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Evaluation of direct and indirect ethanol biomarkers using a likelihood ratio approach to identify chronic alcohol abusers for forensic purposes

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ABSTRACT

The detection of direct ethanol metabolites, such as ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs), in scalp hair is considered the optimal strategy to effectively recognize chronic alcohol misuses by means of specific cut-offs suggested by the Society of Hair Testing. However, several factors (e.g. hair treatments) may alter the correlation between alcohol intake and biomarkers concentrations, possibly introducing bias in the interpretative process and conclusions.

125 subjects with various drinking habits were subjected to blood and hair sampling to determine indirect (e.g. CDT) and direct alcohol biomarkers. The overall data were investigated using several multivariate statistical methods. A likelihood ratio (LR) approach was used for the first time to provide predictive models for the diagnosis of alcohol abuse, based on different combinations of direct and indirect alcohol biomarkers. LR strategies provide a more robust outcome than the plain comparison with cut-off values, where tiny changes in the analytical results can lead to dramatic divergence in the way they are interpreted. An LR model combining EtG and FAEEs hair concentrations proved to discriminate non-chronic from chronic consumers with ideal correct classification rates, whereas the contribution of indirect biomarkers proved to be negligible. Optimal results were observed using a novel approach that associates LR methods with multivariate statistics. In particular, the combination of LR approach with either Principal Component Analysis (PCA) or Linear Discriminant Analysis (LDA) proved successful in discriminating chronic from non-chronic alcohol drinkers. These LR models were subsequently tested on an independent dataset of 43 individuals, which confirmed their high efficiency. These models proved to be less prone to bias than EtG and FAEEs independently considered. In conclusion, LR models may represent an efficient strategy to sustain the diagnosis of chronic alcohol consumption and provide a suitable gradation to support the judgment.

1. Introduction

Alcohol is the most widely abused legal drug in many western countries. Health care expenditures, business and criminal justice costs associated to alcohol-related problems amount to hundreds of billions of dollars yearly, and even a greater economic burden is sustained when alcohol addictive behaviors remain untreated. Over the last decade, numerous scientific studies focused on improving the diagnosis of chronic excessive alcohol consumption to efficiently identify individuals in need of recovery programs, health care, therapeutic monitoring, etc. [1–3].

The selection of appropriate alcohol biomarkers is extremely important for correct diagnosis assessment. In fact, biased results lead to wrong analytical interpretations and consequently to clinical and/or legal errors, which can strongly impact on the life of the involved subjects. Indirect alcohol biomarkers – such as aspartate transferase (AST), alanine transferase (ALT), gamma-

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glutamyl transferase (GGT), mean corpuscular volume of the erythrocytes (MCV) and carbohydrate-deficient-transferrin (CDT) – measured in blood had been traditionally used to distinguish non-chronic alcohol consumers from chronic abusers [4–6]. However, they lack specificity and sensitivity [1,7–9] and have been replaced by direct alcohol biomarkers, such as ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs), that greatly exceed indirect biomarkers in discrimination power [2,8–17]. Moreover, they are detected in the keratin matrix allowing long-term alcohol consumption monitoring.

Consensus documents of the Society of Hair Testing (SoHT) state that (i) the analysis of a 3 cm proximal scalp hair segment provides information on the average alcohol intake over a period of about 3 months, (ii) a scalp hair concentration $\geq$30 pg/mg for EtG and $\geq$0.5 ng/mg for the sum of four FAEEs (i.e. ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate) is indicative of chronic excessive alcohol consumption [11–13,18,19]; and (iii) the use of direct biomarkers in isolation is not advised [18]. Indeed, EtG and FAEEs absorption in hair may be altered by several factors, affecting the correlation between alcohol intake and biomarkers' concentration in hair [20,21]. For example, the hydrophilic EtG and lipophilic FAEEs have different hair incorporation mechanisms, and are differently affected by washing routines, application of alcohol-based hair care products [22], and physical–chemical hair treatments [23,24]. Therefore, their synergic use is recommended to decrease false positive rates [23–25]. Even though the consensus documents list some of the factors that may alter the analytical results and potentially introduce bias in the whole interpretative process, the interpretation of individual biomarkers results based on their respective cut-off values remains unchanged. No recommendations are given on how to interpret discordant results, nor statistical analyses are suggested to include combinations of alcohol biomarker and metadata into a predictive model.

In this study, a likelihood ratio (LR) approach is presented for the first time to better discriminate between non-chronic and chronic alcohol consumers. This approach is extensively exploited in forensics for food authentication [26,27], identification of glass [28–35], car paints [36,37], fire debris [38], inks [39], fibers [40], and DNA profiling [41,42]. LR test ($LR = Pr(E|H_1)/Pr(E|H_2)$) allows one to evaluate analytical data (E, e.g. concentrations of EtG) in the context of two mutually exclusive hypotheses (H_1: the subject is not a chronic alcohol abuser; H_2: the subject is a chronic alcohol abuser), which is what a forensic expert is asked to do in the administration of justice. More aridly, traditional interpretation models relying on cut-off values [25,40,41] are susceptible to the so-called "fall-off-cliff" problem, i.e. even minor deviations from the cut-off can utterly modify the final decision [30]. This problem is not observed when the LR test is applied because LR values not only point out which hypothesis is more consistent on the basis of the experimental evidence, but also provide the magnitude for the decision confidence thanks to the adoption of universally accepted verbal scales [28,43] that convert LR values into statements easily comprehensible by laymen, i.e. people not expert in LR calculations.

In the present study, we tested different LR models using the scalp hair concentrations of EtG and FAEEs as experimental evidences, together with the indirect biomarkers ALT, AST, CDT, GGT, and MCV measured in whole blood. Additional investigated parameters included height, weight, and body mass index (BMI). The main goal was to investigate the discrimination power of an innovative LR approach based on multivariate statistics using different combinations of these biomarkers, in order to corroborate the diagnosis of chronic excessive alcohol consumption. The predictive capabilities of the best LR models were also tested on an independent population of 43 real caseworks individuals, including known or alleged non-chronic alcohol consumers and subjects for whom incoherent FAAEs and EtG results were determined with respect to the accepted cut-offs.

2. Materials and methods

2.1. Study protocol

The data presented herein were recovered from the databases of the Regional Antidoping and Toxicology Center "A. Bertinaria" (Orbassano, Italy). 125 subjects (118 males and 7 females) were included in this study, whose analyses were commissioned by Local Committees for Driving Licences and Alcohol Abuse Treatment Services located in Piedmont, northern Italy. Ethical approval for the study was granted by the Ethical Committee of the Azienda Ospedaliero-Universitaria San Luigi Gonzaga of Orbassano (Protocol Number 0012756). Clinical and toxicological analyses were conducted over a period of 10 months in between years 2014 and 2015. The whole blood was analyzed within 24 h to detect ALT, AST, CDT, GGT, and MCV. Scalp hair was divided into two aliquots and measured, the proximal segment 0–3 cm was cut (no scalp hair shorter than 3 cm were analyzed), then the samples were stored at room temperature and analyzed within 10 days to detect EtG, ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl oleate (E18:0), and ethyl stearate (E18:1). Note that within brackets are indicated the correspondent number of carbons and unsaturations (C:U) for each fatty acid. The final FAEE concentration was calculated as the sum of the four individual concentrations (i.e. E14:0, E16:0, E18:0, and E18:1). Lastly, weight and height were measured to calculate the body mass index (BMI). Only subjects under long-term monitoring at the "A. Bertinaria" Center that consistently showed negative or positive results in hair were selected to represent the population of non-chronic and chronic alcohol abusers. The archived data belonging to the individuals under examination, together with the respective clinical judgment from the medical commission in charge, allowed us to rationally divide them into the "negative" and "positive" classes, i.e. teetotalers and social drinkers (non-chronic alcohol abusers) vs. chronic alcohol abusers. Subjects with doubtful classification were excluded from the study. Descriptive statistics and correlation studies were performed on the data matrix (125×12). All the analytical results are available in the Data-in-Brief [44] article associated with this study.

2.2. Determination of the direct and indirect alcohol biomarkers

Whole blood and scalp hair were collected once from each subject and analyzed within one day – for blood biomarkers – or one week – for hair biomarkers –. BD Vacutainer® EDTA and SST™ specimen tubes were used to collect whole blood samples to measure AST, ALT, GGT, CDT and MCV [45]. One of the two aliquots of hair sample was used to measure EtG. Briefly, hair samples were washed twice with methylene chloride and methanol and let to dry. Then, the samples underwent an overnight extraction step at room temperature with a 35:1 water-methanol solution, followed by sonication. Finally, approximately 100 μL of liquid phase was transferred into a vial for UHPLC–MS/MS analysis. A Shimadzu Nexera UHPLC system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) was employed. EtG was detected in the negative ion mode by electrospray (ESI) ionization [46]. The second aliquot of the hair samples was used to measure FAEEs, following the same sample preparation as described in Pragst et al. [47], Suesse et al. [48] and Albermann et al. [49]. Briefly, hair samples were washed twice with *n*-heptane, dried at room temperature and then cut into segments (1–2 mm in length).