Interpretation of group-level factors from a large population dataset in the determination of ethyl glucuronide in hair

Abstract

Ethyl glucuronide in hair (HEtG) is the most accredited marker to prove chronic alcohol abuse. In this study, we evaluate the comprehensive results of HEtG determination obtained during four years of activity (2009–2013) in our laboratory (Northwestern Italy) – across a large cohort of subjects (over 20 000 subjects mostly undergoing medical examination for driving re-licensing) – to provide a general perspective on HEtG analysis and dependence on group-level factors (e.g. age, gender, site and period of hair collection) that could bias the analytical results. HEtG was measured by liquid chromatography-tandem mass spectrometry. About 12% of the subjects presented HEtG concentrations over 30 pg/mg. Upon non-parametric hypothesis tests, distributions of HEtG in independent populations categorized by age proved statistically different, while no differences were found by considering gender, BMI, and site of sampling (head vs. chest hair). A 'seasonality' factor was evaluated by comparing periods of collection approximately representing the hair growth in winter, spring, summer and autumn, and a seasonal trend was observed showing the highest HEtG levels in winter (16.7%) and minimum levels in summer (8.3%). The experimental HEtG distributions confirm that chest hair sampling can be trusted as an alternative to scalp. Furthermore, among biological and external factors, age and season of sampling may significantly influence the measured HEtG concentration, and this potential source of bias should be taken into account when the results are interpreted.

Keywords:
ethyl glucuronide; alcohol; hair; hypothesis tests; seasonality

Introduction

An important goal of both forensic toxicology and clinical medicine is to identify appropriate biological markers of ethanol consumption to diagnose chronic alcohol abuse or ascertain alcohol abstinence.[1] The methodologies applied in clinical and forensic contexts to support these judgments have seen remarkable modifications over the years. Although the multifaceted approach (consisting of medical examination of symptoms and history of the inspected subject, administration of focused questionnaires, and laboratory determination of biomarkers) to identify a harmful drinking behaviour has remained roughly unaltered, the laboratory analyses towards the determination and quantification of alcohol biomarkers – either indirect or direct – and the interpretation of their diagnostic significance have evolved considerably.[2, 3]

Amongst alcohol biomarkers, the determination of ethyl glucuronide (EtG) in the keratin matrix gained increasing popularity in the last decade, since it demonstrated to provide high sensitivity

and specificity in the discrimination between high-risk and low-risk alcohol drinkers, together with very rare interference from external factors, and minimal bias from individual variability.[4-12] Therefore, the determination of EtG in hair (HEtG) represents nowadays the first choice to support the verification of ethanol use/abstinence in a variety of situations, including workplace testing, driving licence re-granting, minors' adoption, divorce proceeding, withdrawal treatment,[13] and post-mortem or pre-natal alcohol exposure investigation.[14, 15] Consequently, continuous refinement of analytical methods for EtG determination was addressed to improve validation protocols, laboratory throughput, sensitivity and detection capability, and detect potential sources of uncertainty, so as assure solid background to data interpretation.[16-19] Two decades of scientific research led to the development of standardized methods of hair testing, documented in official guidelines, and aimed to help the interpretation of analytical results and the consequent clinical or legal judgment. Specifically, a cut-off value of 30 pg/mg was established to support the diagnosis of chronic alcohol consumption,[10] while a maximum 7 pg/mg EtG concentration was suggested not to contradict a declared alcohol abstinence,[20] and guidelines for good-practice in hair analysis were published.[21] Nevertheless, warnings have been raised about the possible misinterpretation of HEtG results[4, 18, 19, 22, 23] and many aspects of hair analysis still need to be further investigated to gain an exhaustive knowledge.

A major issue of EtG determination is the choice of alternative sources of hair when no scalp hair is available. Several studies proved that axillary and pubic hair are not suitable matrices to assess chronic excessive alcohol consumption,[24-27] whereas chest and leg hair are currently considered as the best alternative to head hair.[12, 26] However, no specific study across large cohorts of subjects has been published yet, in order to clarify this aspect and the role of other inter-individual biological variables (e.g. age, gender, and BMI) as possible sources of bias.

Our laboratory processes every day many hair samples for HEtG analysis, primarily for driving re-licensing (92% of the population) and alcohol abusers' rehabilitation programmes. In this study, we evaluate the comprehensive results obtained during four years of activity (over 20 000 samples studied), taking advantage of the statistical principle that randomization on large cohorts of subjects levels off the individual variability and equalizes their probability distributions. This allowed us to investigate single factors separately, including age, gender, sampling season, and site of hair sampling, in order to highlight potential positive and negative biases.

The individual variability is related to uncontrolled factors, such as the different amount of alcohol consumed, cosmetic hair treatments, hygiene habits, the occurrence of hepatic diseases, etc. Among these, the starting postulate that large cohorts of subjects have similar average intake of alcohol (and similar standard deviation) has been challenged and reconsidered for each studied factor during the forthcoming discussion.

Material and methods

HEtG determination by ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

HEtG analyses were performed with a fully validated method previously reported.[28] Briefly, all hair samples were washed twice using methylene chloride and methanol in sequence. Dried hair was cut into small pieces (about 1 mm) and weighed. EtG was extracted overnight at room temperature with a water–methanol mixture. Then, the samples were sonicated and an aliquot of liquid phase was transferred into a vial for UHPLC-MS/MS analysis. Analyses were performed using a Shimadzu Nexera UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray ionization (ESI) source operating in the negative ion mode. Limits of detection (LOD) and quantification (LOQ) were 3 and 10 pg/mg, respectively. The method was internally validated and accredited in accordance with ISO/IEC 17025:2005 rules. Laboratory performances in HEtG analysis were constantly monitored through regular participation to inter-laboratory proficiency tests organized by the Centre Universitaire Romand de Médecine Légale (University Hospital of Geneva, Switzerland) and the Society of Hair Testing (SoHT).

Study protocol

Over a period of four years (4 September 2009 – 31 December 2013), we analyzed hair samples from over 20 000 subjects who underwent medical examination either for alcohol abuser's rehabilitation programmes or for driving relicensing protocols (92% of the investigated population) in numerous medical commissions located in Piedmont, northern Italy.
All hair samples were cut as close as possible to the scalp or the skin surface, using freshly disinfected scissors. The samples were stored at room temperature and analyzed within 10 working days. Only the proximal 0–3 cm segment was analyzed whenever longer head hair samples were collected. Shorter head hair samples, as well as axillary and chest hair samples, were analyzed in their full length.

Statistical analysis: inter-individual evaluations

HEtG results were repeatedly divided according to different classification criteria – i.e., site of hair sampling, age, gender, BMI and period of sampling – and statistically compared by means of significance tests. As for some samples the entire set of information was not available, and some evaluations only considered a subset of the entire population (e.g. only head hair), the specific

cohort of subjects used to perform each hypothesis test was chosen according to the classification criterion, as reported below.
All statistical analyses were conducted using the software packaging SPSS® (SPSS Inc., Chicago, IL, USA), version 21.0, for Windows.

Site of hair sampling

Information about the site of hair sampling was available for 25 533 samples. The remaining ones, whose origin was not specified at the moment of collection, were excluded from the statistical elaboration. The distribution of admitted samples was: head hair 22 825, axillary hair 762, and chest hair 1946. The assignment of the sampling site (head, chest, and armpit) was based on written statement of the medical personnel. This classification was used to evaluate the differences in HEtG concentration distributions and the percentage of positive samples within each group, according to various cut-off values, including the official one of 30 pg/mg.
Under the hypothesis of independent sample populations, the Yates' chi-square test was selected for conformity assessment. For each couple of keratin matrices under examination, the 2×2 contingency tables were built by listing the number of positive (HEtG > 30 pg/mg) and negative samples. The total percentage of positive samples was considered as the observed value and used to build the expected cell frequency. Lastly, the chi-square test was corrected by the Yates' factor when the compared populations were significantly different. The statistical model is reported elsewhere.[26] When the critical chi-square value at 95% confidential interval (CI) and 1 degree of freedom (df) proved larger than the calculated Yates' chi-square value, the null hypothesis H_0 (no significant differences between two groups) was retained. At 95% CI and 1 df, the critical chi-square value is 3.84.

Gender, age, and BMI

The examined population (n = 20 293) consisted of 18 920 males (93%) and 1373 females (7%). Altogether, 23% (n = 4684) was in the age ranging 18–30 years (group A), 29% (n = 5878) in the range 31–40 years (group B), while the majority (48%, n = 9731) was aged more than 40 years (group C). For a small subset of samples (n = 733), the weight and height data were also collected upon informed consent from the subjects undergoing medical examination, in order to consider the body mass index (BMI) as an additional factor of potential bias. Subjects were divided among underweight (BMI < 18.5, n = 18), normal (18.5 < BMI > 24.9, n = 440), overweight (25 < BMI > 29.9, n = 238), and obese (BMI ≥ 30, n = 37).

Table 1. Observed and expected cell frequency for head, axillary and chest hair

1	Observed cell frequency			Expected cell frequency	
	Head	Axillary	Total	Head	Axillary
Positive	2,603	20	2,623	2,538	85
Negative	20,222	742	20,964	20,287	677
Total	22,825	762	23,587	22,825	762
2 Only males	Observed cell frequency			Expected cell Frequency	
	Head	Chest	Total	Head	Chest
Positive	2,102	192	2,294	2,099	195
Negative	18,871	1,754	20,625	18,874	1,751
Total	20,973	1,946	22,919	20,973	1,946

The Mann–Whitney U hypothesis test was chosen to verify the occurrence of statistically significant differences between two independent populations divided by gender. The null hypothesis H_0 affirms that there are no significant differences between the two populations under examination. A significant level (a two-tailed P-value) of 0.05 (CI = 95%) was chosen for the statistical test. When the P-value proved smaller than the critical limit, the hypothesis H_0 was rejected.

The Kruskal-Wallis non-parametric hypothesis test was chosen to verify the occurrence of statistically significant differences between independent populations divided by ranges of age (18–30; 31–40; > 40 years) and BMI (<18.5; 18.5–24.9; 25–29.9; ≥ 30). The null hypothesis H_0 affirms that there are no significant differences between the independent populations under examination. A significant level (a two-tailed P-value) of 0.05 (CI = 95%) was chosen for the statistical test, as reported above.

Seasonality

Due to the widely variable growth rate and quiescence periods of chest and axillary hair, the correlation between hair growth period and sampling time is possible only for head hair samples. Among all samples, only head hair collected between the 15th and the 30th of the months of March, June, September, and December were considered, so that each 0–3 cm hair lock should approximately be grown in the preceding three months, corresponding to the winter, spring, summer, and autumn seasons, respectively.[29] Between autumn 2009 and autumn 2013, 4503 samples met these inclusion criteria.

The Kruskal-Wallis non-parametric hypothesis test was chosen to verify the occurrence of statistically significant differences between four independent populations divided by season. The null hypothesis H_0 affirms that there are no significant differences between the independent populations under examination. Once again, a significant level (a two tailed P-value) of 0.05 (CI = 95%) was chosen for the statistical test.

Table 2. Distribution of positive results (HEtG > cut-off) for head and chest hair samples at different cut-off values

	Head hair		Chest hair	
Cut-off (pg/mg)	Positive samples	% positive samples	Positive samples	% positive samples
30	2,603	11.4	192	9.9
50	1,469	6.4	101	5.2
100	528	2.3	39	2.0
200	164	0.7	8	0.4
500	19	0.1	1	0.1

Results

Site of hair sampling

The percentage of results classified as positive from the overall head hair samples (n = 22 825) added up to 11%, when a standard 30 pg/mg cut-off value was applied, whereas 6.4% of the samples are above 50 pg/mg. Among axillary hair samples, only 2.6% yielded EtG concentrations above 30 pg/mg. In contrast, positive chest hair samples reached 10%, similarly to head hair. The percentage of samples above 50 pg/mg drops at 5.2%, again a percentage similar to that obtained for head hair, whereas it drops at 1.6% for axillary hair.

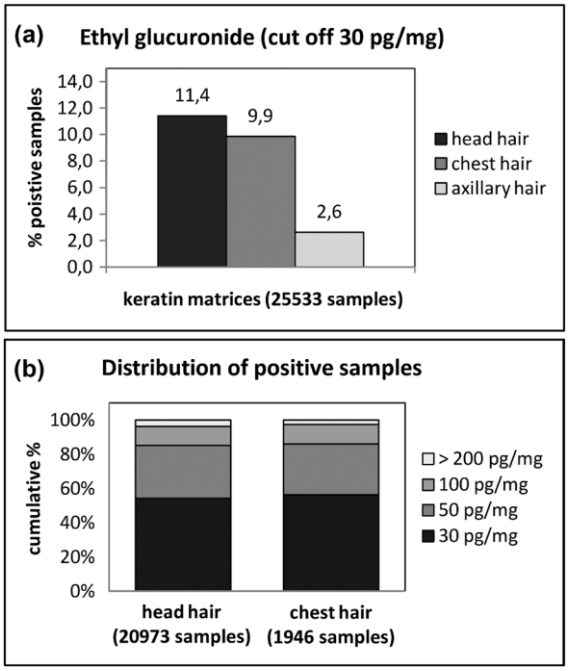

Figure 1. (a) Percentage of positive results (HEtG>30 pg/mg) head, axillary and chest hair. (b) Cumulative percentage of positive results for head and chest hair at 30 pg/mg, 50 pg/mg, 100 pg/mg, and >200 pg/mg.

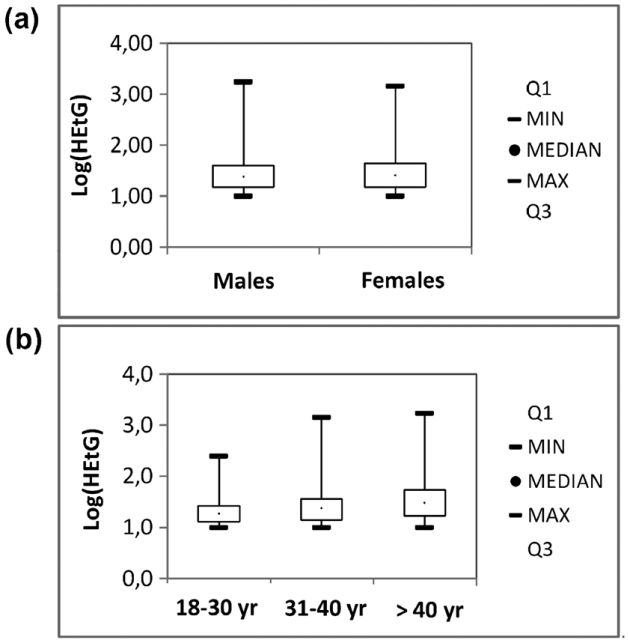

Figure 2. (a) Box plots for the HEtG distributions divided by gender. (b) Box plots for the HEtG distributions divided by age. The data are reported on a logarithmic scale (base 10).

Table 1 shows the 2 × 2 contingency tables for axillary and chest hair samples against head hair. The values obtained from the Yates' chi-square test indicate that the null-hypothesis has to be rejected for axillary hair ($\chi^2 = 51.11$), while it is accepted for chest hair with a χ^2 equal to 3.76 (data not reported in Table 1). Whether only the subset of males is considered, the similarity between chest and hair samples appears to be even stronger ($\chi^2 = 0.04$). As reported later on, the female population (only providing head hair samples) presents a significantly lower percentage of positive results in comparison to males, hence biasing the HEtG results unilaterally in a strict comparison between head and chest hair.

The first contingency table shows the observed and the expected cell frequencies for axillary and head hair. In this case, the observed cell frequency of positive samples (20) is significantly lower than the expected cell frequency (85). After Yates' chi-square test, the null hypothesis H_0 is rejected, confirming that underestimation of the average alcohol consumption is likely to occur when axillary hair is analyzed in place of head hair, as already reported in the scientific literature.[12, 26]

The second contingency table considers the observed and the expected cell frequencies for chest and head hair (only for males). The observed frequencies are very close to the expected ones and the Yates' chi-square test proves that they are not statistically different (the null hypothesis H_0 is accepted).

The strict similarity of HEtG data distributions for chest and head hair is even more evident from the results listed in Table 2 and graphically displayed in Figure 1. The number of samples classified as positive for both head hair and chest hair samples decreases at the same rate when the cut-off value is progressively increased up to 500 pg/mg. Remarkably, the number of subjects who exhibited HEtG concentrations higher than 200 pg/mg is quite large – either from head or chest hair analysis – likely signaling cases of heavy chronic alcohol misuse in the investigated population of subjects aware to be controlled.

Gender, age, and BMI

Among the 25 533 samples analyzed, complete information about gender and age was available for 20 293 specimens. The overall percentage of positive samples was 11.9% (2417), with predominant contribution from male subjects (93% of the total population, n = 18 920), who yielded 12.3% (n = 2327) of hair samples above the 30 pg/mg EtG cut-off value. A significant lower percentage (6.6%) of positive samples was collected from female drivers (n = 1373) tested for re-licensing. However, this percentage increases up to 9.6% if the alternative cut-off of 20 pg/mg would be considered, as recently proposed by Gareri et al. to take into account a gender-related differences in HEtG accumulation.[30] On the other hand, the Mann–Whitney U Test confirmed that the HEtG distribution, for values above LOQ, is not statistically different across the categories (null

hypothesis retained with an experimental P-values equal to 0.183), as also evident in the corresponding box-plot graph (Figure 2a).

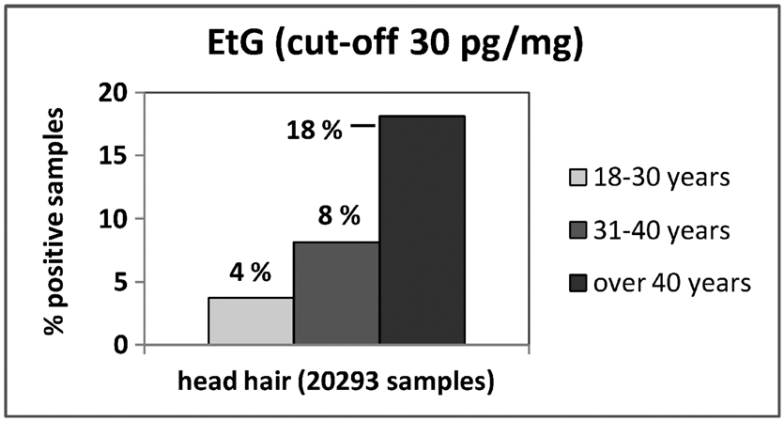

Figure 3. Percentage of positive results (HEtG>30 pg/mg) per age.

Table 3. Descriptive statistics for the HEtG distributions divided by age, gender, and BMI

	Total number of samples	% Positive results	Mean	Standard deviation	Median	IQR	Range (pg/mg)
Gender							
Males	18,920	12	53	158	21	25	LOD-1,726
Females	1,373	7	43	62	23	29	LOD-1,431
Age							
18-30 years	4,684	4	24	21	17	13	LOD-247
31-40 years	5,878	8	35	62	21	22	LOD-1,431
Over 40 years	9,731	18	51	77	27	37	LOD-1,726
BMI							
< 18.5	18	6	—	—	—	—	LOD-54
18.5-24.9	440	12	35	41	21	23	LOD-326
25-29.9	238	9	37	45	20	30	LOD-306
≥ 30	37	16	37	35	22	38	LOD-123

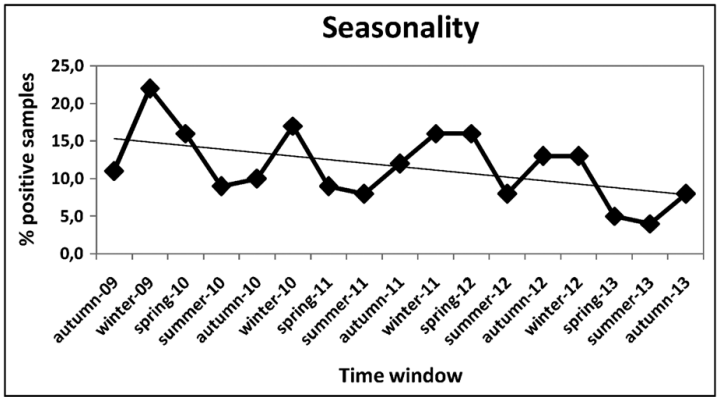

Figure 4. Trend of positive results (HEtG>30 pg/mg) over the time window 2009–2013. Black solid line: interpolation line by means of he least squares method.

The descriptive statistic data are reported in Table 3, including means, standard deviations, medians and inter-quartile ranges (IQR), minimum and maximum values.

Among the positive samples (n = 2417; 11.9%), only 7% (n = 173) belongs to group A (age 18–30), 20% (n = 480) to Group B (age 31–40), while most positive samples (73%; n = 1764) was from the group of oldest subjects (Group C). Likewise, only 4% of the tested samples (n = 4684) turned out positive in Group A; the percentage raised to 8% in Group B (n = 5878), while the highest percentage (18%) of positive samples was found in Group C (n = 9731). The Kruskal-Wallis hypothesis test confirms that the distribution of EtG (for values above LOQ) is different across the categories (null hypothesis rejected with experimental P-values < 0.05). These results are graphically represented in Figure 3, showing the percentages of positive results and Figure 2b where the box-plot graphs are displayed. Descriptive statistic values are reported in Table 3. Lastly, Table 3 and Figure S1 summarize the HEtG distributions (for values > LOQ) in relation to BMI. The cohort of underweight subjects was excluded from the statistical evaluation, since only one subject had HEtG concentrations above LOD. The remaining three independent populations proved not statistically different (P = 0.940) when the distributions of HEtG results higher than 10 pg/mg were compared.

Seasonality

Over the examined period, the average percentage of positive head hair samples, i.e., with HEtG above 30 pg/mg referring to the amount of alcohol consumed during winter, spring, summer, and autumn seasons (see Material and methods), were 17%, 12%, 7%, and 11%, respectively. The percentage of positive results per season is represented in Figure 4. Detailed definitions of central tendency (i.e., mean and median) and dispersion (i.e., standard deviation and interquartile range (IQR) are reported in Table 4.

When only the samples with HEtG concentrations higher than LOQ are considered, data distributions among the four seasons over four years proved statistically different in a few cases, upon application of the Mann–Whitney U test. In detail, winter and autumn do not appear to be statistically different, as for summer vs. spring ($P > 0.05$). On the contrary, the cold seasons checked against the warm seasons proved statistically different ($P < 0.05$). The exact P-values for all paired comparisons are reported in Table 5.

Table 4. Descriptive statistics for the HEtG distributions divided by period of sampling

Seasonality	Total num samples	% Positive samples	Range (pg/mg)	Mean	Standard deviation	Median	IQR
Autumn 2009	100	11	LOD-151	50	41	28	52
Winter 2009	140	22	LOD-209	48	45	31	47
Spring 2010	85	16	LOD-322	58	73	28	67
Summer 2010	112	9	LOD-277	38	50	23	21
Autumn 2010	276	10	LOD-204	38	40	20	26
Winter 2010	103	17	LOD-381	46	81	22	23
Spring 2011	358	9	LOD-335	33	45	19	18
Summer 2011	253	8	LOD-123	25	19	18	22
Autumn 2011	269	12	LOD-179	38	38	23	30
Winter 2011	325	16	LOD-527	50	75	26	35
Spring 2012	360	16	LOD-409	37	47	23	25
Summer 2012	363	8	LOD-355	39	49	22	25
Autumn 2012	289	13	LOD-183	35	32	21	28
Winter 2012	379	13	LOD-345	37	44	26	23
Spring 2013	341	5	LOD-227	28	38	16	17
Summer 2013	366	4	LOD-229	28	32	17	21
Autumn 2013	384	8	LOD-653	38	76	19	21

Table 5. Mann–Whitney U hypothesis tests of HEtG across four seasons: experimental P-values

Seasonality	Autumn	Winter	Spring
Winter	0.2976	—	—
Spring	0.0129*	$1.76 \cdot 10^{-4}$*	—
Summer	0.0066*	$1.29 \cdot 10^{-4}$*	0.8366

Discussion and conclusions

The present study aimed to exploit the results obtained on the determination of HEtG during four years of activity in our toxicology laboratory (located in northwestern Italy), by using this large population dataset to attribute some global trends to common classification parameters. The target population was mainly formed by DUI (driving under the influence) offenders undergoing a re-licensing process. They represent a heterogeneous population that largely overlaps that of social drinkers,[7] within which the frequent alcohol abusers should be singled out. The results reported herein may be used both to examine some features of HEtG as a biological marker of alcohol misuse – and therefore to develop more robust strategies of data interpretation – and to characterize our population of alcohol consumers by means of biological and environmental factors. Whenever an extremely large statistical population is investigated, different cohorts of subjects under examination can be viewed as global entities with specific properties, rather than a collection of independent individuals. This allows the examination of group-to-group variations per se, and the identification of important group-level variables that may influence the outcome under study,[31] viz. HEtG distribution. Moreover, individual behaviors are frequently mediated by the social context, which in turn depends on a biological variable (e.g. age and the gender). Hence, some individual-level variables can be used to categorize people at a group-level and to reveal the specific social determinants that are empirically associated, even if they remain individual-level attributes.[31] In practice, our results obtained from a population of more than 20 000 subjects, each one associated to various individual-level attributes, such as age and gender, may be interpreted from the group-level point of view.

Among the inferences allowed by the present dataset re-evaluation, the most important is possibly the one concerning chest hair, as an alternative to scalp hair to obtain reliable and comparable HEtG results. As a matter of fact, the data reported in Tables 1 and 2 and Figure 1 strongly support the equivalence of the two matrices, even if the chest hair commonly sampled corresponds to a longer growth period than the 3-cm head hair segment routinely analyzed. It is even possible to assume that the slightly lower incidence of positive samples, recorded for chest hair (although not statistically significant), reflects a longer exposure to modest wash-out phenomena. Intra-individual comparison of head and chest hair grown during equivalent periods of time may further support this equivalence, which has already been mentioned in updated consent documents, confirming the possibility to use chest hair as an alternative to scalp hair for the evaluation of excessive alcohol consumption from HEtG determination.

Secondly, although the gender gap in alcohol abuse is narrowing down, several clinical studies noted gender differences in the etiology and clinical characteristics of individuals under alcohol dependence.[30, 32] Indeed, gender differences in alcohol uptake, metabolism and elimination, together with hygiene habits and cosmetic preferences, have been widely described in the

scientific literature.[12, 30] With the limitation that females are consistently underrepresented in the population of DUI offenders,[5, 30] our experimental data suggest that women are still less likely to abuse of alcohol than men. Nevertheless, when HEtG is measurable, its distribution is not relevantly different for males and females, as reported in some studies[33, 34] but controverted in others.[30, 35]

The age of the subjects also appears to represent a discriminant factor, where the average HEtG level is significantly higher for the older groups. Consequently, a higher percentage of subjects among the elders are classified as alcohol abusers. Wurst et al. noticed the same trend by analyzing EtG in urine samples.[36] No unique interpretation is available to elucidate whether the latent factors that affect HEtG are related to different alcohol drinking behaviors, tolerance phenomenon or metabolism; however, some speculations have been made to interpret these results. Concerning metabolic differences, age-related changes in expression of uridine diphosphate glucuronyl transferase, as well as renal and hepatic functions, have been suggested.[12] Different drinking behaviors between young people and elders (i.e., binge-drinking behaviour vs. daily substantial alcohol consumption at mealtime) have also been hypothesized to influence the incorporation of EtG in hair, due to different exposure periods and immediate EtG concentration in blood.[12] Besides, a tolerance phenomenon may develop over time, leading elders to increase their daily alcohol intake. In the present circumstances, where DUI offenders are aware that they should reduce their alcohol intake because they will be controlled, we attribute a major role to the cultural context, making elderly people usually less willing to refrain from alcohol consumption than young.

In opposition, BMI proved not to represent a discriminant factor, although this conclusion is drawn from a limited population. The same result has been reported in studies involving even smaller cohorts of subjects.[6, 33, 34] To date, it can be hypothesized that the BMI factor may be of major importance in interpreting FAEEs concentrations in hair, rather than EtG, being the formers lipophilic alcohol metabolites that accumulate into fat tissues and are secreted in sebum, from which uptake into the keratin structure of hair occurs.

Conversely to age, gender, and BMI, the sampling period is an external parameter that does not have any individual connotations. With the assumption that other individual differences, such as hygiene habits, hair features, use of aggressive cosmetic products, age, gender, presence of hepatic diseases, which may influence the HEtG concentration, are levelled off by the large size of cohorts, a clear seasonal effect on the distribution of the HEtG concentrations is evident. The significantly higher percentage of positive results obtained from hair grown during the winter season may have radically different explanations. This effect can be merely related to an increased quantity and frequency of alcohol usage, and/or the consumption of beverages with higher ethanol-content during the cold season. In contrast, washing-out and sweating effects during the warm months might also be considered a reasonable explanation, since EtG is a hydrophilic compound,

and more frequent showers and seaside bathing are usually taken in summer. Other influencing factors, including seasonal changes of hair growth, are unlikely, also considering the large heterogeneity in the anagen/telogen periods of the growth rate.
Remarkably, the overall decreasing trend ($r = 0.500$) of the positive results percentage over the observation period (Figure 4) proves that the diagnostic performance of HEtG in recognizing alcohol misuse enforces the local control policy and produces a significant deterrent effect.

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