption profiles or due to the inaccurate reporting of alcohol consumption prior to hair sample collection. Evidence for the presence of a linear correlation between hair EtG concentrations and ingested amounts of ethanol was also suggested from rat studies [11,12], where the reporting of alcohol cannot influence correlation measures. However, the extrapolation of animal results

to humans should be done with care: differences in metabolism and hair structure between rat and humans may have implications on the adsorption and accumulation of chemicals.

In this study, we present data of hair EtG concentrations following the controlled consumption of 0 g, 100 g or 150 g of alcohol per week in a randomized 12-week controlled alcohol dosing study in human volunteers matched on age and gender. The primary research goal was to assess whether amounts of alcohol consumed correlated with EtG concentrations in hair. Additionally, the use of the doses of alcohol (reflecting a mean daily consumption of 1.5 to 2 standard alcoholic drinks) allows determining whether the current applied cut-off value of 7 pg/mg hair is adequate to assess the regular consumption of low-to-moderate amounts of alcohol.

## **Materials and methods**

### ***Participants***

A total of 30 participants were recruited through leaflets distributed at the university site of the University of Antwerp (Belgium) and through mouth-to-ear advertisement. Participants were included when aged between 20 – 70 years old, when consuming alcohol on a regular basis (between 2 and 10 alcohol units per week over the past 6 months), and when being able to understand study procedures and sign informed consent. Participants were excluded when: (i) having (a history of) alcohol or other drug abuse or dependence according to DSM-IV criteria, (ii) using pharmacotherapy that could influence the effects of alcohol, alcohol metabolism or alcohol excretion in the past 30 days, (iii) having a severe illness (diseases of the gastro-intestinal system, and/or kidneys, cardiovascular disease) or (a history of) psychiatric treatment that could make participation in the study hazardous, or (iv) having any contra-indication for the use of alcohol (e.g., hypertension, pregnancy). The Alcohol Use Disorders Identification Test (AUDIT) was used to assess harmful and hazardous alcohol use.

The short version of the Drug and Alcohol Screening Test (DAST) was used to additionally assess harmful alcohol or drug use. The study was performed according to Helsinki Declaration on Scientific Research with Humans and approved by the Ethical Committee of the Antwerp University Hospital (B300201215554 – 12/45/361). Participants gave written informed consent.

### ***Study design***

The design was a 12-week controlled alcohol-dosing study with 30 age- and gender-matched healthy individuals assigned to one of 3 study conditions: consuming either no alcohol (N = 10), 100 g (N = 10) or 150 g (N = 10) of alcohol per week for 12 consecutive weeks. The first 8 included participants were attributed to a study group based on their personal preference for one of the 3 study groups, after which other participants were matched for age and gender and attributed to one of the other two groups. Participants consumed alcohol during 5 days per week with 2 alcohol abstinence days per week [13], resulting in daily alcohol consumption of 20 g and 30 g pure alcohol, respectively. The alcohol was provided to the participants as red wine from the same batch. In 3 bottles, the alcohol concentration was measured with a validated analytical method based on head-space gas chromatography with flame ionization detection:  $13.6 \pm 0.3$  (% , v/v). A glass with a measuring line was provided to the study participants to concur with the necessary daily volumes of wine (resp. 186 mL and 279 mL) that had to be consumed. To control for study compliance, urine samples were collected every 3 days and EtG in urine was measured using a liquid chromatography tandem mass spectrometry method (LC-MS/MS) (adapted from [14]). Briefly, 20  $\mu$ L of EtG-D<sub>5</sub> 20  $\mu$ g/mL (internal standard) and 360  $\mu$ L of mobile phase A were added to 20  $\mu$ L of urine sample. After vortexing and centrifugation 100  $\mu$ L of the upper layer was transferred to a LC vial. Ten microliters were then injected on a LC-MS/MS system consisting of an Agilent 6430 liquid

chromatograph equipped with an electrospray interface operating in negative mode. The column used was a Luna® phenyl hexyl column (150 mm x 4.6mm, 5 µm, Phenomenex) operated at 0.6 mL/min with a total run time of 8 min. A linear gradient consisting of mobile phase A (5 mM aqueous ammonium acetate adjusted to pH 5.6) and mobile phase B (methanol) was used. The lower limit of quantification (LLOQ) of the method was 0.1 µg/mL.

At study start, hair samples were collected by fixing approximately 100 mg of hair at the vertex posterior region. The samples were cut as close as possible to the scalp and the proximal 3 cm lengths were analyzed to assess pre-study alcohol consumption. Following the 12-week alcohol dosing regimen, the newly grown hairs at that location (a total length of about 3 cm) were cut and analyzed for concentrations of EtG. This approach minimizes the influence of EtG content that might have been present pre-study in the 6 – 18% non-growing hairs (in catagen and telogen phase; [15]) and reduces the error on the segmentation process when hair growth would not have been exactly 3 cm (i.e., 1 cm/month). Hair bleaching, permanent coloring, hair perming and hair straightening are proposed to affect hair EtG content and were therefore not allowed during the study period [16]. Hair melanin content does not influence the incorporation of EtG in hair [17], therefore no inclusion criteria were set regarding hair color. Participants were instructed not to go to the hairdresser during the study period.

#### ***Ethyl glucuronide in hair***

As presented in Table 1, hair samples were decontaminated with water and acetone and pulverized for 5 min using a ball mill (Type MM 301, Retsch GmbH & Co. KG, Haan, Germany). About 30 mg of pulverized hair was carefully weighted and used for further analysis. Samples were extracted with 2 mL of ultrapure water during 1.5 h ultrasonication,

followed by solid-phase extraction on Oasis MAX cartridges, evaporation of the eluate and derivatization with heptafluorobutyric anhydride (HFBA). Quantification was performed using gas chromatography-tandem mass spectrometry with negative ion chemical ionization (GC-(NICI)MS/MS; Agilent 7000C). The method was linear in the range from 2 to 400 pg/mg hair, and had a limit of detection (LOD) of 0.05 pg/mg and a LLOQ of 0.2 pg/mg. As shown in Table 2, intra- and inter-day accuracy (bias) and precision (coefficient of variation (CV)) for replicated quality control samples were within the acceptance criteria of 15% [18]. Mean overall extraction recovery was 98.8%. For a full description and validation of the analytical method, see [19]. The EtG concentrations obtained from the hair samples collected at 12 weeks was the primary outcome measure.

*== Insert Table 1 about here ==*

*== Insert Table 2 about here ==*

### ***Demographic variables and questionnaires***

The variables assessed included age and gender (for matching purposes), body mass index (BMI), pre-study alcohol consumption, and hair treatment (hair color, pre-study cosmetic treatments) using an in-house questionnaire. The Timeline Follow Back method (TLFB; [20]) was used to keep track of daily alcohol consumption during the 12-weeks trial, as a way of monitoring the obligatory alcohol-free days per week. Regular telephone calls and house visits were made to increase study compliance.

### ***Statistical analyses***



Data were assessed on normality using Shapiro-Wilk tests. Differences between the 3 groups on hair EtG concentrations, demographic variables, and questionnaires were analyzed using repeated measures ANOVA tests or Kruskal-Wallis tests for nonparametric data in SPSS version 22 (Statistical Package for the Social Sciences). Statistically significant differences between 2 groups were addressed post-hoc (using t-tests for parametric and Mann-Whitney U tests for nonparametric data). A Bonferonni correction for multiple testing was applied. According to the distribution of the data, correlations between EtG measures in hair and alcohol (volume) intake was assessed using nonparametric correlation analysis (Spearman's rho). Data are presented as mean  $\pm$  standard error or as median  $\pm$  interquartile range (IQR) where appropriate, with a  $p$ -value  $< 0.05$  considered statistically significant.

## Results

### *Participants*

Participants' characteristics are presented in Table 3. Participants were matched on age and gender. BMI, alcohol consumption in the 3 months prior to study start, hair color and the occurrence of cosmetic hair treatments prior to study start did not differ between groups (see Table 3). Self-reported alcohol consumption in the prior 3 months ranged between 0 and 3300 g alcohol/3 months (see Table 3). Hair EtG concentrations in the hairs cut at study start were  $2.7 \pm 7.7$  pg/mg (range 0 – 83.0 pg/mg; see Table 3) and correlated with the self-reported alcohol consumption of the past 3 months (Spearman's rho  $r = 0.742$ ;  $p < 0.001$ ). The DAST was negative (score 0) in all participants, indicating no harmful drug use. Mean AUDIT scores were  $3.7 \pm 2.6$  and did not differ between groups (ANOVA  $p = 0.234$ ).

= = = *Insert Table 3 about here* = = =

### ***Hair EtG following 12-week controlled alcohol dosing***

EtG concentrations following the 12-week controlled alcohol intake are presented in Table 4. In the non-drinking group, median EtG concentrations were 0.5 pg/mg (IQR 1.7 pg/mg; range < LLOQ – 4.5 pg/mg). In the group of participants consuming 100 g and 150 g alcohol per week, median EtG concentrations were 5.6 pg/mg (IQR 4.7 pg/mg; range 2.0 – 9.8 pg/mg) and 11.3 pg/mg (IQR 5.0 pg/mg; range 7.7 – 38.9 pg/mg), respectively. Hair EtG concentrations were significantly different between the 3 study groups of non-drinkers, 100 g per week alcohol consumers and 150 g per week alcohol consumers (Kruskal-Wallis  $p < 0.001$ ; post-hoc 0 g vs. 100 g:  $p = 0.001$ ; 100 g vs. 150 g:  $p < 0.001$ ; 0 g vs. 150 g:  $p < 0.001$ ; see Figure 1). In the group of participants consuming 150 g alcohol per week, one participant had a high EtG concentration of 38.9 pg/mg (Figure 1).

Urine EtG analysis revealed negative EtG concentrations in the non-drinkers, and provided evidence for alcohol consumption (however with large variation) in the alcohol consumers (see Table 4).

*=== Insert Table 4 about here ===*

*=== Insert Figure 1 about here ===*

### **Discussion**

Our study presents findings of hair EtG concentrations following a controlled alcohol-dosing study in 30 healthy volunteers matched on age and gender, consuming either 0 g, 100 g or 150 g of pure ethanol per week. The latter two groups represent an average daily consumption of 1.5 and 2 alcoholic drinks over several months, being a good representation of daily low-to-moderate- alcohol consumption.

The results of this study provide evidence that EtG concentration in hair increases with increasing doses (amounts) of alcohol consumed, and that a distinction can be made in terms of amounts of alcohol consumed and abstinence, even when low-to-moderate doses of alcohol are consumed. However, still some variation was noted: in participants consuming no alcohol, all but one had very low, but measurable (above the LLOQ of 0.2 pg/mg), EtG concentrations while having consumed no alcohol in the prior months. As the method used in this paper (GC-MS/MS) is an outmost sensitive one, especially in the lower concentration ranges, these EtG concentrations might not have been detected using other methods for the detection of EtG in hair, such as GC-MS and liquid chromatography tandem mass spectrometry (LC-MS/MS) with higher LLOQs. Methods with low LLOQs are published by us and other groups [3,19] and improve the interpretation of results closer to the low cut-off values. With increased sensitivity of the method comes a better assessment at lower concentrations, resulting in lower alcohol use that can be detected as shown in this study.

One controlled study in humans reported on 44 participants that consumed 16 g or 32 g red wine (calculated according to the concentration provided on the wine label) daily for 3 months, but EtG concentrations were detectable in only 5 of 21 alcohol consumers [9]. In that study, all females drank 16 g alcohol per day, while all males drank 32 g alcohol per day, providing a gender bias. Methodologically, low sample weights between 7 and 26 mg hair were used for analysis, and cut hair samples were used instead of pulverized hair, such that EtG from the hair matrix would not have been extracted optimally [21], which may have contributed to the relatively high number of non-detects.

The presence of low amounts of EtG was however noted in abstinent participants. Because hair samples were cut prior to the study start, and the newly re-grown hairs were cut to be analyzed at study end, the impact of possibly alcohol-containing non-growing hairs present pre-study was minimalized. However, below the scalp surface, still a minimal amount

of hair remains, which might explain the presence of EtG concentrations in the group of participants consuming no alcohol during the study. We suggest this could be the case for the outlier #2 and #25, who both consumed relatively large amounts of alcohol pre-study (respectively 1020 g and 3300 g pure alcohol in the 3 months prior to study inclusion). Other possibilities would include non-reported (additional) consumption of alcohol during the study or intake through food. Study adherence is thereby an important factor to take into consideration in lengthy controlled alcohol-dosing human studies. An attempt was made to improve study compliance through the monitoring of urine EtG, although it would have been more interesting to control study compliance by measuring creatinine-corrected urine EtG concentrations. Also, a possible endogen presence of EtG cannot be excluded and should be investigated further.

In all participants consuming no alcohol, all hair EtG values were below 4.5 pg/mg. In participants consuming 100 g pure alcohol per week for 3 months, EtG concentrations lower than 7 pg/mg were still observed in 8 of 10 participants (between 2.7 and 6.9 pg/mg). This raises questions whether the 7 pg/mg hair cut-off value proposed by the SoHT to assess repeated alcohol consumption might not be too high. In light of the results of this study, we suggest the cut-off should be re-evaluated and perhaps be lowered. This deserves further research on a large series of teetotalers.

In support of earlier studies [3,5-7], the analyses of the hairs cut pre-study showed a strong positive correlation between hair EtG values in the proximal 0-3 cm hair segment and the amounts of alcohol consumed in the past 3 months before the study.

In this study, participants were well characterized on the volumes of alcohol consumed prior to study start using a validated questionnaire (TLFB) and were of similar BMI. Also hair color and pre-study cosmetic treatments were mapped in detail and all were matched on

gender and age, such that gender-bias was kept to a minimum. For reasons of feasibility and ethics, only a limited number of dosing groups were investigated.

Summarized, we show that hair EtG concentrations can differentiate between repeated (low-to-moderate) amounts of alcohol consumed over a long time period.

## **Acknowledgements**

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## **Conflict of Interest**

The authors declare that they have no conflict of interest.

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# **Figure Captions**

**Fig. 1** Hair EtG concentrations (pg/mg hair) of participants consuming no alcohol (N = 10), 100 g alcohol per week (N = 10) or 150 g alcohol per week (N = 10) for 12 consecutive weeks.