# Ethyl glucuronide in hair and nail as a biomarker of alcohol consumption

Opportunities and limitations

Thesis for the degree of Philosophiae Doctor (PhD)

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Oslo, November 2020, Jan Toralf Fosen

# Contents

## Abbreviations and definitions

| | |
|---|---|
| AUDIT | Alcohol Use Disorder Test |
| Cosmetic hair treatment | Bleaching, dyeing or perming of hair |
| Chronic excessive alcohol consumption | Average consumption of 60 g or more of pure ethanol per day over several months |
| Direct alcohol biomarkers | Molecules that are created when ethanol is metabolized or reacts with substances in the body |
| EDI | Estimated daily intake of ethanol |
| EtG | Ethyl glucuronide |
| EtS | Ethyl sulphate |
| eGFR | Estimated glomerular filtration rate |
| Indirect alcohol biomarkers | Proteins or cells which undergo typical changes in response to acute or chronic alcohol consumption |
| In vitro | In the laboratory within the confines of a test tube |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| Matrix | Biological fluid or tissue |
| SoHT | Society of Hair Testing |
| TLFB | Time-Line Follow-Back, structured alcohol interview. |

## List of papers

**Paper I**: Fosen JT, Morini L, Sempio C, Ganss R, Morland J, Hoiseth G. Levels of Hair Ethyl Glucuronide in Patients with Decreased Kidney Function: Possibility of Misclassification of Social Drinkers. Alcoholism, clinical and experimental research. 2016;40(3):451–6.

**Paper II**: Fosen JT, Morini L, Sempio C, Giarratana N, Enger A, Morland J, Hoiseth G. Ethyl Glucuronide Elimination Kinetics in Fingernails and Comparison to Levels in Hair. Alcohol and alcoholism. 2017;52(5):580–6.

**Paper III**: Fosen JT, Hoiseth G, Sempio C, Giarratana N, Enger A, Morland J, Morini L. Hair EtG: Alterations in segment levels accompanying hair growth. Drug testing and analysis. 2019;11(1):112–8.

## Summary

### Background/aims

Excessive use of alcohol may have many different harmful effects in health, legal and social perspectives. Underreporting alcohol consumption is a well-known phenomenon when subjects are asked to report their own alcohol use, and objective alcohol biomarkers are needed. The direct alcohol biomarker ethyl glucuronide (EtG) analysed in keratin matrices is used to evaluate the history of ethanol intake. The importance of correct interpretation of EtG concentrations in keratin matrices is underscored by the fact that it is used in clinical decisions, workplace testing and forensic medicine. As a general aim, we wanted to investigate opportunities and limitations of EtG as a biomarker for chronic alcohol consumption in hair and nails. More specifically, we aimed to investigate if decreased renal function may result in different hair EtG levels compared to healthy volunteers and implications of this. We also compared EtG levels in hair and nails among chronic excessive alcohol users and studied EtG elimination kinetics in nails. In addition, we aimed to investigate if hair EtG is stable during hair growth in an alcohol-abstinent period.

### Methods

In paper I, we studied 41 patients with reduced kidney function and compared their hair EtG levels with levels found among 42 healthy volunteers, all reporting moderate alcohol use. In papers II and III we recruited patients from an alcohol rehabilitation clinic. In paper II, we included 40 patients and analysed EtG levels in both hair and nails at inclusion. In addition EtG was analysed in consecutive nail samples during the inpatient period. In paper III, we included 27 patients and for each patient we compared two consecutive hair samples collected with four-week abstinent intervals, analysing EtG concentrations in hair segments roughly representing the same alcohol consumption period.

### Results and conclusions

In paper I, we found significantly higher hair EtG concentrations among patients with reduced kidney function compared to the healthy volunteers, even though the self-reported alcohol consumption did not differ significantly between the two groups. Consequently, interpretation of hair EtG levels among patients with decreased kidney function must be performed with caution to avoid misclassification of amounts of alcohol consumed. In paper II, we found

higher EtG concentrations in nails compared to hair. EtG levels in both matrices correlated with self-reported alcohol consumption. EtG concentrations in nails disappeared faster than would have been expected from nail growth physiology. In paper III, we found decreasing hair EtG concentrations in most subjects during hair growth when comparing the two segments assumed to represent the same period of alcohol intake. Although there are uncontrolled factors present, the results support interpreting EtG in distal hair segments with caution.

In summary, this thesis is a contribution to enhancing knowledge about the interpretation of EtG in hair and nails as a long-term alcohol biomarker. The findings of kidney function affecting hair EtG levels, kinetic data of EtG in nails and the alteration of EtG levels during hair growth might be of great importance for clinicians and toxicologists interpreting EtG levels in keratin matrices.

# 1. Introduction

## 1.1 Harmful use of alcohol

Harmful use of alcohol is one of the leading health risk factors. Alcohol is estimated to have caused approximately 5.3% of all deaths worldwide in 2016, and 5.1% of the global burden of disease is attributable to alcohol consumption (1). In Norway, it is estimated that alcohol causes 1.1% of all deaths and 2.1% of the disease burden (2). According to the World Health Organization (WHO), alcohol consumption has a causal impact on more than 200 health conditions including both diseases and injuries. Alcohol use during pregnancy has been established as a risk factor for several adverse pregnancy outcomes and also increased intrauterine death (1). The negative health effects from alcohol use have been associated with average volume of alcohol consumption, with a dose-response relationship. Any decrease in alcohol consumption, at least down to 10 g/day, has been shown to reduce the annual and lifetime risk of an alcohol-related death (3, 4). WHO's drinking-related, long-term risk levels of serious illness is defined by mean alcohol consumption per day; high (men 61–100, women 41–60 grams), moderate (men 41–60, women 21–40 grams), and low (men 1–40, women 1–20 grams) (5). Statistics on total alcohol per capita consumption from 2016, based on both recorded and estimated unrecorded consumption, show on average of 13.9 grams of pure alcohol per day for the world's population 15 years of age or older and 32.8 grams among current drinkers (consumed alcohol last year) (1). Heavy drinking occasions or binge-drinking is also considered as an important factor in alcohol risk assessment. Heavy episodic drinking (HED) is defined as 60 grams pure alcohol or more on at least one occasion at least once per month and in 2016 the prevalence of HED was 18.2% among the world's population 15 years of age or older and 39.5% among drinkers (1). According to the Society of Hair Testing (SoHT) chronic excessive drinking is defined as average consumption of 60 g or more of pure ethanol per day over several months (6), and this definition will be used in the present thesis.

### 1.1.1 Implications of underreporting alcohol consumption

Global burden of disease calculations on harm rely on an estimation of alcohol consumption that is subject to many uncertainties (7). Underreporting alcohol consumption in self-report studies is a well-known phenomenon, and in several studies, self-reported alcohol consumption accounts for only 30 to 60% of official alcohol sales (8). Important factors for underestimating alcohol consumption are sampling-frame issues (e.g. failing to include subjects with high alcohol intake), non-response bias and under-reporting bias (9). The rate of

underreporting may also vary in a population, reflected by the fact that low socioeconomic status groups tend to report less frequent alcohol consumption and are more likely to drink at extreme levels (10). Underreporting among drinkers in cohort studies compared to alcohol per capita consumption data in population studies will lead to an overestimation of relative risk of alcohol-related problems which is also pointed out by others (11). However, underestimating the number of drinkers will potentially lead to an underestimation of the risk (12). The methods of screening and monitoring of alcohol consumption are therefore important in a health perspective but may also be important in other settings like workplace testing and forensic medicine.

## 1.2 Ways to measure alcohol consumption

The International Statistical Classification of Diseases and Related Health Problems 10th revision (ICD-10) include several diagnostic criteria for alcohol dependence ranging from difficulties in controlling use of alcohol to persistent use of alcohol despite evident presence of harmful consequences (13). This thesis will cover quantitative assessment of long-term alcohol use measured as amount of ethanol consumed per day. Different diagnostic aspects of alcohol abuse are not covered.

### 1.2.1 Interviews as an alcohol consumption screening tool

There are several alternatives for diagnosing problematic alcohol consumption, including self-reporting of alcohol consumption and use of biomarker-based approaches. Different survey alcohol measurement techniques all have various strengths, and different guidelines for alcohol measurement in surveys have been proposed (7, 9). Examples of structured alcohol interviews are the Alcohol Use Disorder Test (AUDIT) (14) and the Time-Line Follow-Back (TLFB) (15). AUDIT-C is a brief alcohol screen that includes three questions about alcohol drinking status/frequency of drinking, volume alcohol on a typical day and frequency of heavy episodic drinking. AUDIT-C is a short version of AUDIT, where AUDIT covers different aspects of alcohol use, including signs of addiction and problems associated with drinking habits. TLFB is a retrospective calendar-based detailed determination of the amount of alcohol consumed over a given period.

As mentioned above, underreporting alcohol consumption is a known problem, and the difficulties of diagnosing harmful alcohol consumption have been addressed in epidemiological studies (16, 17). Underreporting among patient groups can be an important

issue if one it causes a loss of rights contingent on high alcohol consumption, for instance when assessing patients eligible for hepatic transplantation, patients receiving opioid maintenance treatment or during pregnancy (18-21). This underscores the importance of objective biomarkers that properly reflect alcohol consumption.

### 1.2.2 Indirect alcohol biomarkers

Indirect alcohol biomarkers are proteins or cells which undergo typical changes in response to acute or chronic alcohol consumption (22). The indirect biomarkers, often called traditional alcohol biomarkers, are unspecific, ignoring whether they may be potentially influenced by a number of different factors like age, gender, body mass index, diabetes, non-alcoholic liver diseases, pancreatitis, hyperlipidaemia, cardiac insufficiency, severe trauma, medication and renal disease (23).

Blood transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are elevated as a result of organ damage which can be seen as being caused by high alcohol intake. ALT is primarily found in hepatocytes, and AST is found in several organs such as the liver, heart, skeletal muscles, kidneys and brain (24). The sensitivity of these transaminases to detect heavy drinkers with the diagnosis of alcohol dependence has been reported to be about 50% (25, 26). Elevated AST/ALT-ratio $\geq 2$ show a high specificity but low sensitivity for heavy alcohol consumption with alcohol induced liver damage (27).

Gamma-glutamyltransferase (GGT) catalyses gamma-glutamyl to various peptides and the source of GGT activity in blood is mainly from the hepatocyte microtubular system, but GGT is also expressed in other organs (28). GGT is elevated as a result of enzyme induction by chronic alcohol abuse and also shows increased concentration as a result of biliary stasis. False positive tests may be seen as a result of non-alcoholic liver and biliary disease, obesity and use of antiepileptic drugs (27). Using elevated GGT as a marker for heavy drinking shows a specificity of about 80% and a sensitivity of 40–60% (29).

Mean corpuscular volume (MCV) represents an average volume of red blood cells. MCV is a marker of red cell blood cell precursors and is elevated as a result of deficiency of vitamin B12 and folic acid (macrocytic anaemia). As alcohol and alcohol metabolites show toxic effects on the red cell morphology, MCV is often elevated among alcohol-dependent subjects with a specificity above 90% and sensitivity of about 40% (27).

For AST, ALT, GGT and MCV, some studies have found a dose-related relationship with alcohol intake, but no concentration limits have been established that represent defined quantitative measures of alcohol intake (24, 30).

Carbohydrate deficient transferrin (CDT) is often used to diagnose high alcohol consumption. Sialic acid-deficient isoforms of transferrin increases as a result of chronic high alcohol intake and CDT% represents the ratio between sialic acid-deficient transferrin isoforms and the total amount of transferrin. Asialo- and disialotransferrin are both reported to be related to heavy alcohol consumption in a dose-related manner (31). CDT% increases after approximately two weeks of high ethanol intake (60 g/d) and shows a high diagnostic specificity and a sensitivity of about 60–70% (25, 26). A recent standardization of CDT% measurement has concluded on using serum disialotransferrin to total transferrin fraction (31, 32). CDT% levels of ≥1.7% correspond to heavy alcohol use when high-performance liquid chromatography is applied (31).

The combination of several indirect alcohol biomarkers may improve diagnostics of high alcohol consumption. An example is the combination of GGT, MCV and CDT showing a sensitivity higher than 90% detecting alcohol dependence shortly after admittance to a alcoholism treatment programme (29).

## 1.3 Direct alcohol biomarkers and metabolites of ethanol

The short half-life of alcohol in the human body limits the use of ethanol itself as a biomarker to assess recent alcohol intake. Ethanol can be measured in blood in cases involving evidence of alcohol impairment or intoxication, and in urine to show recent intake with a detection window extending up to some hours from detection in blood (33, 34). Transdermal alcohol monitors allow continuous measurement of alcohol consumption, exploiting the fact that about 1% of alcohol is excreted unchanged through sweat (35). This method has been used to study the correspondence to self-reported drinking (36, 37).

### 1.3.1 Oxidative metabolism of ethanol

Less than 10% of ingested ethanol is excreted unchanged in urine, breath and through the skin through sweat (38). Ingested ethanol is mainly metabolized by oxidative pathways. About 90% of the ingested ethanol is converted to acetaldehyde by the enzyme alcohol dehydrogenase (ADH) and acetaldehyde is then metabolized by the enzyme acetaldehyde

dehydrogenase (ALDH) to acetate. A small proportion of the ingested ethanol is metabolized to acetaldehyde by the cytochrome p450 (CYP) system with the enzyme CYP 2E1 as the most important one. CYP 2E1 activity may be affected by nutrition state and certain drugs, and the induction of CYP 2E1 by ethanol itself is one of the mechanisms for accelerated metabolism of ethanol among alcoholics (38).

### 1.3.2 Non-oxidative metabolites of ethanol to be used as direct alcohol biomarkers

A minor but important metabolic pathway to the alcohol biomarker field involves the phase II metabolism resulting in the formation of the conjugated metabolites. Several of these non-oxidative metabolites can be used as direct alcohol biomarkers, which means molecules that are created when ethanol is metabolized or reacts with substances in the body (22). The use of direct alcohol biomarkers shows both high sensitivity and specificity to detect alcohol intake (39).

Enzymatic esterification of ethanol with fatty acids provides fatty acid ethyl esters (FAEEs) and this process is part of the non-oxidative metabolism of ethanol. FAEEs increase rapidly after intake of ethanol and maximum blood concentrations is reached within few hours (40). The FAEEs' elimination half-life from blood is reported to be up to 13–16 hours, and it is believed that FAEEs may accumulate in adipose tissues, heart muscle and the pancreas (41, 42). FAEEs, defined as ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate, can be detected in hair as long-term alcohol biomarkers. Ethyl palmitate above 350 pg/mg in the 0–3 cm proximal segment (450 pg/mg for the 0–6 cm segment) of scalp hair is considered strongly suggestive of chronic excessive alcohol consumption (≥ 60 g/day) (6). FAEEs is incorporated into hair mainly from sebum which may lead to an increase in concentration from proximal to more distal hair segments even subsequent to unchanged drinking behaviour (43). FAEEs concentrations in hair may be influenced by cosmetic treatments and thermal hair straightening, and use of alcohol-containing hair tonic can give false positive results (39).

Another non-oxidative metabolite is phosphatidylethanol (PEth), which is a phospholipid that is formed in cell membranes only in the presence of ethanol catalysed by phospholipase-D enzyme (44). Different fatty acids result in various homologues of PEth, and the PEth homologues 16:0/18:1 and 16:0/18:2 are most prevalent in human blood (39). PEth is formed directly after alcohol intake and is eliminated from blood with a half-life of about 4 to 7 days (39, 45), but a range of 1 to 13 days have been reported in the literature (46). PEth detection time in blood has been reported to be up to three weeks after withdrawal of alcohol among

alcohol abusers (39). In a systematic review by Viel et al. (47) they found that diagnostic sensitivities for chronic alcohol use by the use of PEth in blood were high, varying from 98% to 100% for cut-off concentrations of PEth of 0.22 μM, 0.30 μM or 0.8 μM. PEth 16:0/18:1 values equal to or above 0.3 μM is used in both Norway and Sweden as a cut-off indicating problematic or harmful alcohol consumption, but no clear definition of alcohol consumption with regards to number of grams of alcohol per day is given (39, 48, 49). Although it has been stated that PEth concentrations are found to correlate with the amount of ingested alcohol in alcohol-dependent persons, an experimental study showed a 4-fold interindividual difference between maximum concentration of PEth and the detection time varied from 3 to 12 days among 16 volunteers after a single dose of alcohol estimated to lead to a blood alcohol level of about 1 g/kg (50). Another important weakness is that the presence of ethanol in a blood sample can lead to formation of PEth in vitro (51).

Although there may be large interindividual differences with regards to levels of the mentioned indirect and direct alcohol biomarkers after a certain amount of alcohol intake, they could provide important information when following a person over time with serial biomarker tests (45, 52).

In addition, measurement of ethyl glucuronide (EtG) and ethyl sulphate (EtS) in different matrices is used as direct alcohol biomarkers. The following chapters will describe this in detail, with the main focus on EtG.

## 1.4 EtG as a result of non-oxidative metabolism of ethanol

The conjugated ethanol metabolites EtG and EtS are also formed as part of the phase II metabolism of ethanol. EtG is a product of ethanol and glucuronic acid, catalysed by different uridine diphosphate-glucuronosyltransferase (UGT) isoenzymes (53). The UGT enzymes are located in the microsomal fraction of various tissues, mainly in the liver but also to a minor degree in kidney, skin, lung, gastrointestinal tractus, bile ducts, brain and some other organs/tissues (54). A large number of xenobiotics are eliminated from the human body including many frequently used drugs (e.g. morphine) which are conjugated with glucuronic acid by the same UGT enzyme superfamily responsible for the formation of EtG (55). Previous in vitro studies have found that isoenzymes UGT1A9 and 2B7 are predominantly involved in EtG formation and that UGT enzyme activity can be affected by flavonoids and cannabinoids resulting in altered ethanol glucuronidation (56, 57), but morphine, codeine,

nicotine or cotinine did not show any effect on ethanol glucuronidation (57). Caffeine has recently been shown to increase EtG levels in serum in rats (58), but there are no studies indicating induction of UGT enzymes by ethanol.

EtS, which is often used to verify positive EtG samples, is a product of ethanol and sulphate catalysed by cytosolic sulphotransferase (SULT) which in animal models have been identified in both liver and pulmonary cells (59). Among the SULTs found in human liver tissue several SULTs are involved in the formation of ETS, with SULT1A1 as the most important (60).

EtG and EtS have been proven to be both sensitive and specific biomarkers of ethanol ingestion (61) and EtG, and to some degree EtS, have become a routine analysis in urine. Specialized laboratories analyse EtG in blood/serum/plasma and hair, and in addition nails are also increasingly analysed in some laboratories. Measuring EtS in hair and nails has been challenging and no articles on this topic were published until 2018, when the first analytical method for EtS in hair was published (62).

EtG concentrations in serum have been shown to be dependent on both ethanol dose and drinking exposure time in a pharmacokinetic model (63). Experimental studies have investigated EtG-concentrations in blood after intake of ethanol and found a positive relation between ethanol dose and EtG concentration (64-66). So far, the research suggests a linear relationship between ethanol concentration in plasma and EtG excreted in urine (67), and also between ethanol concentrations in blood and EtG concentrations in blood (68). Accordingly, there is no clear indication of clinically relevant enzyme saturation. Moreover, there are no studies indicating induction of EtG kinetics in chronic excessive drinkers. There may therefore be reason to believe that EtG has a potential as a quantitative alcohol biomarker.

### 1.4.1 EtG elimination

As mentioned above, several xenobiotics are glucuronidated as part of the phase II metabolism, similar to EtG. Once glucuronidation has occurred, the glucuronide conjugates are excreted either in bile or urine (69). EtG is eliminated mainly through urinary excretion (70, 71) and less than 0.1% of the ingested low-to moderate doses of ethanol are excreted as EtG in urine (33, 70, 72-74). The blood elimination half-life of EtG is approximately 2–3 hours (33, 75, 76). Depending on the amount ethanol consumed in a single intake, EtG may be detected in blood some hours after ethanol has been eliminated from the body, and up to about 24 hours after repeated and large ingestions (33, 75, 76). In urine, EtG may be detected

1–2 days after a single alcohol intake (33) and up to four or five days after repeated and large ingestions (77, 78).

Similar to EtG, less than 0.1% of the ingested ethanol dose is excreted as EtS in urine, however the portion of EtS is reported to be somewhat lower than EtG and blood and urine EtS concentrations are most often seen in lower concentrations than EtG (72, 74, 79, 80). Maximum serum and urine concentration of EtS is reached about an hour earlier than for EtG (65, 79) and the detection time for EtS is comparable or a bit shorter than for EtG for both serum and urine (75, 77, 79).

### 1.4.2 EtG stability

EtG is considered to be fairly stable, but it is important to be aware of some important noteworthy aspects. Material containing EtG decomposing at room temperature can result in decreasing EtG concentrations giving false negative results (61, 81-83). Even false positive EtG can be a problem as a result of in vitro production of EtG if urine samples are contaminated by E.coli bacteria in the presence of ethanol, which can be a problem especially in patients with diabetes mellitus (84). In vitro production of EtG has also been shown in post-mortem blood spiked with ethanol (81). However, keeping samples at low temperature and using fluoride preservatives can prevent bacterial growth and ensure stability of EtG (20). The importance of fluoride preservatives has also been emphasized for EtG stability in post-mortem blood samples (83). EtS is reported to be unaffected by bacterial contamination and is therefore important when interpreting urine results (20), except during very extreme conditions not considered relevant for real samples (85). Therefore, EtS will be useful to verify EtG positive samples. However, EtS can be present in non-alcoholic wine and consumption of this could lead to an EtS-positive test and a false assumption of alcohol ingestion (86).

## 1.5 EtG as a biomarker for alcohol consumption

During recent years, EtG has been established as an important alcohol biomarker in workplace testing, forensic medicine and clinical medicine. The use of EtG as a biomarker for alcohol intake may have major implications for the individual and for clinical decisions. Examples are assessment of suitability for a driving license, workplace testing, detection of problematic alcohol use in child custody cases and assessment of patients on waiting list for organ transplantation. EtG is also used to verify if post-mortem ethanol findings are results of

alcohol intake prior to death or because of post-mortem production of ethanol in the human body. EtG is also used in evaluation of blood samples taken from drunk drivers claiming alcohol intake after an incident (hip-flask defence) (87).

This thesis will cover EtG as an ethanol biomarker in hair and nails, matrices that allow long detection windows.

### 1.5.1 Importance of renal function for excretion of EtG

Based on theoretical considerations, renal function could affect the excretion of EtG. Morphine is also known to undergo extensive metabolism by glucuronidation, and accumulation with increased serum concentrations have been shown for the metabolites morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in patients with decreased kidney function as the excretion pathway for the glucuronide metabolites are affected (88-90). Even profound clinical effects with prolonged intoxication reactions have been reported for kidney failure patients treated with morphine (91).

Similar to the reported accumulation of morphine-glucuronides, one could expect accumulation of EtG with higher EtG concentrations in patients with impaired kidney function compared to healthy individuals as a result of EtG's elimination pathway by urinary excretion.

In clinical practice, renal function is mostly measured by creatinine and the calculation of estimated glomerular filtration rate (eGFR). An alternative endogenous filtration marker to creatinine is cystatin C (92). The preferred formula for eGFR is the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, published in 2009, which uses creatinine, age, gender and ethnicity (93). Prior to this, the Modification of Diet in Renal Disease (MDRD) Study equation was used. There are some limitations using eGFR like extremes of muscle mass or body size, special diet and nutritional status and unstable kidney function (acute kidney disease). The gold standard method to measure glomerular filtration rate (GFR) is urinary clearance during continuous intravenous infusion of inulin, but radionuclides and other markers such as iohexol or iothalamate are also considered as accurate methods (92). Many diseases are known to cause decreased renal function, but renal function also decreases with age which is reflected by the formulas of eGFR.

### 1.5.2 Previous studies of EtG concentrations among patients with reduced kidney function

Increased EtG detection time in urine as a result of kidney failure has been shown in a previous study (94). In addition, elevated EtG blood concentrations have previously been reported for one patient with reduced kidney function (76), and a preliminary study by Høiseth et al. (95) reported elevated hair EtG levels among kidney failure patients. The importance of reduced kidney function on EtG as a quantitative alcohol biomarker, however, is still unclear.

## 1.6 EtG in hair and nails as a long-term marker for alcohol consumption.

Hair and nails are keratinous matrices and detection of incorporated EtG offers the opportunity to study alcohol consumption retrospectively for a much longer time compared to blood and urine. In recent years, EtG in hair has been established as a long-term marker for chronic alcohol consumption (96, 97).

### 1.6.1 Hair physiology and incorporation of EtG

Hair grows from hair follicles which is located approximately 3–5 mm below the surface of the skin. A rich capillary system is surrounding the hair follicle, allowing diffusion of molecules into the hair (98). The matrix cells (keratinocytes and melanocytes) undergo rapid mitosis forming the three hair shaft layers medulla, cortex and cuticle. The cuticle, the outer non-pigmented layer, consists of shingle-like cells that protect the hair from light, heat, chemical exposures and physical damage. Next to the cuticle, we find the cortex, which is pigmented by melanin, and the innermost part is the medulla. Hair grows in cycles, known as the anagen, catagen and telogen phases. In the anagen phase the hair grows in length, and this phase can last for several years. Then the hair reaches the catagen or transitional phase where cell division stops and the hair shaft has been fully keratinized. In the telogen phase there is no hair growth. About 10–15% of all head hair is estimated to be in this resting phase, and 85% is in the growth (anagen) phase. Head hair is expected to have a mean growth rate of 1.0 (range 0.60–1.50) cm per month and it has been estimated to take approximately 7–10 days for the growing hair to reach the surface of the scalp (99).

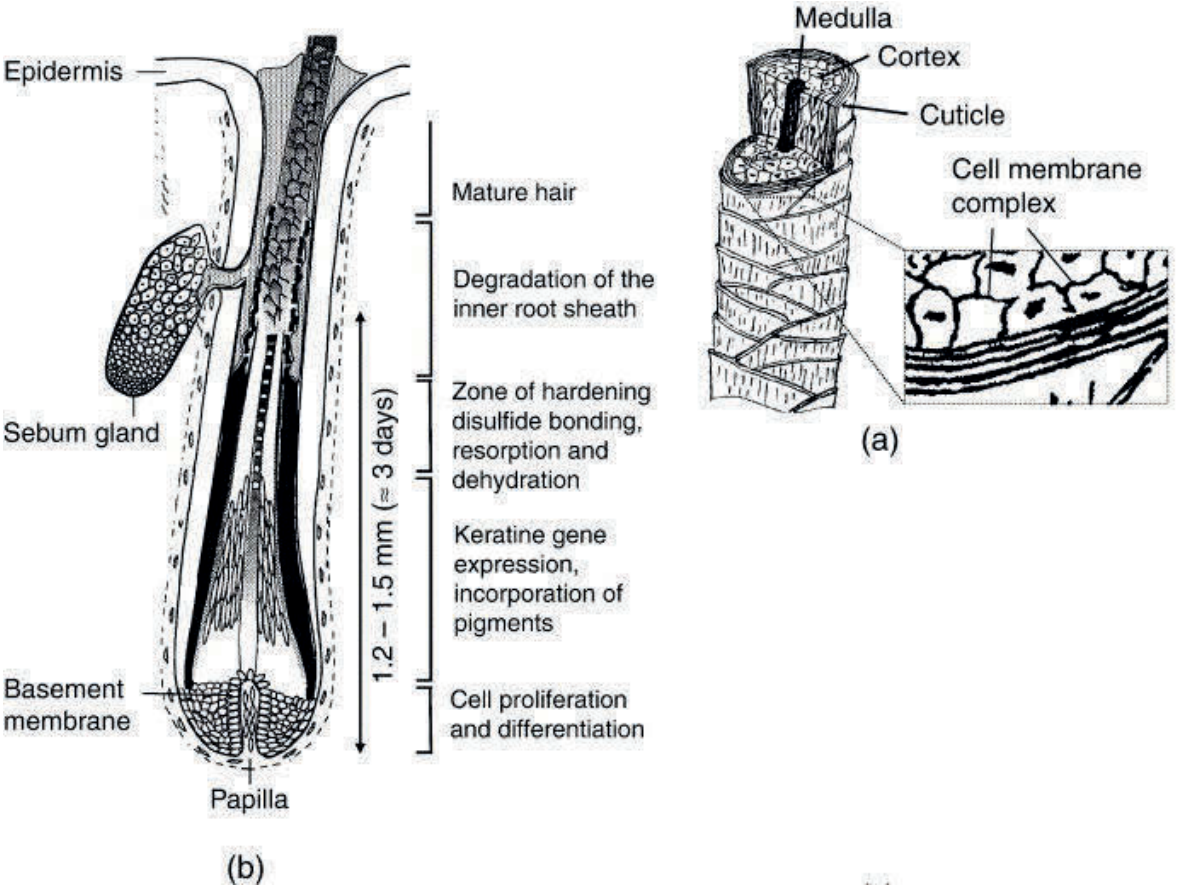


**Figure 1**. a) Structure and constituents of the human hair shaft. b) Formation of hair in a follicle from matrix cells on the basement membrane to the mature hair shaft. Drug incorporation from blood should occur in a 1.2–1.5 mm zone before completion of keratinisation. Adapted from Pragst et al. (98).

Incorporation of EtG into hair is not fully understood. The main mechanism is assumed to be incorporation of molecules into hair by passive diffusion from blood capillaries surrounding growing matrix cells and also to some degree by contamination from sweat and/or sebum into the completed hair shaft (100-102). This implies that in cases where there has been recent ethanol intake, EtG results may also reflect contamination by body fluids (103). However, concentration measured in hair has been proposed to correlate best with the area under the blood concentration versus time curve and the following equation for substance concentrations in hair has been proposed for this main incorporation pathway (42):

$C_{hair,\chi} = ICR_{\chi} \times AUC_{blood,\chi}$

Where $ICR_{\chi}$= incorporation rate of substance $\chi$, $AUC_{blood,\chi}$= area under the blood concentration versus time curve of substance $\chi$.

EtG is a hydrophilic and acidic molecule with pKa=3.21 (98, 104), which means that EtG is rather negatively charged at physiological pH. As a result of intracellular pH of keratinocytes and melanocytes has been found to be more acidic than plasma, the distribution equilibrium is shifted in disfavour of EtG incorporation in the hair root (98). This may be an important cause of low EtG concentrations in hair compared to more lipophilic substances.

Hair EtG is not affected by natural hair colour or pigmentation by melanin (105). According to several studies, gender does not influence EtG incorporation in hair (106-109); however, one study has suggested that there are lower EtG levels in female compared to male samples mediated by hair dyeing (110). An effect of body mass index (BMI) could be theoretically suspected given the close relationship between BMI and ethanol concentrations, and Crunelle et al. (111) found significantly higher hair EtG concentrations among subjects with BMI ≥25 $kg/m^2$ compared to those with BMI <25 $kg/m^2$. However, BMI does not seem to affect diagnostic performance using EtG in hair to detect drinking habits (107, 112). Age is also considered to affect alcohol metabolism and Salomone et al. (109) found higher average EtG concentrations in hair among subjects above 40 years old compared to younger, but this study did not have information about alcohol drinking history. However, in several studies, age was not found to affect hair EtG test performance (107, 113).

It is important to be aware of the fact that certain hair care products can contain EtG which may result in false positive hair EtG test (114). Some studies (115, 116) have reported no significant increase of EtG in hair after regular use of hair care products containing ethanol, but Morini et al. (117) demonstrated false positive hair EtG after prolonged use of alcohol-based perfumes in hair.

### 1.6.2 Nail physiology and incorporation of EtG

About 80% of nail growth happens in the germinal matrix (see figure 2), where keratin cells are formed and give horizontal growth of the nail plate. In addition, approximately 20% of the nail plate is a result of vertical growth from the nail bed giving increased thickness to the nail (118, 119). The nail lacks melanin as a result of very few melanocytes involved in the growth process. An important difference compared to hair is that nails show continuous growth (118). The growth speed of fingernails is about 0.1 mm per day or 3 mm per month (range 1.9–4.4 mm per month) (120), resulting in a potential detection window usually of about 3–5 months in fingernail and even longer in toenails (up to 14 months) as a result of slower growth speed (118).

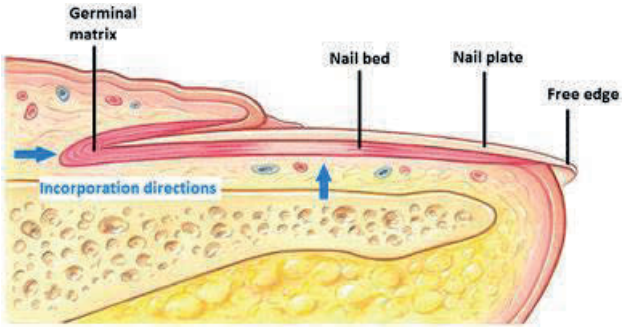


**Figure 2**: Structure of the nail: a sagittal section of the fingertip. Figure from Cappelle et al. (119).

The incorporation mechanisms in the nail are still unclear. However, the main incorporation mechanism of substances into nails is believed to happen mainly through diffusion from the rich blood supply both in the germinal matrix and the nail bed, thus allowing incorporation in both a vertical and horizontal way during nail formation (119). In addition, sweat and sebum-mediated transport of molecules is also believed to contribute to the incorporation of substances in the nail. This latter mechanism can explain why substances can be found by analysis of the nail already within the first 8–24 hours after administering a drug (121, 122). External contamination by direct contact with substances also have to be considered as a potential source for incorporated molecules (123).

Both nail clippings of the nail overhang and scrapings obtained by abrasion of the upper layers of the nail can be used for analysis. Post-mortem removal of the nail during an autopsy allows the entire nail to be collected for analysis (118).

### 1.6.3 Previous studies of hair EtG compared with alcohol consumption

A meta-analysis from 2013 reported that subjects with average consumption exceeding 60 grams alcohol per day, showed a mean hair EtG of 142.7 pg/mg (95% confidence interval 99.9–185.5 pg/mg). For subjects with EDI above 0 and up to 60 grams alcohol per day, there was a mean hair EtG of 7.5 pg/mg (95% confidence interval 4.7–10.2 pg/mg) (124).

Another meta-analysis was published in 2014 showing pooled analysis of raw data from several studies where subjects reporting average consumption exceeding 60 grams alcohol per day were included. The results of the pooled analysis showed a sensitivity of 0.85 and a specificity of 0.97 for hair EtG with a 30 pg/mg cut-off. The same study found a parabolic (less increase in hair EtG at the highest EDIs) direct correlation between hair EtG level and

estimated daily intake of ethanol (EDI) (Spearman's ρ=0.79; 95% confidence interval 0.69–0.87) (100).

Several primary studies have investigated hair EtG as a long-term alcohol biomarker and compared hair EtG concentrations with alcohol consumption (106, 107, 113, 125-137). Many of these studies are part of the knowledge base for international guidelines on this topic. Organizations like European Workplace testing and SoHT have implemented use of hair EtG in their guidelines and specific EtG concentration limits have been defined to represent different amounts of alcohol intake (138, 139). The latest SoHT consensus on alcohol markers in hair define a concentration cut-off level to detect chronic excessive alcohol consumption which corresponds to an average consumption of 60 g or more of pure ethanol per day over several months. This cut-off level has been set equal to or greater than 30 pg/mg EtG in the proximal head hair with a segment length of 3 cm and up to 6 cm. Hair EtG concentrations above 5 pg/mg strongly suggests repeated alcohol consumption and hair EtG concentration lower than or equal to 5 pg/mg does not contradict self-reported abstinence (6).

### 1.6.4 Previous studies of nail EtG compared to hair EtG

Analysis of drugs in nail has during the latest years been proven to be a promising alternative to hair, also by using EtG as a long-term marker for chronic excessive alcohol consumption (118, 119, 140). However, only a few studies have compared EtG concentrations in hair and nail (128, 141, 142). Jones et al. (141) found that EtG in fingernails were about three times higher than EtG in hair. Berger et al. (128) reported high sensitivity and specificity for EtG in both fingernails and hair as long-term alcohol markers and suggested a possible cut-off of 56 pg/mg for chronic excessive consumption, which is higher than the established 30 pg/mg cut-off in hair (6). Morini et al. (142) studied EtG in fingernails but also compared hair and nail samples for one of the subjects and concluded that EtG accumulates at a higher rate in nails than in hair and that EtG in nails could be a potential marker of both chronic alcohol abuse and binge drinking habits.

### 1.6.5 Previous studies of EtG kinetics in nail

To our knowledge, no studies have investigated longitudinal concentrations of EtG in nail after alcohol drinking cessation. The knowledge of nail EtG elimination kinetics is important when interpreting EtG concentrations in nail, both with respect to the amount of ethanol intake but also how long one could expect to find EtG in nails. There are a few studies which

have used a longitudinal design to evaluate non-ethanol-related drug concentrations in nails and compared to concentrations found in hair (121, 143-145).

For nails, no EtG guidelines are currently established due to the sparse amount of research.

### 1.6.6 Stability of EtG after incorporation into hair and nails

Segmental hair analysis of EtG is widely used. The segmental hair analysis is performed by analysing EtG concentration in a hair segment, which – based on hair growth speed – can be calculated to represent a specific period of time for alcohol consumption. Interpreting EtG in different hair segments assumes that EtG remains quite stable in hair after incorporation into the hair strands. Cosmetic hair treatment like bleaching, perming and dyeing of hair may lead to lower concentrations of hair EtG or false negative results (6, 146-149). Bleaching and perming are reported to remove a significant amount of EtG incorporated in hair with a mean reduction of 73.5% and 95.7%, respectively (147). Temporary dyeing seems to be less aggressive to hair with no important change in hair EtG compared to permanent dyeing, which shows a marked decrease in hair EtG (147, 149). Thermal hair straightening has also been shown to reduce EtG levels in vitro (150). Studies of photo-degradation of EtG in hair by sunlight are sparse, but one study found both increasing and decreasing EtG concentrations in hair irradiated by artificial sunlight (151). Regarding stability after collection of hair samples, in vitro experiments have concluded that EtG remains stable in hair even after accelerated ageing by storage at high temperatures (152, 153).

Correspondingly for nails, and due to lack of scientific studies, one can hypothesize that external contamination, chemical influence by nail care products and even hand-washing routines can be of interest when interpreting nail EtG results. Regarding the stability of EtG in nail clippings after sample collection, EtG concentrations were found to be constant in EtG-positive nail samples analysed regularly for a five-week period (142).

### 1.6.7 Previous studies of stability of EtG after incorporation into hair

According to the SoHT's consensus from 2019, analysing EtG in hair segments less than 3 cm or greater than 6 cm should be interpreted with caution (6), indicating that EtG concentrations in these segments might not be representative for the corresponding alcohol consumption period.

Only a few studies have tried to address the question of whether EtG remains stable after incorporation into hair and if EtG concentrations remain representative for the alcohol intake

in the corresponding more distal hair segment (42, 103, 126, 154-156). The results of these studies are conflicting in terms of whether EtG in distal hair segments is representative for the alcohol consumption in the corresponding time period. Agius et al. (103) and Appenzeller et al. (126) found that hair EtG was stable or matched drinking history over several months (up to 12 months) when comparing different hair segments from single time-point hair sample. Meier et al. (155), Pragst et al. (42) and Tsanaclics et al. (156) found declining EtG concentrations from proximal to more distal hair segments. However, none of these studies were designed to evaluate changes in hair EtG with a longitudinal design, as they all used samples collected at one single time-point which was segmented before analysis. The in-vitro experiments of Luginbühl et al. (154) investigating the effects on EtG by incubating hair samples in water, showed decreasing EtG concentrations for both deionized- and chlorinated water. The extent to which hair EtG during abstinence is stable after incorporation and whether EtG in hair is representative for alcohol consumption in the corresponding more distal hair segments are therefore issues that are important to address in a longitudinal study design.

## 1.7 Summary

In summary, hair EtG analysis is an established method as long-term biomarker for alcohol with cut-off limits for different amounts of alcohol intake. However, some aspects such as importance of reduced renal function and the stability of EtG during hair growth are important to clarify. The use of nail EtG analysis is still in the experimental stage and increased knowledge about EtG kinetics and EtG levels in nails is needed.

# 2. Aims of the thesis

## 2.1 General aim

The general aim of this thesis was to study the ethanol metabolite EtG in nail and hair considering opportunities and limitations as a marker of chronic alcohol intake.

## 2.2 Specific aims

I decided to focus on the following, more specific aims:

1. To investigate whether renal disease can impair EtG excretion sufficiently to increase hair EtG levels, disturbing its potential as a marker of chronic excessive drinking (paper I).

2a. To investigate the relationship between EtG concentrations in hair and nails related to alcohol consumption (paper II).

2b. To investigate the elimination kinetics and detection times of EtG in fingernails during an alcohol abstinence period (paper II).

3. To investigate change of EtG levels during hair-growth in an alcohol abstinence period, and whether this potentially may imply limitations in the use of EtG in hair segments as a quantitative measure of alcohol intake (paper III).

# 3. Methodological considerations

Division of Forensic Sciences, Norwegian Institute of Public Health was responsible for studies performed up until 1 January 2017. As of this date, the sections and departments where transferred to Oslo University Hospital and were given new names. The current name is Department of Forensic Sciences, Oslo University Hospital.

## 3.1 Study design

A preferred study design for our aims would ideally be a randomized controlled intervention study with predefined daily alcohol doses for a longer period, but this would have been difficult to conduct due to large alcohol doses (in paper II and III) and ethical concerns regarding this. Instead, all studies were designed as prospective observation studies based on measurements of EtG and self-reported alcohol consumption.

In paper I, we recruited patients from a kidney failure clinic and compared hair EtG concentrations with subjects from age-matched control group of healthy volunteers. Both groups had self-reported moderate alcohol consumption. The hair EtG results from 12 of the patients included in this study was also published as a case series in a preliminary study by Høiseth et al. (95) but the results were only compared to other previously published data of healthy volunteers from an Italian population (135). In paper I, we decided to include a higher number of patients with reduced kidney function and include an age-matched Norwegian control group.

To minimize age-biased results we recruited the same number of subjects in the study group and the control group with the following age intervals: below 59 years, 60 to 69 years, 70 to 79 years, and 80 to 89 years. It was difficult to get enough control subjects matched with the patients in all age intervals. After advertising (email and bulletin board) at the Norwegian Institute of Public Health and the University of Oslo, we recruited participants. Also some acquaintances of these and some colleagues were included in the control group. None of the colleagues were employed in the same section or were directly subordinate or superior to any of the persons in the project group.

One inclusion criterion for the patients participating in the study was a diagnosis of decreased kidney function but without a need for dialysis, according to the patient's journal. In addition, we used the criterion of serum creatinine value >90 µmol/L in women and >100 µmol/L in

men and estimated glomerulus filtration rate (eGFR) <60 mL/min/1.73 $m^2$. We assumed that the combination of the clinical diagnosis, creatinine and eGFR values would ensure that no subjects with normal kidney function would be included in the patient group. For both the patient and control group there was an inclusion criterion of moderate alcohol intake (0< EDI <60 g/day) during the past three months prior to inclusion. For the control group, known kidney disease was an exclusion criterion.

In papers II and III, patients from an alcohol rehabilitation clinic were included. Sixteen of the 40 patients in paper II were also included in paper III; however, the data obtained were different in the two studies. Hair and nail samples were collected for analysis of EtG, and EDI was estimated based on self-reported alcohol consumption during the past 3 months before inclusion. Primary inclusion criteria included alcohol dependence according to ICD-10 (13). In addition, we used a criterion of alcohol consumption during the last three weeks prior to attendance. It is debatable whether using EDI as an inclusion criterion might have been more suitable, as the amount of alcohol consumed was more relevant in this pharmacokinetic study than the diagnostic criteria for alcohol dependence. However, the various drinking patterns during the past days or even weeks prior to inclusion complicated the use of EDI as an inclusion criterion. It must be considered a weakness in both papers II and III due to the unequal period of abstinence before inclusion among different patients. The median number of abstinence days were 8.5 days (range: 2–18) in paper II and 7 days (range: 2–16) in paper III.

All hair samples were collected from the posterior vertex of the scalp. A recent publication shows large differences in EtG concentrations depending on the collection site from the scalp and also very different distribution of EtG between individuals (157). This emphasizes the importance of sample collection from the same area, especially in paper III, where two hair samples were collected with a time interval of about one month. The sample collectors were instructed to use the posterior vertex for all samples, but the sample collection site was not systematically registered. In paper III, we analysed EtG for each patient in the 0–3 cm hair segment (named T1) from the hair sample taken at inclusion and the 1–4 cm hair segment (named T2) collected approximately one month later. With the assumption of hair growth speed of one cm per month, the T1 and T2 segment should roughly represent the same period of alcohol intake. This study design, however, does have some important limitations. Firstly, there was no control of the growth speed of the hair. In addition there were some difference of the time period between collection of the first (T1) and the second (T2) hair sample with a

median of 28 days and above 33 days for four of the patients (interquartile range: 28–33), which is somewhat problematic in light of the assumed growth speed of 1 cm per month and using EtG results from the 1–4 cm T2 segment in comparison to the 0–3 cm T1 segment. Analysis of the second hair sample's 0–1 cm hair segment could have provided important information on this, but there was not sufficient hair material for analysis of this segment. A problem arises as a result of the differences in time between collection of the first and second hair sample and a possible difference in hair growth speed. In cases of more than one month (30.5 days) between sampling and/or hair growth speed exceeding 1.0 cm per month, a part of the 3 cm T2 segment subjected to the analysis refers to the abstinence period which will dilute EtG concentration in the T2 segment (negative bias). An estimation of this possible negative bias was not reported in paper III, but in an attempt to estimate this we calculated a dilution factor for all individuals. The dilution factor was calculated as follows:

$$\frac{[\text{Assumed hair growth between T1 and T2 segment collection}] - 1}{[Hair\ segment\ length]}$$

This results in the following calculation in case of a hair growth speed of 1.3 cm per month:

$$\frac{\frac{1.3\ cm}{30.5\ days} * [Number\ of\ days\ from\ T1\ and\ T2\ segment\ collection] - 1}{[Hair\ segment\ length]}$$

Assuming a hair growth speed of 1.3 cm for all individuals, the calculated dilution factor was ≤5% for 9 patients, 6–10% for 12 patients, 11–15% for 3 patients and 3 patients had a dilution factor >15% (17, 20 and 25%). After correction of the T2 segment EtG results using this dilution factor for all individual subjects in the study, there was still significant change in EtG concentration from T1 to T2 (p=0.008) and the median change in EtG from T1 to T2 went from -46.0 to -42.0%. Moreover, after calculating a dilution factor based on an extreme hair growth speed of 1.5 cm per month, there was a significant change in EtG concentration from T1 to T2 (p=0.025) with a median change of -37.9%.

In total, it should be noted that the studies were not designed to find the effect of possible confounders like hair washing and cosmetic hair treatment. Nevertheless, these factors may be important, and we therefore recorded cosmetic hair treatment (bleaching, dyeing, perming) in all studies. These results are considered in the papers. Regarding hair washing routines, we have also recorded activity in the three-month period before inclusion in papers II and III, but these results are not considered in the papers. In paper I, information about hair washing

routines was inadequate. After reassessment of the data in paper II, we found no correlation between hair EtG/EDI and hair washing frequency (Spearman's $\rho$=-0.259 p=0.117). In the study of paper II, we also registered cosmetic nail treatment and hand-washing routines and these results were not considered in the paper. There were four response options when reporting hand washing, but the patients reported only two of the four different choices of handwashing frequency (twice or more hand washings per day, handwashing prior to every meal). These two choices represented hygienic routines with the largest number of hand washings per day. The questions were not optimal as it may be difficult to understand the differences between the response options. We found significantly higher nail EtG/EDI at inclusion for those reporting hand washing twice or more daily compared to hand washing prior to every meal (p=0.028). There was no significant difference in nail EtG/EDI among patients who had used nail-polish and patients who did not use nail-polish during the three months immediately prior to inclusion (p=0.052), but it is difficult to draw a conclusion about this because of the small number (n=3) of patients who reported using nail-polish. Regarding papers II and III, there was a weakness resulting from not registering this type of information during the inpatient programme, but visual inspection of the samples yielded no cause to suspect that cosmetic hair and nail treatment occurred during the study period.

### 3.2 Self-reporting of alcohol consumption and use of questionnaires

Ideally, we would have used an objective quantitative biomarker as a gold standard assessing the alcohol consumption on individual basis. In the absence of this, all studies used self-reported alcohol consumption in comparison with the EtG results. For all subjects, an EDI was calculated on the basis of self-reported alcohol consumption as a mean for the last three months. In the papers, we have related our alcohol consumption data to the SoHT's definition of chronic excessive drinking which means average consumption of 60 g or more of pure ethanol per day over several months (6). In the papers, we used some different terms, e.g. "heavy drinkers" for EDI of 60 g/d or more and "social drinkers" for EDI between 0 and 60 g/d. The use of these terms was not optimal because they are not well defined in the SoHT's guidelines or elsewhere in the scientific literature. Underreporting of alcohol consumption has been reported to be a problem of concern when trying to estimate drinking habits in alcohol surveys (7, 158). The magnitude of this skewed reporting is unknown and indirect alcohol biomarkers have been used only to a very limited degree to try to address this question (159). Analysis of previous population studies have been performed using high-density lipoprotein cholesterol (HDL-C) as an objective measurement of alcohol consumption on a population

basis, and HDL-C concentrations strongly suggest underreporting in these studies (10, 17). Although underreporting alcohol consumption in these types of epidemiological studies cannot be transferred directly to our studies, it is important to be aware of this possible bias. Validated questionnaires like TLFB (15) in paper I and AUDIT-C (14) in papers II and III were used when interviewing the included subjects in an attempt to minimize the problem of underreporting. The TLFB test is designed to obtain retrospective information about the drinking pattern, frequency and quantity consumed based on a calendar-based approach have been considered as a reliable validated tool (160, 161). In paper I, the TLFB-test procedure was considered to be a suitable tool to obtain EDI for both the patient group and the control group. This detailed approach was chosen because of the population with low-to-moderate alcohol intake (EDI <60 g/day). In papers II and III, our co-authors in Pavia suggested the use of AUDIT-C questionnaire (162) modified with additional questions about hair and hand washing and cosmetic treatment routines as the best tool when evaluating the EDI. As AUDIT-C is a brief screening test for problem drinking and contains only three questions regarding alcohol consumption, the clinical staff were trained to ask additional questions about drinking habits with the goal of obtaining an EDI representing the mean intake during the prior three months. A weakness of this method was that we did not have detailed insight into the process by which the clinical staff retrieved information about consumption and the calculation of the EDI.

The AUDIT-C scores were not reported in papers II and III, but the scores show that nearly all patients were in the high-to-severe risk category of alcohol dependency (maximum possible score is 12 points) (163). In paper II, all patients showed AUDIT-C scores in the high risk (6–7) or severe risk (8–12) category for alcohol dependency except for two patients with a score of 5 (moderate risk). The median AUDIT-C score was 12.0 (range 5–12). In paper III, all patients showed AUDIT-C scores in the high risk or severe risk category with a median score of 11.0 (range 7–12). The skewness of the AUDIT-C scores (67.5% of the patients in paper II and 63.0% of the patients in paper III scored 10–12 points) limited the use of statistics. The EDI, which showed a wider range of values, was therefore considered to be the best choice for statistical analysis. The extent to which potential underreporting can be expected in our studies using the questionnaires in these clinical settings is not known. In paper I, one might speculate that underreporting may be a problem as the kidney-failure patient group possibly is given clinical advice to avoid high alcohol intake because of their kidney disease. The healthy volunteer control group included some colleagues, which was not

optimal with regard to self-reporting of alcohol consumption. The inclusion of some colleagues may have increased underreporting in the control group. However, the passive advertising of the project (email and bulletin board) would most likely not attract subjects intending deliberately to underreport alcohol intake. All included subjects in the control group showed hair EtG below the limit of detection (LOD) except one person with a hair EtG of 10 pg/mg (EDI=17.1 g/d). The large majority of negative hair EtG samples in the control group indicate that underreporting is a minor problem. The problem of underreporting could be an important issue in paper I if the underreporting bias in the patient group is more extensive than in the control group, which could imply that underreporting may explain some of the difference shown in hair EtG results compared to reported alcohol consumption. However, there was no indication of excessive alcohol consumption from the indirect alcohol biomarkers (ALT, AST, GGT) analysed in the patient group.

In papers II and III, the included patients were attending an alcohol detoxification clinic, all with the diagnosis of alcohol abuse, where underreporting supposedly is less likely to be a major problem at inclusion of the study. However, as mentioned above, the calculation of EDI has some limitations. A possible underreporting bias is of less concern in light of the aims in paper II and III. Underreporting, however, could be a problem if a cut-off limit for EtG in nails is being explored by comparison to EDI, but this is not the case if comparing EtG levels in nails to concentrations in hair with the assumption of a reliable hair EtG cut-off limit for drinking habits.

With regard to the calculated EDI values in papers II and III, some of the alcohol-dependent patients had EDIs below 60 g/day (14 subjects in paper II, 9 in paper III). Underreporting could be one possible explanation for this. The AUDIT-C scores (reported above) and the fact that these patients were attending an alcohol rehabilitation programme and were diagnosed with alcohol dependence supports the premise that this is a population with high alcohol consumption. The calculation of EDI represents the mean alcohol consumption per day for the last three months, which means low EDIs could be a result of periods of low alcohol intake or abstinence prior to inclusion. The latter can also partially explain some of the low hair EtG concentrations (<30 pg/mg) among the alcohol dependent subjects (12 subjects in paper II, 5 in paper III). In addition, cosmetic hair treatment (bleaching, dyeing or perming) could affect some of the results. In paper II, there were five patients with hair EtG <30 pg/mg among patients reporting EDI ≥60 g/day. Among these five patients, there were two subjects who reported cosmetic hair treatment (dyeing) during the three months prior to inclusion. In paper

III, there was one subject who had hair EtG <30 pg/mg and EDI ≥60 g/day at inclusion, but this person did not report any cosmetic hair treatment. Low hair EtG levels among some alcohol-dependent subjects attending rehabilitation programmes have previously also been reported by others (49).

Another limitation in papers II and III was that relapse was not systematically investigated during the study period. No investigation other than standard procedure during such treatment was performed. However, breath ethanol was measured on suspicion of relapse and if patients had left the clinic for shorter periods. We think that most patients having a relapse during the study period would have been discovered as part of close follow-up in the inpatient programme, but we cannot totally exclude the chance of undetected relapses.

## 3.3 Analytical methods

EtG analysis in hair in our laboratory at Department of Forensic Sciences was not deemed to meet the demands of these studies, as a result of very high limit of quantification (LOQ) (17 pg/mg) when the studies were performed and no nail analysis was available. All analyses of EtG in hair and nails were instead performed in the laboratory at Department of Public Health, Experimental and Forensic Medicine at the University of Pavia in Italy. In this laboratory, LOQ was ≤ 5 pg/mg for EtG in hair. A study of stability of EtG during shipment was not performed and EtG concentrations are assumed to be stable during shipment. All samples were handled in the same way with regard to storage, wrapping and dispatch by air mail. EtG in keratin matrix is documented to be stable at room temperature during long-term storage (152, 153). Shipment may involve other conditions, and different results of a stability study could not be ruled out. However, investigating stability would have required extra sample material and this would have been difficult to obtain.

The clinical chemical analyses in blood were received from the medical records and were not collected as part of the studies. In paper I, laboratory parameters from blood analysis (creatinine, eGFR, ALT, AST, and GGT) were performed at the medical biochemistry laboratory of Akershus University Hospital, and in papers II and III, the blood analyses (creatinine, ALT, AST, GGT, and CDT) were sent to Fürst Medical Laboratory in Oslo, Norway. Both mentioned laboratories are accredited. CDT% analysis was based on the ratio between sialic acid-deficient transferrin isoforms (disialo- and asialotransferrin) and the total

amount of transferrin, which is not in accordance to the recent standardization of CDT% measurement using serum disialotransferrin to total transferrin fraction (32, 164).

### 3.3.1 Measurement of kidney function

As mentioned, the inclusion of patients with reduced kidney function in paper I relied on both the diagnosis of kidney failure, creatinine and eGFR. The eGFR was automatically calculated in the laboratories from creatinine using the Modification of Diet in Renal Disease (MDRD) study equation including age, gender and ethnicity (165), and not the newer CKD-EPI equation which show less bias than the MDRD Study equation, especially when eGFR ≥ 60 ml/min/1.73m$^2$ (92). The use of the MDRD equation was considered satisfactory as all included patients had eGFR <60 ml/min/1.73m$^2$. Calculations of eGFR on our data using the CKD-EPI equation show that all patients have eGFR <60 ml/min/1.73m$^2$ and that there is still significant correlation between the level of EtG in hair (corrected for EDI) and the eGFR (Spearman's $\rho$=-0.434, p=0.005). The use of more accurate and invasive methods measuring GFR (e.g. clearance of iohexol) was not part of the standard monitoring programme for this patient group and was not considered necessary for the purpose of the study. Using an objective continuous quantitative measure like eGFR was important when studying if there is a concentration dependent relation to hair EtG in the patient group.

One important limitation of study in paper I was that we did not measure kidney function (eGFR) in the control group of healthy volunteers. We considered that demanding collection of blood samples from this group would have made it difficult to recruit subjects to the study and that self-reporting of health conditions like diseases (including kidney diseases) and medication was sufficient to ensure that subjects with significantly reduced kidney function were not included. Knowing that kidney function is influenced by age, we ensured an age-matched control group.

### 3.3.2 Measurement of EtG in hair

The hair samples were collected from the posterior vertex of the head by cutting as close as possible to the skin. All hair EtG analysis was performed by a fully validated method (166). In paper I, the LOD and LOQ was 2.0 and 3.0 pg/mg, respectively. An even lower LOD and LOQ could have given further information knowing that most subjects in this study have low EDI and show hair EtG levels below LOD. In papers II and III, we used a LOD of 3.0 and the LOQ was 5.0 pg/mg, which were considered acceptable for the purposes of the studies.

After a washing step with dichloromethane and methanol, the hair samples was cut (1–2 mm) by scissors and were incubated in an aqueous solution with penta-deuterated EtG internal standard solution overnight, followed by an ultrasonication step for 2 hours before the sample was analysed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). The pre-analytical procedure was similar in papers I, II and III.

Pulverization or powdering of hair instead of using hair cuttings (manual cutting of hair in small ~1–2 mm pieces) might have improved the extraction efficiency of EtG and is the preferred method as stated in SoHT consensus (6). Therefore it could be argued that the use of hair cuttings instead of a pulverization technique is a weakness in this method. Higher extraction efficiency using pulverization compared to using hair cuttings has been shown in several studies (132, 167-172). For instance, Salomone et al. (171) observed a median increase of about 42% in EtG concentrations when switching from cutting to milling of the hair samples. Mueller et al. (170) also confirmed that pulverization is one of the most important pre-analytical factors regarding extraction efficiency but also showed the importance of incubation time, ultrasonication and other extraction conditions. The authors emphasized that the effect of pulverization depends on the settings of the other pre-analytical factors and that increased extraction of EtG by pulverization can almost be compensated by other extraction conditions. The possibility of partial compensation by other pre-analytical factors has also been demonstrated by Kronstrand et al. (132) who has shown that extraction of EtG from hair reaches a plateau already after four hours of incubation in water when the hair sample has been automatically pulverized, compared to 18 hours for intact hair (132). Considerable differences of extraction efficiency have been shown for different pulverization techniques, and a weak pulverization has been shown by Kummer et al. (173) to be comparable with hair cuttings. Standardization of pre-analytical conditions among laboratories has been suggested (146) and even the use of different cut-offs for chronic excessive alcohol consumption dependent on the pre-analytical conditions used (171). The hair EtG 30 pg/mg cut-off for chronic excessive alcohol consumption from SoHT was established on basis on studies using different pre-analytical conditions, some using hair cuttings and others using pulverization (43, 113, 127, 137, 174). In addition, our colleagues in Pavia performing the analysis have shown that extraction is constant and highly reproducible over time under the conditions stated. The validation of the method showed concentration dependent accuracy and precision constantly below 3 and 7%, respectively (166). On this

basis, we considered the pre-analytical method for hair EtG suitable to the purpose of the studies, but the importance of pulverization should be investigated in future studies.

The analytical method used approximately 50 mg hair in papers II and III and 100 mg hair in paper I (lowest accepted weight was 20 mg), which is fairly high. The analytical results were corrected for sample weight. In paper III, the amount of hair in the proximal 3 cm segment was too small for analysis for some of the subjects (n=15). For these subjects, another 1 cm of hair (up to 3 extra) was included in both T1 and T2. This was a weakness of the study as the study subjects self-reported alcohol only for the previous 3 months, roughly corresponding to the EtG found in the proximal 0–3 cm. However, we did not find any significant correlation between length of hair segment at T1 and change in EtG concentrations from T1 to T2 (Spearman's $\rho$=-0.188, p=0.380).

#### 3.3.3 Measurement of EtG in nails

In paper II, nail samples were obtained by cutting the nail overhang from the thumbs and the index fingers on both hands.

All nail EtG analysis was performed using a fully validated LC-MS/MS method (142). The LOD (6.0 pg/mg) and LOQ (10 pg/mg) values were somewhat high, but we believe the method was sensitive enough to answer the aims of paper II. It could be argued that even lower LOD and LOQ possibly could have given more detailed information about the last phase of elimination kinetics in nails, but for the purpose of comparing EtG concentrations in hair and nails, the LOD and LOQ were considered satisfactory.

About 10–20 mg (lowest accepted weight was 5 mg) of nail material was used in the analysis. After a washing step (dichloromethane and methanol) and drying of the nail clippings, an aqueous solution with penta-deuterated EtG internal standard solution was added. After an overnight incubation, it was then ultrasonicated for two hours. After these pre-analytical steps, the samples were analysed by LC-MS/MS. No pulverization of the nail clippings was performed. As mentioned for the analysis of hair, pulverization could have improved extraction of EtG. Cappelle et al. (175) showed from a 2-fold to 10-fold increase of EtG concentrations in pulverized nail samples compared to non-pulverized nails. The use of pulverization instead of nail cuttings in paper II could possibly have given more detailed information in the longitudinal study of nail kinetics, but it should be noted that we considered it important that the pre-analytical procedure was similar for both hair and nail,

using cutting of hair and nail. It is therefore important to be aware of this limitation when comparing EtG concentrations with other nail studies.

### 3.4 Statistics

Parametric tests were considered but based on the distribution of the data and the fact that there were relatively few subjects included, we decided to use non-parametric tests. In paper I, all EtG results below LOD were set to zero in the statistical analysis. However, there were no EtG concentrations between LOD and LOQ. In papers II and III, all EtG results below LOQ were set to zero. P values below 0.05 were considered as statistically significant.

In general, the number of included subjects in the present studies was not large enough to study the effect of possible covariates, and this was also not the primary aim of the studies. Multiple regression analyses are not performed in any of the papers. We have therefore not corrected for the effect of age and gender. In paper I, the control group was age-matched with no difference in age ($p=0.39$) and correction for age was therefore not necessary. Moreover, there was no difference between EDI, BMI, bleaching, perming or the use of hair care products between the study group and the control group. However, we found a difference between gender as well as hair dyeing between the study and the control group. After considerations, the statistical analyses in paper I were performed without inclusion of covariates. When re-analysing the data in a multi-variate model (linear regression) including both gender and hair dyeing as covariates, we still found significant difference in the EtG/EDI ratio between the patient group and the control group ($p=0.004$). However, there was no significant effect of gender ($p=0.505$) and hair dyeing ($p=0.828$) in this model, but the study was not designed to find such effects of covariates.

In paper III, correction for patient characteristics was not relevant, as the same subjects were studied at the two time points. However, an alternative might be to include the number of days between last alcohol intake until collection of sample T2 and the use of hair treatment prior to the study as covariates in a multiple regression model, but that aspect was handled separately.

It was a weakness not to do any power analysis prior to conducting the studies of papers I and III. Paper I was an explorative study of EtG kinetics with limited prior knowledge of how reduced kidney function would affect the EtG concentrations in hair, and as a result of this,

we did not assume any possible effect size. The preliminary study by Høiseth et al. (95) had already shown significantly higher (p=0.009) hair EtG levels among kidney-failure patients compared to previously published results for healthy volunteers, but we wanted to compare kidney-failure patients with a comparable age-matched Norwegian control group. The EtG concentrations in hair among the kidney failure patients were also extremely variable in the preliminary study and this further complicated power analysis. Paper II was a kinetic study and power calculations were therefore less relevant. In paper III, we wanted to explore possible changes in EtG concentrations during hair growth. We could have calculated the sample size necessary to show a statistically significant difference from T1 to T2, but we considered a descriptive approach to be more important. All statistical analyses were performed after inclusion of all subjects in the studies.

## 3.5 Ethical considerations

All studies were approved by the Regional Committee for Medical and Health Research Ethics (REK) in Norway (paper I: reference number 2009/4109, papers II and II: reference number 2013/1222). For paper I, the local ethics committee at Akershus University Hospital also approved the study and for papers II and III, the clinical board at Trasoppklinikken also accepted the studies.

All biological samples were destroyed after analysis and accordingly, approval of bank for biological samples was not required in the opinion of the juridical department at the Norwegian Institute of Public Health. It should be noted that the REK approval includes a biobank acceptance, but this is related to a part of the project (urine samples) that is not included in the present thesis.

All patients and controls signed an informed consent before participating in the studies. Based on the aims in papers II and III, it was considered unfavourable with a period of abstinence before inclusion because of the difficulty in standardizing the length of such an abstinence period. All patients reported two days or more since their last alcohol intake. With regards to ethics, an abstinence period was important to allow all patients the ability to give a valid informed consent. One might argue that some of the patients could have experienced alcohol withdrawal symptoms during the period in which they gave their consent, but the clinical staff considered all patients capable of providing valid informed consent.

None of the studies intervened with patient treatment and the hair and nail samples collection process was considered minimally invasive to the patients. In papers II and III, this was considered especially important, as these subjects attended a long treatment programme where relationships with the treatment staff was essential. It is debatable whether a better study design might have had no treatment staff members as recruiters of participants or collectors of samples. This was the case in paper I, where only the project group recruited patients and controls and interviewed the subjects about alcohol consumption. None of the clinical treatment team members were involved in this aspect. In papers II and III, clinical staff recruited patients, collected samples and interviewed participants about alcohol consumption both prior to and during the inpatient programme. Investigating alcohol consumption was considered as part of the patient treatment programme but this was performed in a more systematic manner (using the AUDIT-C and calculating an EDI) as a part of the study. However, there was an ethical concern regarding asking the patients about possible relapse during the study because this might have implications for the patient's treatment. We decided not to ask the patients about this apart from what was a part of the normal clinical treatment programme, and suspicion of a relapse was based on the clinic's standard routines to identify relapse (breath alcohol tests, clinical suspicion).

The data collected in all studies were de-identified by using unique identifier numbers and electronic data were saved on a secured research server at the Norwegian Institute of Public Health until our department was administratively transferred to Oslo University hospital in January 2017. The data was then secured on servers reserved for person-sensitive data in accordance with the hospital requirements. The signed informed consents contained the unique identifier number and were stored in a locked cabinet during the period approved by REK.

## 3.6 Concluding methodological remarks

The most important methodological weakness in paper I seems to be the possibility of various degrees of underreporting alcohol consumption between the patient and the control group. In paper II, the lack of control of relapse during the inpatient programme is important. In paper III, the inaccuracy in the segments caused by different duration of the abstinence phase before collection of the last sample (T2 segment) is an important weakness.

Overall in this thesis, the lack of controlled studies with regards to alcohol consumption, relying on self-reporting of alcohol intake, is an important aspect to be aware of.

# 4. Summaries of the results

## 4.1 Paper I

**Levels of hair ethyl glucuronide in patients with decreased kidney function: Possibility of misclassification of social drinkers**

The main purpose of this study was to compare levels of hair EtG in patients with decreased kidney function and healthy volunteers, both groups with a moderate alcohol intake. We also wanted to evaluate whether subjects from the patient group could obtain a false-positive diagnosis of chronic excessive drinking (≥60 g/day).

A total of 41 patients were recruited from a clinic that treats kidney failure; all participants with a creatinine value >90 μmol/l and eGFR <60 ml/min/1.73 $m^2$ and 42 age-matched healthy volunteers were included for comparison. EDI was calculated on the basis of self-reported alcohol consumption during the previous 3 months using a TLFB-test and hair samples was obtained from the posterior vertex of the head. The proximal 0–3 cm hair segment were analysed for EtG.

The median EDI was 2.8 g/day (range 0.1–28.0) in the patient group and 4.0 g/day (range 0.1–24.0) in the control group, with no statistical difference between the groups (p=0.58). The hair EtG concentrations in the patient group ranged between <LOD and 331 pg/mg with a mean value of 25.1 and median 0.0. In comparison, the healthy volunteers had hair EtG concentrations of <LOD-10 pg/mg showing a mean value of 0.2 and median 0.0. The levels of hair EtG (corrected for EDI) were significantly higher among the subjects with decreased kidney function (p<0.001). Eight patients (20%) had hair EtG concentrations above the 30 pg/mg limit for chronic excessive alcohol consumption, despite all EDI levels were <60 g/day. There was significant correlation between hair EtG (corrected for EDI) and both eGFR (Spearman's ρ=-0.413, p=0.007) and serum creatinine levels (Spearman's ρ=0.420, p=0.006) in the patient group.

The results from paper I show higher hair EtG concentrations in the patient group compared to healthy volunteers, as could be expected from theoretical considerations knowing that EtG's main elimination pathway is through urinary excretion. The results also showed the possibility of misclassification of drinking habits among subjects with reduced kidney function.

## 4.2 Paper II

**Ethyl glucuronide elimination kinetics in fingernails and comparison to levels in hair**

In paper II, we compared levels of EtG in nails to the levels in hair among alcohol-dependent patients at the beginning of alcohol treatment programme. We also wanted to investigate the kinetics of EtG in fingernails of these patients during an alcohol abstinence period of up to 12 weeks.

Overall, 40 patients diagnosed with alcohol dependence were included and an EDI was calculated based on self-reported alcohol consumption during the previous three months using a modified AUDIT-C-test. Hair samples and nail clippings were obtained at inclusion followed by collection of nail samples every 7th–10th day during the treatment programme for up to 12 weeks.

The self-reported alcohol consumption representing the average intake during the previous 3 months showed a median EDI of 92.5 g/day (range: 8.0–455.0). Median EtG concentrations in nail was 252.7 pg/mg (range: 27.1–2267) for the nail samples collected at inclusion. For the hair samples obtained at the same time as these nail samples there was a median EtG of 49.1 pg/mg (range: <LOQ-718.5). All patients showed higher nail EtG/EDI ratios compared to hair EtG/EDI ratios ($p<0.001$), and the median value of the ratios between nail EtG and hair EtG was 5.0 (range: 1.07–56.1). There was a significant correlation between nail EtG and EDI (Spearman's $\rho=0.440$, $p=0.004$) and hair EtG and EDI also showed a significant correlation (Spearman's $\rho=0.519$, $p=0.001$). A significant correlation was also found between nail EtG/EDI and hair EtG/EDI (Spearman's $\rho=0.638$, $p<0.001$).

In the longitudinal part of the study, the EtG concentrations in nails fell below LOQ in most cases within about 2 months. The calculated median EtG half-life in nail clippings was 13.3 days (range: 5.5–29.0). There was a significant correlation between the time elapsed until the last positive sample and the EtG levels in nail at inclusion (Spearman's $\rho=0.449$, $p=0.004$). However, there was no significant correlation between time for the last positive sample and the reported EDI (Spearman's $\rho=-0.017$, $p=0.918$).

The results of the comparison between nail and hair EtG show that EtG in nail could be a potentially good alternative to hair matrix and that EtG cut-off level in nail should be higher than the established 30 pg/mg cut-off for EtG in hair representing chronic excessive drinking.

Furthermore, based on the fact that growth time from the germinal matrix to the nail's free edge is about 3–5 months, EtG concentrations in nails obtained during the inpatient period disappeared faster than what would be expected.

## 4.3 Paper III

**Hair EtG: Alterations in segment levels accompanying hair growth**

In this paper we wanted to use longitudinal hair EtG measures to study change of EtG levels during hair growth in an alcohol abstinence period.

Twenty-seven patients diagnosed with alcohol dependence were recruited at admission for an inpatient withdrawal treatment programme. The patients were interviewed about alcohol consumption during the last three months using a modified AUDIT-test, which was used for calculation of EDI. Hair samples were collected at inclusion and the proximal 0–3 cm hair segment (T1) was analysed. Another hair sample was collected about four weeks later and the proximal 1–4 cm hair segment (T2) was used for EtG analysis. In case of low sample weight, additional distal cm was added to both T1 and T2. Assuming a hair growth rate of about one cm per month, the T1 and T2 segment should roughly represent the same period of alcohol exposure.

The patients reported a median EDI of 150 g/day (range 8.0–350). The median hair EtG concentration in the T1 segment was 100 pg/mg (range: 7.7–1320) and the median concentration in the T2 segment was 53.4 pg/mg (range: <LOQ-692). There was a significant decrease in EtG concentrations from T1 to T2 ($p=0.003$) and the median change in EtG concentrations was -46.0%. There were also declining EtG/EDI ratios from T1 to T2. Among the 27 patients, 18 showed decreasing EtG concentrations from T1 to T2 and in six of these the value at T2 was below LOQ. Eight of the patients had stable or smaller change in EtG from T1 to T2, which was considered within analytical variation. One of the patients had a considerable increase in EtG concentration from T1 to T2, this patient also showed increasing EtG concentrations in nail in the same period and relapse could therefore be suspected.

The results of this study indicate that EtG concentrations might not be stable in hair during growth in an abstinence period. The precise mechanism underlying this change in concentrations is not known, and whether this is a result of wash-out effects or other

mechanisms is as yet unanswered. The findings in this study support the suggestion from SoHT to interpret EtG in distal hair segments with caution.

# 5. General discussion

This thesis has investigated the ethanol metabolite EtG in hair and nail with regard to opportunities and limitations as a biomarker of chronic alcohol intake. We have demonstrated that reduced kidney function may substantially increase hair EtG concentrations leading to a possible misclassification of alcohol drinking habits. Another main finding is that EtG in nails shows higher concentrations than in hair, and a correlation of EtG to alcohol consumption is shown for both hair and nail. EtG in nail disappeared in most cases within about 2 months of alcohol abstinence and this disappearance is faster than would be expected from nail growth. Furthermore, we found decreasing EtG concentrations in hair segments during growth in an alcohol abstinent period among chronic excessive drinkers.

## 5.1 Aim 1, paper I: To investigate whether renal disease can impair EtG excretion sufficiently to increase hair EtG levels, disturbing its potential as a marker of chronic excessive drinking.

As reported in paper I, we found significantly higher hair EtG concentration among patients with reduced kidney function compared to the control group of healthy volunteers, and we found that 8 subjects (20%) of the kidney-failure patients had hair EtG concentration above the SoHT`s 30 pg/mg cut-off for chronic excessive drinkers, even though both patients and control group self-reported a non-significant (p=0.58) difference in alcohol consumption with a mean EDI of 5.5 and 6.3 g/day, respectively.

Elevated EtG concentrations in blood and hair among subjects with reduced kidney function could be expected from theoretical considerations as well as studies reporting elevation of serum concentrations of other glucuronides in this patient group (88-90). Furthermore, the preliminary study by Høiseth et al. (95) reported elevated hair EtG levels among kidney-failure patients. In addition, prolonged EtG detection time in urine among patients with kidney failure have been shown in a previous study (94). It should be noted that increased concentrations of EtG in blood during kidney failure is not documented, only indicated in one subject without verified kidney disease (76). However, the large effect on hair EtG levels in paper I strongly supports the fact that the effect of kidney failure on blood EtG concentrations is large and mimics the documented effect shown for morphine glucuronides (88-90). Mosebach et al. (176) have recently published a study which show elevated hair EtG

concentrations among patients with advanced renal dysfunction (GFR <30 mL/min). This group of patients, mostly on dialysis, showed significantly higher hair EtG concentrations in relation to EDI compared to patients with compensated renal function (GFR >60 mL/min) (p=0.0049) and impaired renal function (GFR 30–60 mL/min) (p=0.016). However, there was no difference between patients with compensated renal function and patients with impaired renal function. For those patients with a positive hair EtG sample test there was a median hair EtG/EDI ratio of 48.2 for the patients with advanced renal dysfunction and 3.1 for the group of impaired renal function. After reassessment of the data in paper II, there was a median hair EtG/EDI ratio of 7.8 (range 0.8–25.3) among the kidney-failure patients showing a positive hair EtG sample test (n=19), which is comparable to the study of Mosebach et al.

Knowing that the main excretion path for EtG is by urine, it is expected that EtG concentrations in blood would increase if kidney function is compromised. Generally the glomerular filtration rate approximates the renal excretion of many substances. In the absence of tubular secretion or reabsorption, the rate of elimination of a substance is theoretically proportional to the glomerular filtration rate (177). To our knowledge, the renal excretion mechanisms of EtG have not yet been studied, but a net effect of retention of EtG resulting in increased EtG blood levels among subjects with reduced kidney function is important to consider understanding the increased levels of EtG in hair. It is reported that concentration of a compound in hair correlates best with the area under the concentration versus time curve in blood, and increased blood concentration over time will then lead to elevated levels in hair (42). Apart from kidney failure, we could not totally exclude other causes of elevated EtG concentrations. For instance, the study group could have used drugs that can cause interactions which increase glucuronidation. The study in paper I was, however, not designed to discover the mechanisms of the difference observed in EtG between the patient- and the control groups.

As mentioned in “Methodological considerations”, the study of paper I has limitations like possible differences in the degree of underreporting of alcohol consumption between the patient and the control group and lack of kidney-function testing for the control group. Another limitation is that there are so few studies on this topic which could be discussed in the light of paper I. The validity of our study should therefore be confirmed by other studies, preferably by other designs. It would be valuable to determine if alcohol consumption could be predicted by using hair EtG measurements in the kidney-failure population as well. A

controlled alcohol dosing study performed in a larger population with reduced kidney function could provide important information about this.

However, there are many reasons to believe the conclusions of our findings. The results are expected from theory and urine EtG findings in a prior study with kidney failure patients also support the main finding in paper I. The hypothesis of a quantitative relation between EtG concentrations in blood and hair and thus a relation between alcohol intake and hair EtG is also supported by the fact that there was a significant correlation between levels of EtG in hair (corrected for EDI) and both eGFR and serum creatinine levels in the patient group. This correlation also supports causality between the reduced kidney function and the elevated hair EtG concentrations according to Bradford Hill's criteria for causality (178).

The elevated hair EtG concentrations (corrected for EDI) among the kidney-failure patients compared to the healthy volunteers may have important implications for the interpretation of the hair EtG results. After the publication of paper I, Pragst et al. (179) have stated that information about kidney function must be available to avoid misclassification of alcohol usage. One might also question whether this also should be stated in the SoHT's guidelines.

The effect of kidney failure will be different when using hair EtG as a biomarker for long-term alcohol consumption depending on the population that is investigated. When analysing hair EtG in samples from a large population with a suspected low incidence of reduced kidney function, only individual cases of false positive hair EtG ≥30 pg/mg could be expected. An example of this could be a population where hair EtG is being used as documentation for re-granting of a driving licence after chronic alcohol abuse. Hair EtG analysis in patient populations could be more questionable, e.g. being used in connection with organ transplantation where the risk of comorbidity of kidney disease has to be taken into consideration. In the study of paper I, 20% of the participants were misclassified as chronic excessive drinkers, even though none of the patients had reached the stage at which they needed dialysis. Accordingly, the use of hair EtG among patients to be considered for kidney transplantation would be very problematic because of the risk that several patients could be falsely diagnosed with chronic alcohol abuse.

## 5.2 Aim 2a, paper II: To investigate the relationship between EtG concentrations in hair and nails related to alcohol consumption.

As presented in paper II, the EtG concentrations in nails was higher than the concentrations found in hair for all subjects in this study and a significant correlation between EtG concentrations and EDI was shown for both hair and nails.

The median ratio between EtG in nail and hair in paper II was 5.0 (range: 1.07–56.1) and this is comparable to prior studies which have compared EtG concentrations in hair and nail (141, 142). Jones et al. (141) reported EtG levels in fingernails that were three times higher than EtG levels in hair. Morini et al. compared EtG in hair and nail for only one subject and also reported higher EtG concentration in nail (142).

The opposite relation, with higher concentrations found in hair than in nail is previously published for zolpidem, codeine, haloperidol, cocaine and MDMA (143-145, 180, 181). This difference in relation between concentrations in hair and nail could be explained by different physiochemical properties for the substances. One explanation of the differences is that incorporation of many substances into hair is positively correlated with the content of melanin found in hair (182, 183), while nails lack melanin (120). Incorporation of EtG differs from many other drugs in the way that EtG deposition in hair is not affected by melanin content (105). The fact that EtG is a hydrophilic and acidic molecule with pKa=3.21 (104), while many of the medicinal molecules are basophilic, may also be important to explain the differences from other substances with regard to incorporation in hair and nail. A higher incorporation rate for EtG into nail, thus a higher sensitivity compared to hair as a matrix, has been highlighted in the literature as an advantage to distinguish between low levels of drinking and complete abstinence and also to detect binge drinking (108, 142).

In paper II, we did not describe the diagnostic sensitivity and specificity of the EtG results in hair and nail as tests for chronic excessive alcohol consumption (≥60 g/day). It should be noted that this is somewhat complicated by the fact that EDI is calculated as an average of the alcohol consumption over a period of 90 days, the abstinence period and lack of detailed consumption pattern in a 90-day period prior to inclusion. The sensitivity of hair EtG for detecting the patients reporting EDI ≥60 g/day using the SoHT`s 30 pg/mg cut-off for EtG in hair was only 0.77 and the specificity was 0.50. This is seen in table 1. This sensitivity and specificity could be considered surprisingly low but could be caused by the abstinence period

before inclusion. Underreporting alcohol consumption could also not be excluded as a factor explaining this.

**Table 1**. Number of subjects with EDI ≥60 g/day and hair EtG ≥30 pg/mg.

| | | Hair EtG test result, cut-off ≥30 pg/mg | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| EDI ≥60 g/day | Positive | 20 | 6 | 26 |
| | Negative | 6 | 6 | 12 |
| | Total | 26 | 12 | 38 |

In paper II, we decided not to suggest a possible cut-off for nail EtG representing chronic excessive alcohol consumption because of the low number of included subjects. It has previously been suggested that the EtG cut-off for chronic excessive consumption should be higher in nails compared to hair (128, 184). Cappelle et al. (184) suggested a possible nail EtG cut-off >123 pg/mg for chronic excessive drinking, while Berger et al. (128) proposed 56 pg/mg as possible threshold for high-risk drinkers (≥60 gram per day). The number of subjects in paper II is too limited to provide any adequate statistical approach, but an indication of which nail EtG levels that corresponds to EDI ≥60 g/d could be obtained from our results. If studying those subjects with EDI above the limit for chronic excessive alcohol consumption (≥60 g/day), a possible nail EtG cut-off of 100 pg/mg provides a sensitivity of 0.85 and a specificity of 0.21 (table 2). An EtG nail EtG cut-off of 260 pg/mg will instead provide a sensitivity of 0.58 and a specificity of 0.79 (table 3).

**Table 2**. Number of subjects with EDI ≥60 g/day and nail EtG >100 pg/mg.

| | | Nail EtG test result, cut-off >100 pg/mg | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| EDI ≥60 g/day | Positive | 22 | 4 | 26 |
| | Negative | 11 | 3 | 14 |
| | Total | 33 | 7 | 40 |

**Table 3**. Number of subjects with EDI ≥60 g/day and nail EtG >260 pg/mg.

| | | Nail EtG test result, cut-off >260 pg/mg | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| EDI ≥60 g/day | Positive | 15 | 11 | 26 |
| | Negative | 3 | 11 | 14 |
| | Total | 18 | 22 | 40 |

Alternatively, a cut-off for EtG in nail could be evaluated based on the reported hair EtG concentrations. The fact that 12 patients had EDI <60 g/day implies that present material is not perfect for setting a cut-off for EtG in nail, when using hair EtG as the reference value. If studying those subjects with hair EtG ≥30 pg/mg, a possible cut-off of 100 pg/mg in nails provides a sensitivity of 1.0 and a specificity of 0.58 (table 4). By increasing the cut-off to 260 pg/mg, we measured a sensitivity of 0.65 and a specificity of 1.0 (table 5).

**Table 4**. Number of subjects with hair EtG ≥30 g/day and nail EtG >100 pg/mg.

| | | Nail EtG test result, cut-off >100 pg/mg | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Hair EtG ≥30 pg/mg | Positive | 26 | 0 | 26 |
| | Negative | 5 | 7 | 12 |
| | Total | 31 | 7 | 38 |

**Table 5**. Number of subjects with hair EtG ≥30 g/day and nail EtG >260 pg/mg.

| | | Nail EtG test result, cut-off >260 pg/mg | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| Hair EtG ≥30 pg/mg | Positive | 17 | 9 | 26 |
| | Negative | 0 | 12 | 12 |
| | Total | 17 | 21 | 38 |

The present data therefore suggest that EtG cut-off in nails for chronic excessive drinking should be higher compared to EtG cut-off in hair, and maybe established in the range 100–

260 pg/mg. This suggested cut-off is also consistent with the median EtG nail/hair ratio of 5.0 observed in the present study, which would imply a cut-off of 150 pg/mg. Larger studies are needed to conclude on nail EtG cut-off limits for alcohol consumption. Studies with controlled dosing of alcohol are preferred, but self-reported data on alcohol consumption can provide adequate information to decide on a cut-off limit.

Another outcome of the present study of paper II was the demonstration of a relatively good correlation (Spearman's $\rho$= 0.440, p=0.004) between levels of EtG in nails and EDI. Paul et al. (185) found an even better correlation between nail EtG levels and EDI (Spearman's $\rho$= 0.665, p<0.001), while Berger et al. (128) and Morini (142) also found a god correlation between nail EtG content and EDI. In paper II, we also found a significant correlation between nail EtG/EDI and hair EtG/EDI (Spearman's $\rho$ = 0.638, p<0.001). The fact that a high correlation is seen between EDI-corrected EtG levels in hair and nail indicates that there are some common aspects of the EtG incorporation in these two different matrices. Significant and positive correlation between hair and nail as matrices has also been demonstrated by others for EtG (141, 184) and for several other substances (186). Correlation between EtG in nail and EDI and also the good correlation between EtG concentrations in nail and hair both support nail as an alternative matrix to hair.

Correlation between hair EtG and EDI is an important premise for the SoHT's cut-off limits for different levels of alcohol consumption (6) and the present results add knowledge to this. Good correlation between hair EtG and EDI is also shown in a large pooled-data analysis of several studies (100). The cut-off limits for EtG levels in hair including a dose-related relationship between EDI and EtG levels in hair is an important advantage compared to some other alcohol biomarkers and also compared to hair measurements for drugs other than alcohol. The data of EtG in nail is still too limited to conclude if the correlation between nail EtG and EDI is sufficient to make cut-off limits corresponding to different levels of alcohol consumption similar to what is defined for hair EtG in the SoHT's guidelines.

## 5.3 Aim 2b, paper II: To investigate the elimination kinetics and detection times of EtG in fingernails during an alcohol abstinence period.

In paper II, we also investigated longitudinal data on EtG in nail after cessation of alcohol drinking, which was missing in the literature. Better knowledge of the kinetics of EtG in nails

is important to understand the complex incorporation of EtG into nails. One could argue that there are two important reasons for better understanding the kinetics in nails. Firstly, this will help us to make a more precise cut-off for EtG in nail. Secondly, if longitudinal samples are used and an increase in concentrations is interpreted as a new intake, it is important to know if this is caused only by increased consumption. For hair, analysing different hair segments from single time-point samples are most used by far.

As seen in figure 3 in paper II, there were several individuals with increasing nail EtG concentrations during the study period. Four of these patients were selected to illustrate increasing nail EtG concentrations. Individual longitudinal data for EtG in nail for these four patients are shown below in figure 3–6. Two of the 40 patients left the study already after only one nail sample collection. Thirty of the 38 included patients with longitudinal data available showed maximum EtG concentrations in nails at inclusion time. Eight of the 38 patients showed maximum nail EtG concentrations later than the inclusion day.

The fact that some of the patients had maximum EtG concentrations in nails later than the inclusion day could be a result of incorporation mechanisms involved. Madry et al. (121) tried to characterize incorporation of zolpidem into nails and found three concentration peaks after a single intake of 10 mg of the substance. The first peak was found in nail overhang 24 hours after intake and was also the highest peak concentration. This peak was suggested to be a result of sweat and sebum-mediated incorporation of the substance. The second peak was reached two–three weeks after intake, and the authors suggested incorporation through the nail bed as an explanation of this peak. Peak three appeared after 10–18 weeks after intake and was presumably caused by incorporation via germinal nail matrix (121). One could hypothesize that the difference in when maximum EtG concentration is observed and some of the fluctuations in EtG concentrations seen for some of the patients could be a result of the different mechanisms involved in incorporation of EtG, also depending on the pattern of alcohol consumption prior to inclusion in the study. As the previous study of Madry et al. (121) investigated single intake and a different substance, the chronic use of alcohol might not show a similar pattern.

Although a peak in nail EtG concentration could also be caused by relapse, the use of longitudinal EtG measurements should be interpreted with caution until more data are available that can help us discriminate peaks caused by relapse and peaks as a result of different incorporation mechanisms. Although we observe some patients showing small

increases in nail EtG after cessation of drinking, our data indicates that at least large increases in concentrations and also increased concentrations during multiple measurements strongly indicate relapse.

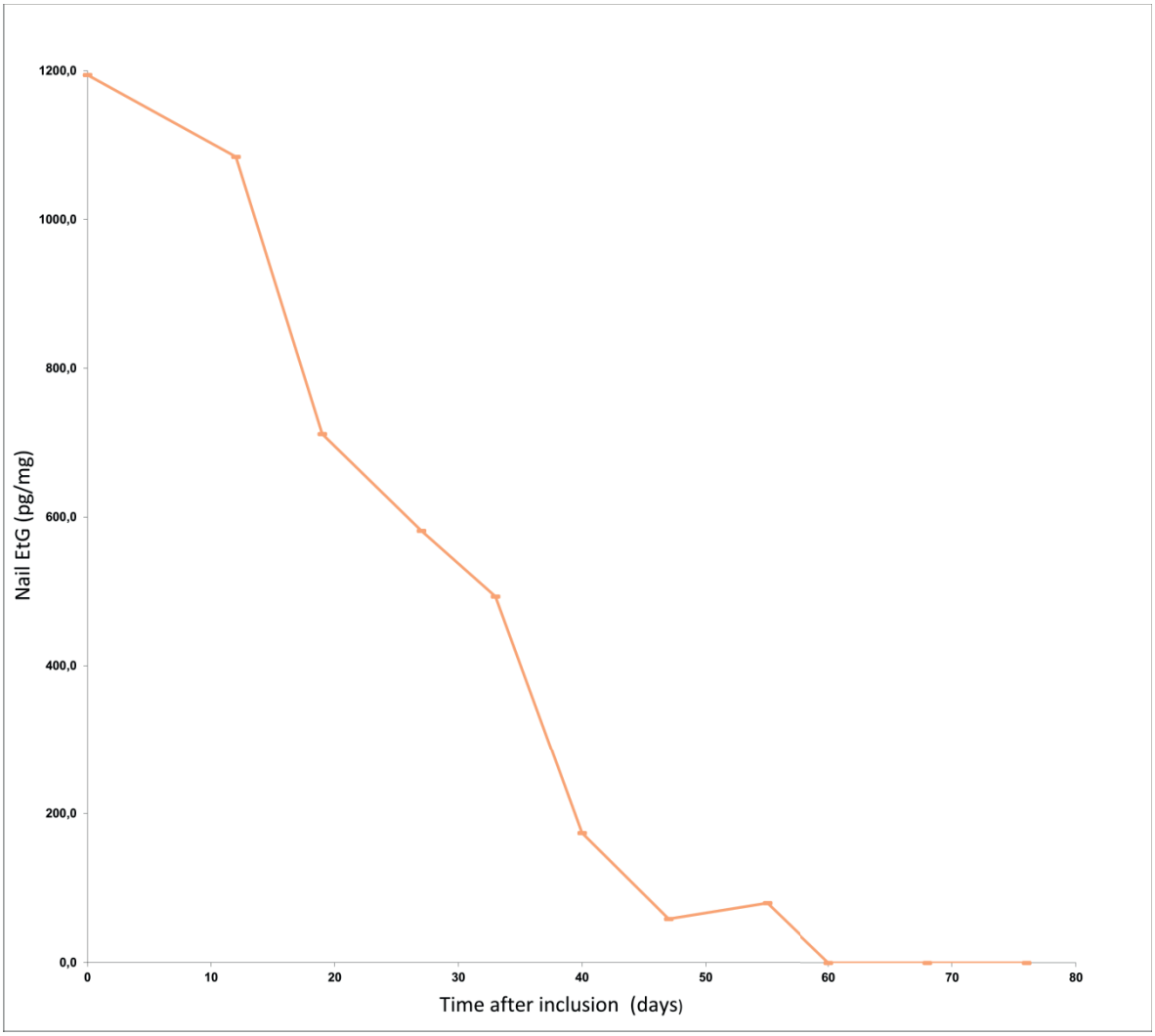


**Figure 3**: Nail EtG concentrations for patient 32 (according to table 1 and figure 3, paper II). There was no suspicion of relapse during the study period. EDI was 180 g/day and hair EtG at time of inclusion was 226.7 pg/mg. A small peak was observed 55 days after inclusion, but it should be noted that the increases in concentration was within analytical variation.

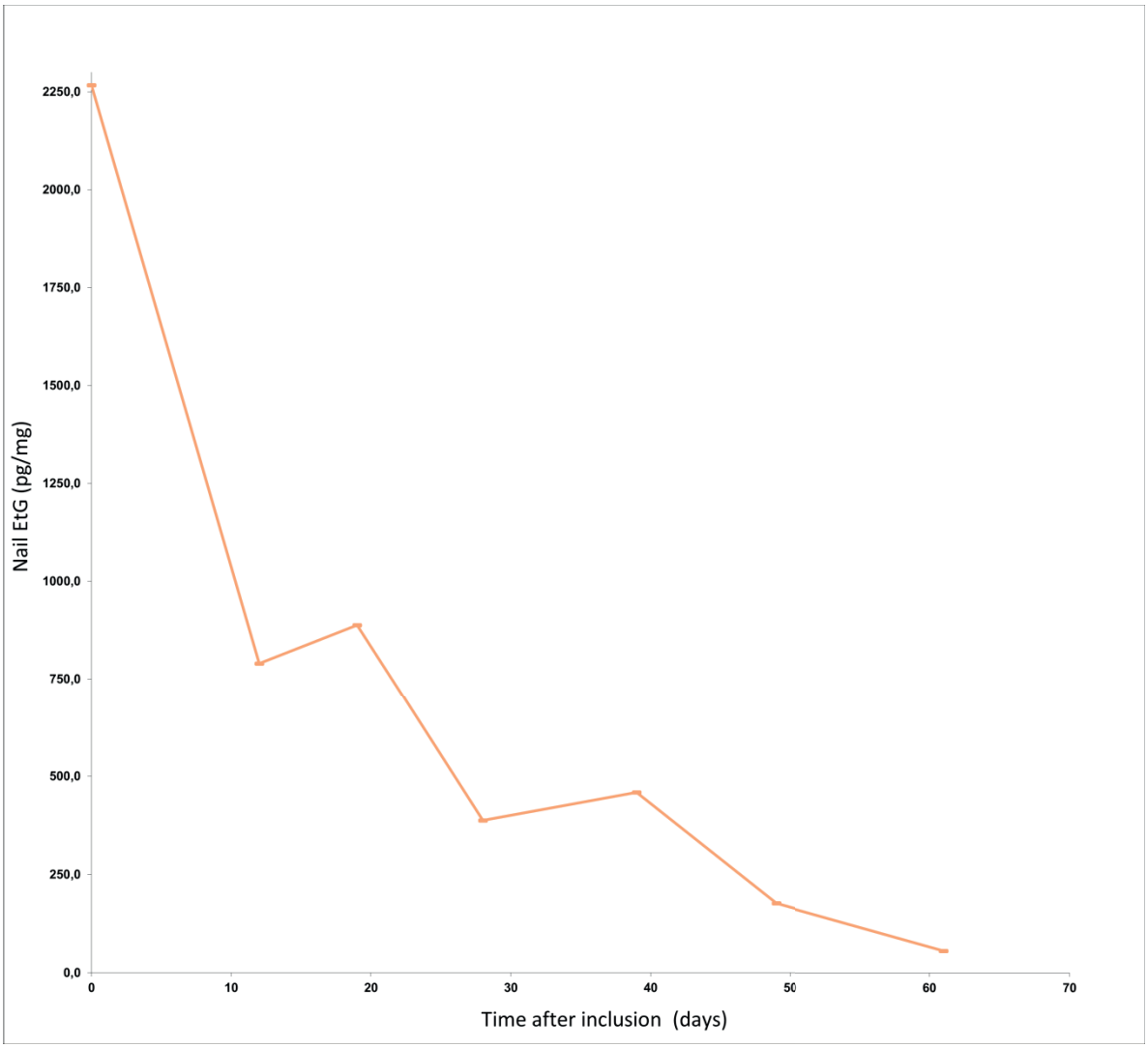


**Figure 4**: Nail EtG concentrations for patient 18 (according to table 1 and figure 3, paper II). There was no suspicion of relapse during the study period. EDI was 72 g/day and hair EtG at time of inclusion was 179.8 pg/mg. A second and a third peak were observed 19 and 39 days after inclusion, respectively, but it should be noted that the increases in concentrations were within analytical variation.

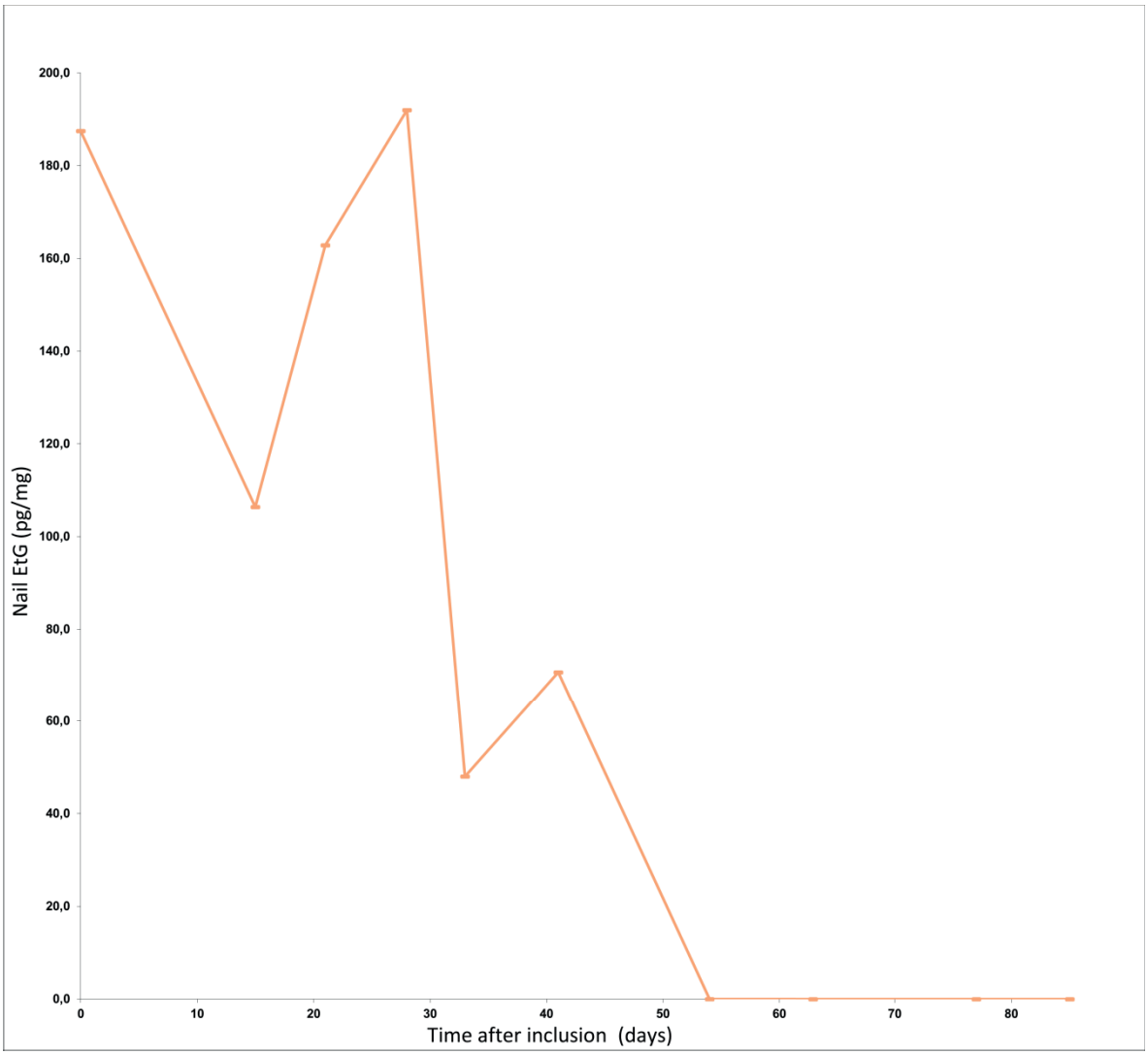


**Figure 5**: Nail EtG concentrations for patient 12 (according to table 1 and figure 3, paper II). There was no suspicion of relapse during the study period. EDI was 51.0 g/d and hair EtG at time of inclusion was 14.2 pg/mg. A second (maximum concentration) and a third peak were observed 28 and 41 days after inclusion, respectively, and only the second peak exceeded analytical variation.

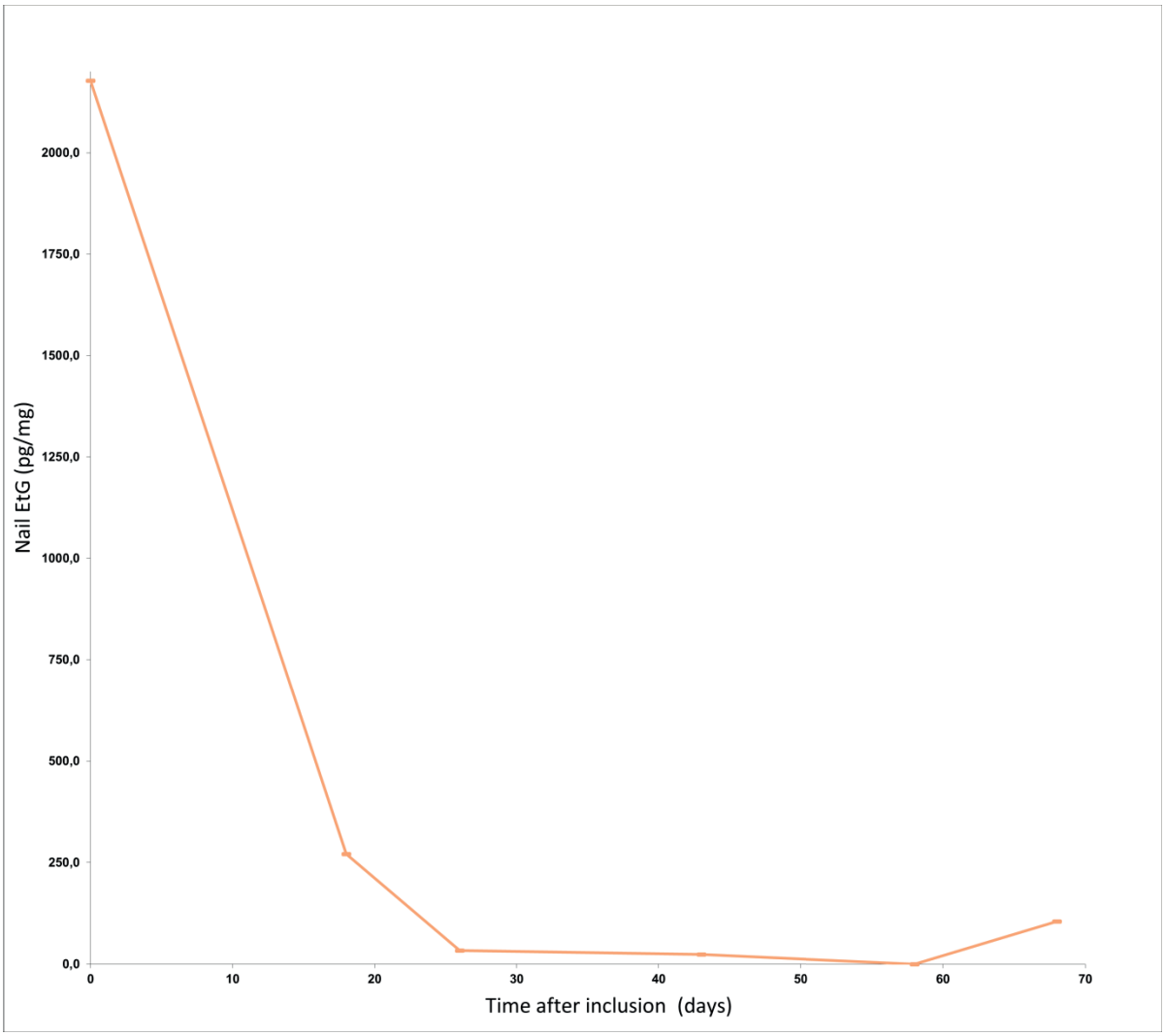


**Figure 6**: Nail EtG concentrations for patient 16 (according to table 1 and figure 3, paper II). There was suspected relapse prior to the last nail sample which showed increased EtG concentration (105.4 pg/mg). EDI was 66.0 g/d and hair EtG at time of inclusion was 38.8 pg/mg.

Fingernails regenerate with a growth time from the germinal matrix to the nail's free edge of about 3–5 months (119). This limits the potential detection window for a substance using nail as a matrix. One of the main findings in paper II was that the disappearance of EtG in nail was faster than expected from nail growth physiology. Eighteen of the 40 patients did not achieve EtG concentrations below LOQ during the study period, and median time from inclusion to the last positive sample was 34.5 days (range: 0–81). For 22 of the 40 patients EtG concentration in nail reached below LOQ within two months, with a median time of 47.0 days (range: 13–60). The calculated half-life (median: 13.3 days, range 5.5–29.0) also indicates that EtG disappears from nails faster than would be expected. A relatively short window of detection has also been shown for amphetamine and methamphetamine by Lin et al. (144). In

this study, fingernail clippings from eight drug users under treatment were collected every fourth week for a 12-week period. These eight subjects all showed high amphetamine/methamphetamine concentrations in nails at inclusion and all nail samples were below LOD (0.2 ng/mg) after 12 weeks. Four of the eight subjects were negative already eight weeks after inclusion. Longer detection time in nails have been shown after single intake of 10 mg zolpidem in two different studies, showing a detection window from about 3.5 months and up to 7.5 months (121, 143). The early disappearance of nail EtG shown in paper II should be confirmed by other studies, preferable in studies with controlled alcohol doses.

As a result of this early disappearance of EtG from nail in paper II, it is reasonable to hypothesize that EtG molecules have been removed from the nail matrix. The extent of removal and the removal mechanism that is most important is not known. Possible removal mechanisms or wash-out effects could be a substance extraction into blood circulating through the nail bed and also by external influence though extraction during hand washing. Madry et al. (121) showed a peak concentration of zolpidem 24 hours after a single intake of the substance and a declining concentration to the second sample (one week after zolpidem intake), suggesting a wash-out effect during this period. However, the study of paper II was not designed to investigate the mechanisms of the early disappearance of EtG, and this should be addressed in future studies.

When interpreting hair EtG concentrations, different hair segments have traditionally been considered as representative for the alcohol consumption in the corresponding hair growth period. The early disappearance of EtG in nail found in paper II, in addition to the complex incorporation mechanisms into the nail matrix, suggests that it may be difficult to use nail analysis related to a time-corresponding interpretation. As a result of this, a cut-off limit for EtG in nails could possibly only be established for general individual alcohol consumption and not a cut-off corresponding to a specific time period of consumption.

The results of this kinetic study of EtG in nails should be addressed further in new studies, both to confirm our results but also to investigate the incorporation mechanisms of EtG into nails. The knowledge is crucial when assessing EtG concentrations in this matrix.

## 5.4 Aim 3, paper III: To investigate change of EtG levels during hair-growth in an alcohol abstinence period, and whether this potentially may imply limitations in the use of EtG in hair segments as a quantitative measure of alcohol intake.

In paper III, the main finding was a significant (p=0.003) change in EtG concentrations in the hair samples collected at inclusion compared to the levels found in hair after one month of hair growth, although segments analysed (T1 and T2) should roughly represent the same alcohol consumption period. The median change of EtG concentrations was -46.0%.

An important question to ask, in light of our findings, is whether segmental hair analysis of EtG in hair is representative for the corresponding period of alcohol consumption. SoHT consensus report for the use of alcohol markers in hair from 2019 emphasize that if samples less than 3 cm or longer than 6 cm are used, the results should be interpreted with caution (6). Some studies have previously tried to address this question concerning segmental analysis of EtG in hair (103, 126, 155, 156). Both Appenzeller et al. (126) and Agius et al. (103) stated that EtG concentrations found by segmental analysis of distal hair segments seemed to be representative for the alcohol consumption, even in hair segments up to 12 cm. Meier et al. (155) and Tsanaclis et al. (156) both found decreasing hair EtG concentrations from the 0–1 cm segments to the 2–3 cm segments with a median ratio from proximal to distal segment of 2.0 and a mean ratio of 2.2, respectively. These studies of segmental hair analysis are more thoroughly discussed in paper III and new articles on this topic were not identified.

In paper III, we did not calculate the ratio between T1 and T2. The ratios found in the studies of Tsanaclis et al. (156) and Meier et al. (155) are comparable to the ratio between T1 and T2 in paper III. In paper III, six of the included subjects had a total loss of hair EtG from T1 to T2. By setting these six T2 concentrations equal to LOD (3 pg/mg), it is possible to calculate a ratio between T1 and T2. The median ratio for all patients (n=40) was 1.85 (range 0.09–33.3).

After publication of papers II and III, we wanted to compare individual data of EtG half-life in nails (paper II) with change of hair EtG from T1 to T2 (paper III) for the 16 patients who were included in both papers. If individuals with rapid decreasing EtG concentrations in nail also showed a large decline in EtG concentrations in hair from T1 to T2, it might indicate some common characteristics among these subjects (e.g. low affinity EtG-keratin binding). However, we found no significant correlation between EtG half-life in nails compared to change in hair EtG concentration from T1 to T2 (Spearman's $\rho$=0.030, p=0.912). No

connection between rapid decrease in EtG concentrations in nail and hair could be caused by the fact that affinity is different in nail and hair or it may be caused by wash-out being the most important elimination factor. The lack of statistical power due to the low number of subjects could also be an explanation of the lack of significant correlation.

It is well-known that cosmetic hair treatment like bleaching, perming and dyeing of hair may lead to lower concentrations of hair EtG or false negative results (6, 146-149). In paper III, there was still a significant ($p=0.013$) decrease in hair EtG concentration from T1 to T2 when excluding all ten patients reporting any kind of cosmetic hair treatment prior to inclusion ($n=8$) or between T1 and T2 sample collection ($n=2$).

There could be several reasons of reduced hair EtG concentration from proximal to more distal segments during hair growth. A degradation of EtG during storage has been considered not to be a problem in the literature (152, 153) and in our study a possible degradation would only have led to lower T1 concentrations as these were collected prior to T2, but analysed simultaneously. Differences in EtG concentrations in sweat and sebum surrounding different hair segments in the collected samples is not expected to affect the results as these EtG molecules, not yet incorporated into hair, are expected to be removed by the pre-analytical washing procedure.

A wash-out effect from regular water contact (e.g. hair washing) is a plausible explanation of the loss of EtG from T1 to T2. Wash-out effects in hair with reduced EtG concentrations in hair incubated in chlorinated and ionized water have been shown by Luginbühl et al. (154). The extent to which these results can be transferred to a real-life situation of normal hair washing routines is unknown, but the fact that EtG is a hydrophilic substance makes it vulnerable for wash-out in contact with water. Another wash-out mechanism could be the presence of sweat without EtG in a period of abstinence, with a possible extraction of EtG molecules from hair to sweat, as suggested by Pianta et al. (187). A reduction of EtG due to wash-out effects could be of greater clinical relevance than for many other substances, as a result of the chemical properties of EtG resulting in a low incorporation rate in addition to vulnerability for wash-out.

When observing wash-out effects, the horizontal study design of paper III differs somewhat from the situation seen when analysing different hair segments from one hair sample taken at a single time-point. In the abstinence period from T1 to T2 there should be no EtG incorporated into hair strands through diffusion from blood or from sweat or sebum. In a

situation of fairly stable alcohol consumption prior to collection of a single time-point hair sample, incorporation of EtG into hair by sweat may in fact disguise some of the possible wash-out effect by hair washing in the different hair segments. This is the most probable situation in real-life samples. The concentration gradient between hair and sweat containing EtG is probably of importance to the extent of this possible compensation of EtG incorporated by sweat.

There are two situations where the results from paper III are of considerable importance. Firstly, interpretation of distal hair segments could be influenced by the results shown in paper III, although the study design in paper III differs from a real-life case including several segments sampled simultaneously.

This can be illustrated by a case involving a hair sample received for analysis at our institute. A patient was admitted to hospital with suspicion of forced alcohol intake relatively shortly before admittance. Segmental hair analysis showed concentrations of EtG of approximately 25 pg/mg in the proximal 0–1 cm segment (close to the scalp), 15 pg/mg in the next 1–2 cm segment, 7 pg/mg in the 2–3 cm segment and 3 pg/mg in the 3–4 cm segment. The question was whether this pattern of EtG segmental analysis supports a forced alcohol intake in a short period prior to admittance. Alternatively, could wash-out effects explain this reduction of EtG from proximal to distal segment making the result consistent with high alcohol intake during the total period? The results seen in paper III could indicate that some of the declining EtG concentrations seen in this case could possibly be explained by wash-out effects. However in the case presented, there is greater difference in EtG concentration from proximal to distal hair segments compared to the median difference reported in paper III, although such high losses were seen. Although the collection of samples differ from that of the study in paper III, the drop in EtG concentrations from the proximal to the distal hair segments is so large that it probably also indicates differences in alcohol consumption for the corresponding hair segment. In real-life cases, where a single time-point hair sample is cut into different hair segments, the conditions do not change as a result of different sample collection time. In the presented case, it was concluded that the EtG segmental analysis could support increased alcohol intake relatively shortly (within 1 month) prior to admittance compared to the 2–4 months period prior to admittance.

Another situation where the results from paper III could be informative is cases with information of e.g. one month of abstinence before sample collection. In such cases with

abstinence prior to sample collection, e.g. criminal offenders in prison, an EtG concentration of e.g. 20 pg/mg in a hair segment corresponding to the period prior to the abstinence period does not necessarily reflect a moderate alcohol intake but could be underestimated. The timing of sample collection related abstinence length may, as result of this, be of importance. An alternative direct biomarker like phosphatidylethanol (PEth) would also show lower blood concentration (decline of about 50% in a few days) after an abstinence period (188).

Our data are limited and are not suitable to provide clear clinical advice. Notwithstanding the methodological weaknesses mentioned earlier under "Methodological considerations", our data indicate that possible EtG wash-out effects may have clinical importance already after one month of abstinence before sample collection. This is also supported by the physiology of EtG and incorporation into hair and a limited number of studies on this topic.

Overall, the study of paper III supports the suggestion from SoHT to interpret distal hair segments with caution, but our results needs to be confirmed by other studies.

# 6. Conclusions

A major finding in this thesis is that reduced kidney function may result in substantially higher hair EtG concentrations than the levels seen in healthy volunteers. One implication of this is that use of hair EtG among kidney-failure patients could lead to misclassification of drinking habits with the risk of having a false-positive diagnosis of heavy drinking.

We also found that patients attending an alcohol rehabilitation clinic had higher EtG concentrations in nails compared to the concentrations in hair and there was also a significant correlation between nail EtG/EDI and hair EtG/EDI. When studying nail EtG concentrations during the inpatient period of abstinence, we found that most individuals showed declining EtG levels extending below LOQ within about two months after inclusion. This rapid decline of EtG concentrations in nails is faster than what would be expected based on normal nail growth rate. Further studies of nail EtG kinetics is needed to understand incorporation mechanisms and to what extent EtG molecules may leach out from the nail matrix as a result of wash-out mechanisms.

Another major finding was the reduction of hair EtG concentrations in two different hair segments among most of the alcohol-dependent subjects during hair growth in an alcohol abstinence period, even though the two hair segments analysed should have represented roughly the same alcohol consumption period. Although the study has some important limitations, our results support the SoHT's advice to be cautious when interpreting distal hair segments. Further studies are needed to confirm our results and to understand wash-out mechanisms as a possible cause of declining hair EtG concentration during hair growth.

## 7. Future perspectives

There are some unanswered questions regarding the use of hair EtG as an alcohol biomarker among patients with reduced kidney function. One important question to be answered is whether alcohol consumption level could be predicted by using hair EtG in this population. An important aspect is whether it is possible to calculate or normalize hair EtG, for instance by using eGFR, to get a reliable hair EtG test using already established cut-off levels representative for amount of alcohol consumption. Alternatively, what is the lower limit of eGFR to get a reliable hair EtG test? A controlled alcohol dosing study performed using a larger population with reduced kidney function could provide important information about this.

An advantage of using nail as a matrix instead of hair is the simplicity of the sample collection and its availability in subjects who lack head hair material; however, the limited knowledge of EtG in nail suggests caution when interpreting test results. Further kinetic studies of EtG in nail are needed to better understand the incorporation mechanisms and the extent to which nail EtG is representative for certain periods of alcohol consumption. Knowledge about the stability of incorporated EtG in nail is also important to enhance, particularly in terms of whether EtG in nails is affected by being exposed to different nail cosmetics and its frequent contact with water. Controlled studies are needed to compare EtG concentrations in nail and hair and to evaluate cut-off limits for EtG in nail that represent the amount of alcohol consumed and the period of consumption.

Interpretation of EtG in different hair segments relies on the stability of EtG after incorporation into hair. Enhanced knowledge about the stability of EtG is important when interpreting EtG levels in different hair segments. When studying wash-out effects, controlled alcohol dosing studies are preferred with thorough registration of abstinence period, but also conditions like hair washing and hair cosmetics. This could further help us to ascertain if the current cut-off limits can be trusted in distal segments.

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# 9. Papers I–III

# I

Alcoholism: Clinical and Experimental Research
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# Levels of Hair Ethyl Glucuronide in Patients with Decreased Kidney Function: Possibility of Misclassification of Social Drinkers

Jan Toralf Fosen, Luca Morini, Cristina Sempio, Rudiger Ganss, Jørg Mørland, and Gudrun Høiseth

**Background:** The use of hair ethyl glucuronide (EtG) levels as a biomarker for chronic high intake of ethanol (EtOH) is increasing, and misclassification of alcohol consumption may have large implications for the patient. The aim of this study was to compare levels of hair EtG in patients with reduced kidney function to levels seen in a comparable control group and to investigate whether the hair EtG levels among kidney failure patients who are social drinkers may lead to a false-positive diagnosis of heavy drinking.

**Methods:** A total of 41 patients with reduced kidney function and 42 healthy volunteers were included in the study. Both patients and the healthy volunteers reported moderate alcohol intake. The levels of EtG in hair (corrected for estimated daily intake of EtOH [EDI]) were compared between the 2 groups.

**Results:** There was no significant difference between the groups regarding EDI. Despite this, there were significant higher levels of hair EtG (corrected for EDI) in the patient group compared to the control group ($p < 0.001$). Eight subjects (20%) in the patient group showed EtG levels in hair above 30 pg/mg, in contrast to no subjects among healthy volunteers ($p = 0.002$). In the patient group, there was significant correlation between levels of EtG in hair and both estimated glomerulus filtration rate and serum creatinine levels.

**Conclusions:** This study documents an increased risk of obtaining a false-positive diagnosis of heavy drinking among renal disease patients who are social drinkers. Interpretation of EtG levels in hair among patients with reduced kidney function must be performed with caution.

**Key Words:** Alcohol, Ethyl Glucuronide, Hair, Kidney Function.

THE ALCOHOL METABOLITE ethyl glucuronide (EtG) has in recent years become an important biomarker for ethanol (EtOH) intake. EtG, which is the product of EtOH and glucuronic acid catalyzed by the enzyme uridine 5′-diphospho-glucuronosyl transferase, is a minor metabolite in the EtOH metabolic pathway, representing below 0.1% of the ingested EtOH (Foti and Fisher, 2005).

EtG has been established as a direct EtOH marker in urine and blood, but the latest years, the use of EtG in hair as a marker of chronic alcohol consumption has been investigated (Crunelle et al., 2014). Studies have indicated that EtG in hair shows high diagnostic sensitivity and specificity for detection of chronic heavy alcohol consumption compared to other traditional EtOH biomarkers (Hoiseth et al., 2009b; Politi et al., 2006). The use of EtG in hair as a marker for chronic, high intake of EtOH is increasing, and in several countries, EtG in hair is used to assess suitability to hold a driving license after chronic alcohol abuse. The society of hair testing has recently published a consensus on alcohol markers in hair. A cutoff level for EtG in hair of 30 pg/mg in the proximal 3-cm hair segment is recommended to detect excessive drinking intake of EtOH (above 60 g/d) (Kintz, 2015). This recommendation corresponds to findings in previously published studies (Appenzeller et al., 2007a; Morini et al., 2009).

Renal excretion is the major elimination route for glucuronides (Osborne et al., 1993), and also for EtG (Hoiseth et al., 2007) and after an identical intake of alcohol, one can expect higher blood concentrations of EtG and prolonged urinary detection window in patients with decreased kidney function compared to healthy volunteers. Higher EtG blood concentrations were seen in 1 subject with reduced kidney function in a previous study (Hoiseth et al., 2009a), and prolonged urinary detection times for EtG in

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renal failure patients have previously been published (Hoiseth et al., 2012).

Increased incorporation of EtG in hair could be suspected as a result of increased blood levels of EtG among patients with decreased kidney function compared to healthy volunteers. In these patients, levels of EtG in hair indicating excessive alcohol use could be seen in social drinkers. This was indicated in a preliminary, small study in patients with decreased kidney function, showing higher levels of EtG in hair of renal disease patients (Hoiseth et al., 2013). In this study, the small number of Norwegian patients was compared to previously published data from an Italian population. The patient and control group was therefore not comparable in respect to hair pigmentation and environmental conditions like exposure to sunlight. It has been reported that hair pigmentation influences the deposition of drugs in hair (Reid et al., 1996; Scheidweiler et al., 2005); however, it has not been shown differences in hair EtG as a result of hair pigmentation (Appenzeller et al., 2007b; Kharbouche et al., 2010). Recent studies have indicated that different exposure to sunlight might affect the levels of drugs in hair (Favretto et al., 2014). Hair bleaching, coloring, and perming may also reduce levels of hair EtG (Crunelle et al., 2015; Kerekes and Yegles, 2013; Morini et al., 2010; Suesse et al., 2012). It was therefore important to include a control group comparable with respect to environmental factors. Also, kidney function decreases with age, and to study the effect of renal disease as such, it was also important to include a control group matched on age.

The aim of this study was to compare levels of hair EtG in a larger material of patients with decreased kidney function to EtG levels seen in a comparable, age-matched population of healthy volunteers and answer the following question: Is it possible to obtain a false-positive diagnosis of heavy drinking among renal disease patients who are social drinkers?

## MATERIALS AND METHODS

### *Study Protocol*

This study includes a patient group with decreased renal function and a control group of healthy volunteers. For the patient group, nondialyzed renal disease patients were recruited from the renal failure clinic at Akershus University Hospital, Lørenskog, Norway. Inclusion criteria were serum creatinine value >90 $\mu$mol/l in women and >100 $\mu$mol/l in men (Rustad et al., 2004), estimated glomerulus filtration rate (GFR) <60 ml/min/1.73 $m^2$ (automatically calculated from creatinine using the Modification of Diet in Renal Disease formula including age and sex) (Rule, 2007), and a diagnosis of decreased kidney function in the patient's journal. Only patients having a moderate use of alcohol were included in our study which was approved by the National Committee for Research Ethics in Norway, the Norwegian directorate of health and the local ethics committee at Akershus University Hospital. All patients signed informed consent before attending the study. Medical information was received from the patient's medical record. This included the diagnosis of decreased renal function, serum creatinine value, and the estimated GFR as objective measurements of the individual renal function. Values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) were also collected, as objective measures of possible alcohol overconsumption.

For both the patients and the control group, an estimated daily intake of EtOH (EDI) (calculated on the basis of the timeline follow-back procedure) for the last 3 months was obtained by interviewing the participants carefully about type of beverage and amount consumed on a typical day using a validated questionnaire (EMCDDA, 2004).

Hair was collected by cutting a hair specimen as close as possible to the skin at the posterior vertex. The 3-cm proximal hair segment, roughly representing the last 3 months, was submitted for EtG analysis. Information about hair bleaching, coloring, perming, and the use of hair care products (hair spray, styling gel, etc.) was obtained by interview.

For the control group, individuals with no known kidney failure were recruited. The control group was age matched with the study group, as the same number of subjects in the following age intervals was included: below 59 years, 60 to 69 years, 70 to 79 years, and 80 to 89 years. Information about diseases which could possibly affect kidney function was obtained, based on self-report. No information about creatinine levels and GFR or other blood parameters was available for the control group.

Also, time for last alcohol ingestion, height, weight, age, and sex was recorded for each participant both in the patient group and in the control group.

### *Analytical Methods*

EtG in hair was determined using a previously published method (Morini et al., 2006). Briefly about 100 mg (20 mg is the lowest accepted weight) of hair was washed with 2 different organic solvents (dichloromethane and methanol), dried under nitrogen stream, and cut into small pieces (1 to 2 mm length segments). Samples were then soaked into 700 µl of deionized water with penta-deuterated EtG ($D_5$-EtG) as internal standard and ultrasonicated for 2 hours and centrifuged at 10,800×$g$. Finally, 10 $\mu$l was injected in the liquid chromatography-tandem mass spectrometry system. The chromatographic separation was carried out in reversed phase, isocratic mode, using a mobile phase containing a very high water percentage (99% water + 1% acetonitrile). A triple quad was used was used in multiple reaction monitoring mode and negative polarization. Two transitions for each analyte were chosen for identification, while the most intense was used for quantification purposes. A 5-point calibration curve ranging from 3 to 50 pg/mg was used for quantification purposes. Samples exceeding calibration range were diluted accordingly and re-injected. The limit of detection (LOD) was 2 pg/mg, and the lower limit of quantification was 3 pg/mg.

### *Statistics*

Differences in baseline characteristics such as age, body mass index (BMI), and EDI were studied using independent-samples $t$-test. Differences in categorical variables were analyzed using Pearson chi-square test in the case of no cells with expected counts <5; otherwise Fischer's exact test was used. Differences in respect to hair levels of EtG corrected for EDI (ratio EtG/EDI) in the patient group compared to the healthy volunteers were analyzed using the nonparametric Mann–Whitney $U$-test. Correlation between hair levels of EtG and GFR or serum creatinine was assessed using Spearman's nonparametric rank correlation test. A $p$-value below 0.05 was considered significant.

## RESULTS

A total of 41 patients with decreased kidney function (30 men and 11 women) and 42 age-matched healthy volunteers

(13 men and 29 women) were included in the present study. Patient characteristics are presented in Table 1. All subjects in the patient group had ALT, AST, and GGT within the normal range (≤50 U/l for ALT and AST, ≤60 U/l for GGT) (Klauke et al., 1993; Rustad et al., 2004; Szasz, 1974). According to the inclusion criteria, all patients showed levels of creatinine and GFR outside the normal range. All patients also had a diagnosis of decreased renal function in their medical journal. Four patients reported use of hair bleaching, coloring, or perming last 3 months, and 7 patients reported use of hair care products.

**Table 1.** Patient Characteristics for the Patient Group and the Control Group and a *p*-Value for the Difference Between the Groups

| | Patient group ($n$ = 41) | Control group ($n$ = 42) | $p$ |
|---|---|---|---|
| *N* | 41 | 42 | |
| Age, years (mean, SD) | 64.8 (15.1) | 61.7 (17.1) | 0.39 |
| Sex (female) ($n$, %) | 11 (26.8) | 29 (69.0) | <0.001 |
| BMI, kg/m$^2$ (mean, SD) | 26.5 (4.5) | 25.1 (2.9) | 0.095 |
| Estimated daily intake of ethanol, g (mean, SD) | 5.5 (6.3) | 6.3 (7.0) | 0.58 |
| Creatinine, $\mu$mol/l (mean, SD) | 293.3 (123.9) | – | – |
| Glomerulus filtration rate, ml/min/1.73 m$^2$ (mean, SD) | 21.6 (10.6) | – | – |
| Hair coloring ($n$, %) | 2 (4.9) | 12 (28.6) | 0.004 |
| Hair bleaching ($n$, %) | 1 (2.4) | 2 (4.8) | 1.00 |
| Hair perming ($n$, %) | 1 (2.4) | 0 (0) | 0.49 |
| Hair care products ($n$, %) | 7 (17.1) | 12 (28.6) | 0.21 |

From self-report, no subjects in the control group had kidney disease and no subjects reported diseases which were expected to affect kidney function, except from 2 subjects suffering from diabetes. They reported that their creatinine and GFR values were subject to control and within the normal range. Fourteen controls reported use of hair bleaching, coloring, or perming last 3 months, and 12 controls reported use of hair care products.

There was no statistical significance between the groups with respect to age, EDI, and BMI. There were a higher number of females in the control group ($p < 0.001$). There were significant more participants in the control group reporting hair coloring ($p = 0.004$), but there was no significant difference between the groups with respect to use of hair care products, bleaching, or perming.

The levels of hair EtG ranged between <LOD and 331 pg/mg (mean: 25.1, median: 0.0) in the patient group and between <LOD and 10.0 pg/mg (mean: 0.2, median: 0.0) in the control group. The EDI ranged between 0.1 and 28.0 g/d (mean: 5.5, median: 2.8) in the patient group and 0.1 and 24.0 g/d (mean: 6.3, median: 4.0) in the control group. Individual values of hair EtG related to EDI for the patient

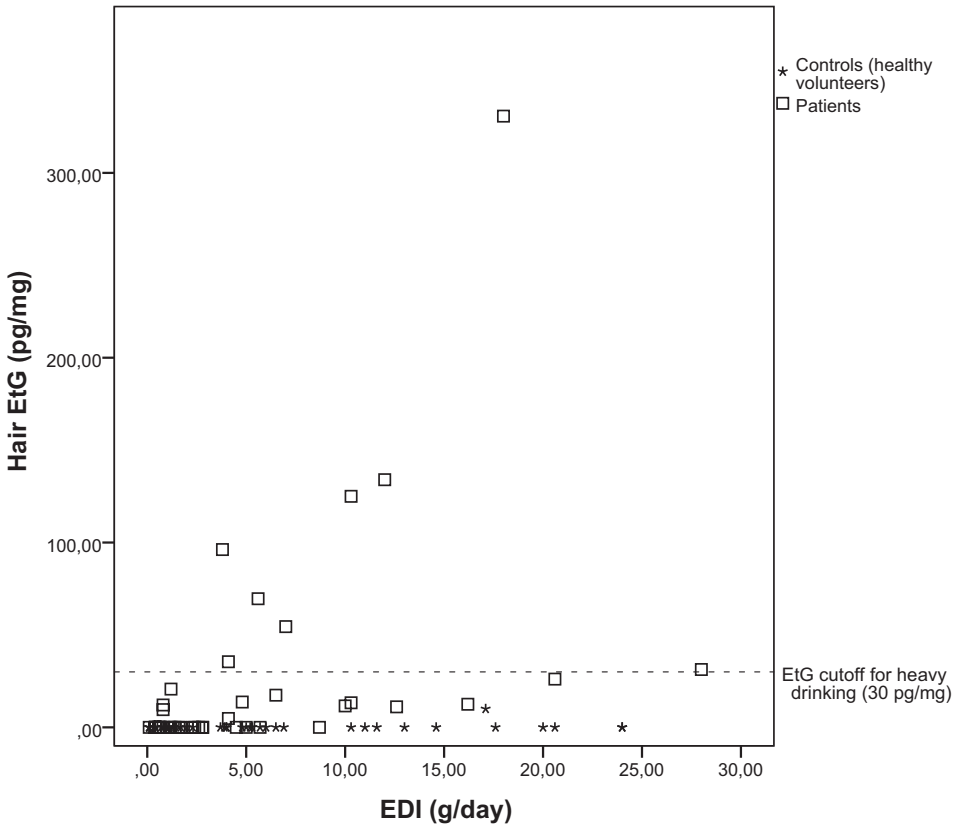


**Fig. 1.** Individual levels of estimated daily intake of ethanol and hair ethyl glucuronide in patients ($n$ = 41) and controls (healthy volunteers) ($n$ = 42).

group and the control group are shown in Fig. 1. The ratio between hair EtG and EDI ranged between 0 and 25.3 (pg/mg)/(mg/d) (mean: 3.7, median: 0.0) in the patient group and between 0 and 0.58 (pg/mg)/(mg/d) (mean: 0.01, median: 0.0) in the control group. The levels of hair EtG (corrected for EDI) in the patient group were significantly higher than the levels seen in the control group ($p < 0.001$). This is shown in Fig. 2.

There were no subjects showing EtG levels in hair above 30 pg/mg in the control group, in contrast to 8 subjects (20%) in the patient group ($p = 0.002$). The subjects in the patient group with levels above 30 pg/mg showed concentrations in the range 31 to 331 pg/mg. The individual levels of hair EtG, EDI, creatinine, and GFR of these subjects are shown in Table 2.

In the patient group, there was a significant, negative correlation between the level of EtG in hair (corrected for EDI) and the GFR (Spearman's $\rho = -0.413$, $p = 0.007$). There was also a statistically significant positive correlation between hair EtG (corrected for EDI) and serum creatinine (Spearman's $\rho = 0.420$, $p = 0.006$). The relation between hair EtG/EDI and GFR is shown in Fig. 3.

## DISCUSSION

This study showed that within a group of patients suffering from decreased kidney function, hair EtG levels were higher compared to a healthy control group with an equal EDI. Social drinkers with decreased kidney function might therefore exceed the cutoff for heavy drinking and thereby be misclassified as heavy drinkers.

The findings are expected from theoretical considerations. Renal excretion is the major elimination route for EtG and from studies of other glucuronides like morphine-6-glucuronide (Bodd et al., 1990), and we know that decreased kidney function impairs excretion. Prolonged detection time in urine is also seen for EtG (Hoiseth et al., 2012). Increased incorporation of EtG in hair could be suspected as a result of

**Table 2.** Individual Levels of Ethyl Glucuronide (EtG) in Hair, Estimated Daily Intake of Ethanol (EDI), Serum Creatinine, and Glomerulus Filtration Rate (GFR) in the 8 Patients (Social Drinkers) Exceeding the Hair EtG Cutoff for Heavy Drinking

| Case number | Hair EtG (pg/mg) | EDI (g/d) | Serum creatinine ($\mu$mol/l) | GFR (ml/min/1.73 $m^2$) |
|---|---|---|---|---|
| 1 | 330.7 | 18.0 | 288 | 18.0 |
| 2 | 134.0 | 12.0 | 500 | 10.3 |
| 3 | 125.0 | 10.3 | 350 | 14.9 |
| 4 | 96.2 | 3.8 | 370 | 13.6 |
| 5 | 69.6 | 5.6 | 670 | 8.0 |
| 6 | 54.5 | 7.0 | 178 | 23.6 |
| 7 | 35.5 | 4.1 | 254 | 22.9 |
| 8 | 31.3 | 28.0 | 440 | 11.9 |

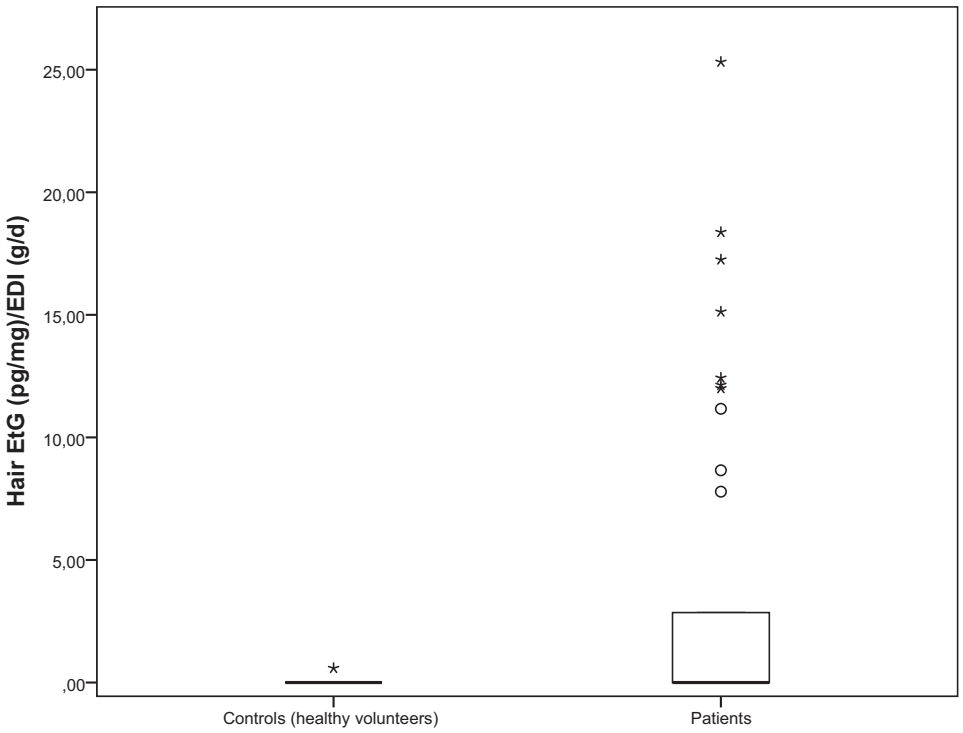


**Fig. 2.** Levels of ethyl glucuronide (EtG) in hair corrected for estimated daily intake of ethanol (EDI) in patients with decreased kidney function ($n = 41$) compared to controls (healthy volunteers) ($n = 42$). The box length is the interquartile range of hair EtG/EDI. The line across the inside of the box represents the median value. Circles and asterisks represent values exceeding 1.5 and 3 times the interquartile range, respectively.

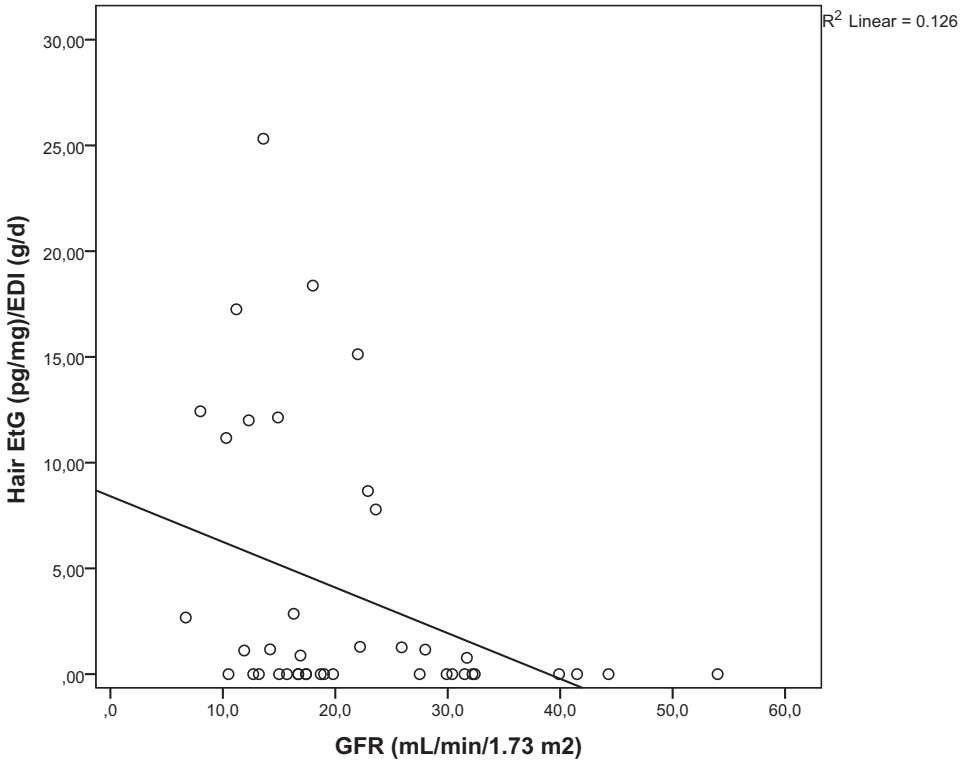


**Fig. 3.** Individual levels of glomerulus filtration rate compared to ethyl glucuronide (EtG) in hair corrected for estimated daily intake of ethanol (EDI) in patients with decreased kidney function ($n$ = 41).

increased blood levels. The present study verified the very preliminary, uncontrolled previous study (Hoiseth et al., 2013), which indicated that higher EtG levels in hair could be seen as a result of decreased kidney function, probably through a mechanism of higher blood levels of EtG.

Previous studies indicate that hair EtG levels above 30 pg/mg are highly consistent with a chronic excessive alcohol consumption habit (Alt et al., 2000; Appenzeller et al., 2007a; Pragst and Yegles, 2008; Yegles et al., 2004). All previous studies are, however, to the best of our knowledge performed on healthy populations. In the present study, both the control group and the patient group are low consumers, with EDI well below the definition of heavy drinking. In the control group, all subjects showed negative, or very low levels of EtG, as expected from the estimated EDI (Kronstrand et al., 2012; Politi et al., 2006). In the patient group, with an equally low EDI, 8 subjects (20%) showed levels of EtG in hair above the suggested cutoff for defining heavy drinking (30 pg/mg). Our results therefore show that this suggested cutoff is not trustable when it comes to patients with kidney disease.

It should be noticed that a dose–response relationship between degree of kidney impairment and hair EtG is indicated from the significant correlation between serum creatinine and GFR, respectively, and hair EtG corrected for EDI. This supports the theory of a causal relation, as the dose–response relationship being one of the most important criteria when assessing causality (Hill, 1965).

As a result of the fact that kidney function decreases with age, it is important that the control group was age matched with the patient group. The control group was not matched on sex, as we consider sex differences to be less important concerning kidney function. Another important strength of this study was the objective measures of both kidney disease and alcohol over consumption in the patient group and the use of a comparable control group with respect to factors such as hair pigmentation and sun bleaching and also comparable EDI values.

There are some limitations/weaknesses of this study. EDI was based on self-reported alcohol consumption, which is can be biased when studying at-risk alcohol consumers (Toneatto et al., 1992). Clinical advices of reducing alcohol consumption or at least avoiding heavy intake because of their kidney disease may lead to underreporting of EDI among the patients. To try to minimize this bias and to obtain equal results from both groups, a validated questionnaire (EMCDDA, 2004) was used both for the patient and the control group. Also, values of AST, ALT, and GGT in the patient group suggest that there was no heavy alcohol consumption among the patients, but these parameters were not available for the control group. Underreporting of alcohol consumption may also happen in the control group, but a marked difference in the extent of underreporting between the groups is not expected. There were more subjects reporting hair coloring in the control group compared to the patient group. Some

previous studies have shown decreased concentrations of EtG in hair after coloring, while another study found no such difference. If excluding subjects with hair coloring before analyses, the levels of EtG in hair corrected for EDI were still higher in the patient group than the control group ($p < 0.001$).

In conclusion, this study verified the risk to have a false-positive diagnosis of heavy drinking among renal disease patients who only drink socially. Interpretation of hair EtG levels among patients with decreased kidney function must be performed with caution.

## CONFLICT OF INTEREST

None of the authors have any conflict of interests.

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RESEARCH ARTICLE

WILEY

# Hair EtG: Alterations in segment levels accompanying hair growth

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## Abstract

Hair levels of the direct ethanol biomarker ethyl glucuronide (EtG) are used to evaluate history of alcohol intake. The proximal 3 cm of the hair sample is most often analyzed and this is assumed to represent the intake of ethanol over approximately the past three months. The aim of this study was to investigate change of EtG levels during hair growth in an ethanol-abstinent period. Twenty-seven patients were recruited from an alcohol treatment clinic. A hair sample was collected after hospitalization and EtG was analyzed in the 0–3 cm hair segment (T1). Another hair sample was collected after one month of abstinence and EtG was analyzed in the 1–4 cm hair segment (T2), discarding the proximal 1 cm (0–1 cm) of the segment. As a result of the segment choice and assuming a hair growth rate of 1 cm per month, T2 should represent roughly the same time of alcohol exposure as segment T1. The median concentration of EtG in T1 was 100 pg/mg (range 7.7–1320) and the median concentration in T2 was 53.4 pg/mg (range < LOQ-692). EtG concentrations decreased significantly from T1 to T2 ($p$ = 0.003) and the median change in EtG from T1 to T2 was −46.0%. This study shows decreasing EtG concentrations in most subjects in a hair segment during growth when comparing two segments that is assumed to represent roughly the same period of alcohol intake. Further research is needed to confirm if this observed decline of EtG is a result of wash-out-effects of EtG or other factors.

**KEYWORDS**

alcohol marker, ethyl glucuronide, hair, stability

## 1 | INTRODUCTION

Analyzing ethyl glucuronide (EtG) in hair is widely used in both forensic and clinical settings. For the diagnosis of heavy drinking (above 60 grams per day[1]) and according to Society of Hair Testing (SoHT) guidelines, a cut-off at 30 pg/mg EtG in hair is used.[2] The proximal 3 cm of the hair are most often analyzed. This is assumed to represent the intake of ethanol over the past three months. Hair growth cycle is subject to different growth speed depending on the growth phase, but a mean growth rate of 1 cm (range 0.60–1.50) per month is expected in head hair.[3] This is widely accepted in practice[4] and is an assumption for the interpretation of the results when head hair from the vertex region of the scalp is collected for analysis. SoHT has also recommended interpreting EtG-results from hair segments less than 3 cm or more than 6 cm with caution.[2]

EtG, which is a conjugate of ethanol and glucuronic acid catalyzed by the enzyme uridine 5′-diphospho-glucuronosyl transferase, is a minor metabolite in the ethanol metabolic pathway, representing below 0.1% of the ingested ethanol.[5] EtG is a hydrophilic and acidic substance[6] with pKa = 3.21.[7] EtG is accordingly rather negatively charged at physiological pH. The fact that intracellular pH of keratinocytes and melanocytes has been found to be more acidic than plasma means that the distribution equilibrium is shifted in disadvantage of EtG-incorporation in the hair root.[6] This seems to be an

  

important cause of the low EtG-concentrations in hair compared to more lipophilic substances. The precise mechanisms involved in the incorporation of EtG into hair are not fully understood, but experimental data suggest that the most important route is incorporation from blood and that transfer from sweat and sebum is less important.[8-10]

In cases where there has been recent ethanol intake, EtG results may also reflect contamination by body fluids, as suggested by Agius et al.[11] Cosmetic hair treatment like bleaching and perming may remove a substantial amount of the previously incorporated EtG.[12] Wash-out effects, as a result of hair wash, for instance, have also been discussed in several studies with conflicting results as whether they can lead to reduced EtG levels in hair.[11,13-16] A previous study has indicated that segmental hair analysis can show alcohol consumption trends for several months and show a relationship between the EtG concentration in hair and the amount of alcohol intake.[17]

To the best of our knowledge, no studies have been designed to investigate changes in EtG concentrations in hair after a period of abstinence in segments representing the same growth period. A period of abstinence gives the opportunity to study if the EtG levels are unchanged after cessation of drinking. The aim of this study was to use longitudinal data to study change of EtG levels during hair-growth in an ethanol abstinent period among patients admitted to an alcohol treatment clinic.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design and protocol

Patients were recruited from a 12-step treatment clinic: Trasoppklinikken in Oslo, Norway. The clinic is an open unit staffed with specialized medical and nursing personnel. The clinical board at Trasoppklinikken approved the study. All participants signed informed consent before attending the study which was approved by the National Committee for Research Ethics in Norway (ref. no 2013/1222). For the first 16 of the 27 patients included in the present study, longitudinal nail EtG data were also collected.[18]

The inpatient treatment program at Trasoppklinikken usually lasts 10 weeks, ranging from detoxification to completing educative, cognitive behavioral, and 12-step therapy. Most patients use only alcohol, but there are no restrictions concerning other drug use/dependences. Breath ethanol is measured several times weekly on a regular and random basis to control for abstinence. Positive breath ethanol results were recorded as part of the present study. Serum creatinine (normal range 45–90 μmol/L for women and 60–105 μmol/L for men), aspartate aminotransferase (AST) (normal range < 35 U/L for women, < 45 U/L for men), alanine aminotransferase (ALT) (normal range < 45 U/L for women, <70 U/L for men), gamma-glutamyl transferase (GGT) (normal range < 45 U/L for women 0–39 years, < 75 U/L for women above 40 years, < 80 U/L for men 0–39 years, < 115 U/L for men above 40 years), and carbohydrate deficient transferrin (CDT) (normal range < 1.7%)[19] were measured relatively close to admission and were received for 89%–96% of the patients from their medical records.

The patients received a preliminary informal invitation to the study as outpatients, before admission to the clinic. After the admission, all patients were formally invited to join the study and signed the informed consent.

The inclusion criterion for the present study was a history of alcohol ingestion within the last three weeks before the first sample collection. Twenty-seven patients were included in the study. All patients met the ICD-10 criteria for alcohol dependence.[20] All patients were interviewed about alcohol consumption using a modified AUDIT-test,[21] and an estimated daily intake of ethanol (EDI) was calculated on the basis of the information obtained (representing an average consumption over the last 90 days). Time of last alcohol intake was given special attention during the interview, and this date was recorded. In the modified AUDIT-interview, information about washing/treatment of hair was obtained.

A hair sample was collected after hospitalization (the day of inclusion) by cutting a hair specimen as close as possible to the skin at the posterior vertex and EtG was analyzed in the 0–3 cm proximal hair segment (T1), roughly representing hair growth the last three months. Another hair sample was collected approximately four weeks after inclusion and EtG was analyzed in the 1–4 cm hair segment (T2), discarding the proximal 1 cm of the segment (too low sample amount for reliable analysis). The choice of hair segments in the two different hair samples is shown in Figure 1. As a result of the segment choice and assuming a mean hair growth rate of 1 cm per month, T2 should represent roughly the same time of alcohol exposure as segment T1. In cases where the amount of hair in the proximal 3 cm segment was too small for analysis ($n$ = 15), another 1 cm of hair (up to 3 extra) was included in both T1 and T2. Hair segment T1 and T2 were analyzed simultaneously. EtG-test results were neither disclosed to the patients nor to the treatment staff.

All subjects were inpatients during the total study period. It was also noted if the clinic staff suspected alcohol intake since collection of the last sample. This could be based on a result of breath ethanol analysis or for other clinically valid reasons.

### 2.2 | Analytical methods

The analysis of hair has been fully validated and is previously published.[22] Limit of detection (LOD) and limit of quantification (LOQ) were 3.0 and 5.0 pg/mg, respectively. Results below LOQ were set to zero and overlapping EtG levels T1 ± 20% and T2 ± 20% were considered within analytical variation.


**FIGURE 1** T1 is the 0–3-cm hair segment from the hair samples taken at inclusion. T2 is the 1–4-cm hair segment from the hair samples taken one month later than inclusion (the first cm was discharged from this sample). Assuming a mean hair growth rate of 1 cm per month, EtG concentrations in segment T1 and T2 should then roughly represent the same time period of alcohol intake. In case of low sample weight additional distal cm was included in both T1 and T2 [Colour figure can be viewed at wileyonlinelibrary.com]

### 2.2.1 | Chemicals and materials

EtG and $D_5$-ethyl glucuronide ($D_5$-EtG) were obtained from LGC Standards (Milan, Italy). Formic acid for mass spectrometry was purchased from Sigma-Aldrich (St Louis, MI, USA). HPLC-grade methanol and acetonitrile were obtained from Panreac (NovaChimica, Milan, Italy).

### 2.2.2 | Sample preparation

Approximately 50 mg hair samples were washed with organic solvents (dichloromethane and methanol). They were dried and cut into small pieces (1–2 mm length). Samples were then extracted with 700 µL of water, and 20 µL of internal standard ($D_5$-EtG) aqueous solution (0.01 mg/L), incubated overnight and sonicated up to two hours. Finally, 8 µL of supernatant was injected in the liquid chromatography–tandem mass spectrometry (LC–MS/MS) system.

### 2.2.3 | Instrumentation

LC–MS/MS analyses were carried out through an Agilent 1100/1200 Series system (Agilent Technologies, Palo Alto, CA, USA) interfaced to a 4000 Q-TRAP (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) with an electrospray (ESI) Turbo V ™Ion Source. The Kinetex C18 column (100 x 2.1 mm i.d., 5-µm particle size, Phenomenex, Castelmaggiore, Italy) was kept at 25°C during the analysis. Analytes were eluted in isocratic mode, using a mobile phase consisting of 99% formic acid (0.1%) and 1% acetonitrile, with a flow rate of 0.2 mL/min. A post-column infusion of 0.1 mL/min of acetonitrile was used to enhance sensitivity.

The ESI source settings were: ion-spray voltage, 4200 V; source temperature, 400°C; nebulization and heating gas (air), 40 psi and 50 psi, respectively. Multiple reaction monitoring was optimized using nitrogen as collision gas and a dwell time of 100 ms.

The transitions m/z 221 →75 and m/z 226 →75 were used as quantifiers for EtG and D5-EtG, and the transitions 221→85 and m/z 226 →85 were used as qualifiers for EtG and D5-EtG respectively. Data acquisition and elaboration were provided by Analyst®software (version 1.5.1 AB SCIEX).

## 2.3 | Statistics

The time of inclusion (upon admission to the clinic when the first hair sample was collected) was set as time zero and intervals between the two hair samples (T1 and T2) were reported as days after the first sample. Concentrations of EtG in hair were not normally distributed. For all results, median and range values were reported for continuous variables and frequency distribution is reported for categorical variables.

Correlation between continuous variables was assessed using the non-parametric Spearman's rank correlation test. Differences between dependent variables like concentrations in hair T1 and T2 within each subject were studied using the non-parametric Wilcoxon signed ranks test.

# 3 | RESULTS

Twenty-seven patients (63% men) with a median age of 46 years (range 25–68) were included in the study. The median number of days between collection of the hair samples at T1 and T2 was 28 (range 25–48).

Based on self-reports of average alcohol intake during the last three months, the median EDI was 150 g/day (range 8.0–350). A median time of 7 days (range 2–16) had passed since the last alcohol intake and inclusion in the present study. Values above the normal range[19] were seen for 12 patients for CDT%, 11 patients for AST, 5 patients for ALT, 13 patients for GGT, and 0 patients for creatinine.

At inclusion, all patients reported hair washing at least once a week, 15 of these reported hair washing twice to three times a week, and 7 reported daily washing. Eight patients reported hair bleaching, coloring, or perming in the last three months before inclusion (known for 96% of the patients). Of these eight patients, treatment was specified as coloring in three patients, and hair bleaching in two patients. For the last three patients the hair treatment was not specified. For the period between T1 and T2, one patient (patient 20) reported use of hair toner and one patient (patient 27) reported unspecified hair treatment (known for 41% of the patients, but visual inspection of the hair samples did not reveal suspicion of treatment in any other patients). There was no information regarding change of hair washing routines between T1 and T2.

The median concentration of EtG at T1 was 100.0 pg/mg (range 7.7–1320) and the median concentration at T2 was 53.4 pg/mg (range < LOQ-692). There was a significant decrease in concentration of EtG from T1 to T2 ($p = 0.003$). There was still a significant decrease ($p = 0.013$) in EtG concentrations from T1 to T2 if excluding all the 10 patients reporting any kind of cosmetic hair treatment. There was a significant correlation between the level of EtG in hair T1 and EDI (Spearman's $\rho = 0.834$, $p < 0.001$). A significant correlation was also found for the level of EtG in hair T2 and EDI (Spearman's $\rho = 0.702$, $p < 0.001$).

Individual EtG concentrations of T1 and T2 related to EDI and the % change from T1 to T2 are shown in Table 1. The median values of EtG/EDI-ratio for T1 and T2 was 1.50 (range 0.09–4.40) and 0.75 (range 0–3.14), respectively. There was also a significant decrease in EtG/EDI ratios ($p = 0.001$) from T1 to T2.

Individual EtG values in hair segment T1 and T2 for all patients are shown in Table 1 and Figure 2. Of the 27 patients, 18 showed decreasing EtG concentrations from T1 to T2, and in six of these, the value at T2 was below LOQ. In another eight of the 27 patients, the EtG values were stable, or a smaller change was observed, that was considered within analytical variation (~20%). In one of the patients, a considerable increase in EtG hair concentrations from T1 to T2 was observed (patient 1, in Table 1). Nail samples from this patient (data not shown) from the same time period also showed increased levels of EtG and relapse could be suspected. The median change in EtG concentrations from T1 to T2 was −46.0% (mean = −9.7%). If excluding patient 1 from the analysis, the median change in EtG concentrations from T1 to T2 was −46.7% (mean = −48.4%).

There was no significant correlation between days of abstinence before inclusion in the study and the change in EtG concentrations from T1 to T2 (Spearman's $\rho = -0.011$, $p = 0.957$), and there was no significant correlation between length of hair segment at T1 and change in EtG concentrations from T1 to T2 (Spearman's $\rho = -0.188$, $p = 0.380$). In addition, there was no significant

**TABLE 1** Individual data of levels of ethyl glucuronide (EtG) in hair segment T1 (inclusion) and T2 (one month after inclusion), estimated daily intake of ethanol (EDI), abstinent period before inclusion, and percentage change from T1 to T2. LOQ = limit of quantification

| Patient No. | Hair EtG Segment T1 (pg/mg) | Hair EtG Segment T2 (pg/mg) | EDI[a] (g/day) | Cosmetic Hair Treatment Within 3 months Prior to T1. | Abstinent Period Before Inclusion (days) | No. of Days Between T1 and T2 | % Change From T1 to T2 |
|---|---|---|---|---|---|---|---|
| 1 | 14.2 | 156 | 51 | Coloring | 6 | 33 | 999[c] |
| 2 | 78.0 | 36.0 | 123 | None | 4 | 33 | −53.8 |
| 3 | 47.5 | 53.3 | 71 | None | 13 | 28 | 12.2[b] |
| 4 | 17.4 | <LOQ | 50 | None | 14 | 28 | Total loss |
| 5 | 100 | <LOQ | 51 | None | 12 | 29 | Total loss |
| 6 | 8.0 | <LOQ | 27 | Bleaching | 8 | 48 | Total loss |
| 7 | 12.0 | <LOQ | 8 | Unspecified treatment | 4 | 41 | Total loss |
| 8 | 54.5 | <LOQ | 57 | Coloring | 7 | 28 | Total loss |
| 9 | 7.7 | <LOQ | 85 | None | 11 | 41 | Total loss |
| 10 | 94.5 | 23.9 | 150 | Unspecified treatment | 5 | 33 | −74.7 |
| 11 | 40.4 | 53.4 | 50 | Unspecified treatment | 2 | 34 | 32.2[b] |
| 12 | 265 | 157 | 200 | NA | 3 | 32 | −40.8 |
| 13 | 59.6 | 37.8 | 28 | None | 13 | 32 | −36.6 |
| 14 | 31.1 | 16.8 | 17 | Coloring | 5 | 32 | −46.0 |
| 15 | 279 | 44.1 | 160 | None | 3 | 29 | −84.2 |
| 16 | 48.2 | 71.1 | 100 | None | 11 | 29 | 47.5[b] |
| 17 | 512 | 525 | 300 | None | 3 | 28 | 2.5[b] |
| 18 | 1320 | 692 | 300 | None | 2 | 28 | −47.6 |
| 19 | 640 | 175 | 200 | None | 8 | 28 | −72.7 |
| 20 | 121 | 82.2 | 350 | None | 9 | 28 | −32.1[b] |
| 21 | 244 | 277 | 150 | None | 11 | 28 | 13.5 [b] |
| 22 | 300 | 230 | 200 | None | 8 | 28 | −23.3[b] |
| 23 | 913 | 531 | 250 | None | 3 | 28 | −41.8 |
| 24 | 450 | 471 | 150 | None | 16 | 28 | 4.7[b] |
| 25 | 715 | 410 | 163 | Bleaching | 4 | 25 | −42.7 |
| 26 | 158 | 11.4 | 200 | None | 9 | 28 | −92.8 |
| 27 | 467 | 84.5 | 200 | None | 4 | 26 | −81.9 |

[a]Average over the past 90 days.

[b]% change from T1 to T2 within analytical variation (overlapping EtG levels T1 ± 20% and T2 ± 20%).

[c]Increased concentration and suspicion of relapse (increased nail EtG concentrations).

NA = not available

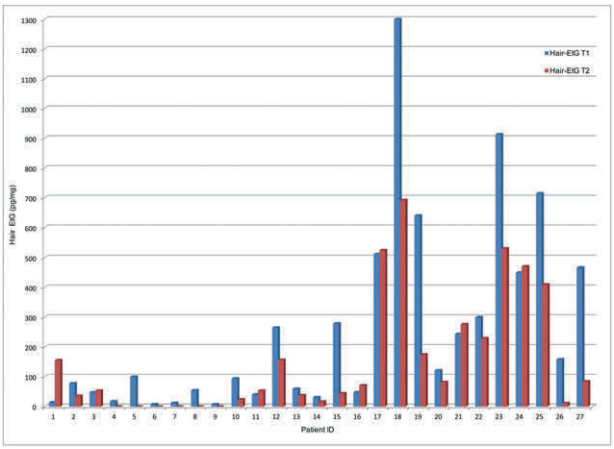


**FIGURE 2** Levels of EtG in hair segment T1 compared to segment T2 [Colour figure can be viewed at wileyonlinelibrary.com]

correlation between the reported frequency of hair washing and change in EtG concentrations from segment T1 to T2 (Spearman's $\rho = 0.162$, $p = 0.420$).

## 4 | DISCUSSION

The aim of this study was to use longitudinal hair data to evaluate EtG levels in two hair segments, representing the same period of alcohol intake, in hair samples collected with a one-month interval. EtG levels in hair segment T2 were significantly lower than in segment T1, although the two segments should roughly represent the same time of exposure.

The study results showed a median reduction of 46% in EtG concentrations from T1 to T2 and in 18 of 27 patients there were

declining EtG concentrations. A smaller change that was considered within analytical variation was observed in eight patients, and a substantial increase in EtG concentration was observed in one patient. Significant correlations between EDI and EtG were seen at both T1 and T2, but the correlation was weaker at T2.

According to the SoHT cut-offs,[2] 5 of 22 patients diagnosed with chronic excessive alcohol consumption (> 30 pg/mg) on basis of the T1 concentrations would instead be classified as patients with repeated alcohol intake (≥ 7, < 30 pg/mg) when interpreting the corresponding T2 concentrations. In 4 of 5 patients who would be diagnosed with repeated alcohol intake (≥ 7, < 30 pg/mg) on basis of T1, the T2 EtG concentrations was below 7 pg/mg. Our results therefore support the suggestion from SoHT of interpreting EtG in distal hair segments with caution.[2] The present study might also indicate that 6 cm segments, corresponding to the assumed consumption period of six months is too long for accurate interpretation of a hydrophilic substance like EtG.

According to a previous study, degradation of EtG during storage is a minor problem,[23] and the analytical washing procedures would expectedly remove EtG from sweat surrounding the hair, but not yet incorporated. There are, however, possible other mechanisms for the loss of EtG during the one-month interval from T1 to T2. First, EtG may decrease as a result of EtG molecules leaching from the hair matrix during the period. The poor incorporation of EtG into hair compared to lipophilic basic substances is known, and the question is to what extent EtG disappears as a result of wash-out by normal hair hygiene. Such EtG wash-out effects have been proposed in previous studies[13-16,24] as a mechanism of EtG elimination from hair. Potential wash-out of EtG in nail have also been proposed as a possible explanation in a previously published study[18] which showed that EtG disappeared from nails faster than it was otherwise assumed from nail growth only. Although that study used nail as a matrix, the incorporation and elimination of mechanisms is to some extent comparable to hair.

During normal hair growth when drinking is continued, the leaching of EtG from the hair matrix could possibly be balanced by contribution to incorporation from EtG containing sweat. In the present study, abstinence could lead to lack of such contribution, and the EtG-negative sweat could also possibly lead to an increased efflux of EtG from the hair, as suggested by Pianta et al.[24]

The mechanisms of incorporation might be important for the degree of wash-out effects. Incorporated substances are assumed to mainly enter hair by passive diffusion from blood capillaries into growing cells and also to some degree by diffusion from sweat and/or sebum into the completed hair shaft.[6,10] However, as previously indicated the hydrophilic and acidic properties of EtG make it more likely to be incorporated from sweat than from sebum.[9,11] Although the precise distribution of the EtG molecules in hair is not known for the different incorporation mechanisms, deposition of EtG by sweat may possibly lead to more EtG in the peripheral part of the hair (cuticle/cortex), while blood-incorporated EtG might lead to higher or equal concentrations in the center of the hair (medulla).[25] An important question is the reason for some subjects showing larger decreases in concentrations compared to others. Degree of wash-out effects will also be influenced by how available the incorporated EtG molecules are for wash-out. This could be caused by the quality of the hair, where hair damaged by mechanical stress becomes more susceptible for affection by cosmetic treatment.[6] Hair cosmetic treatment may also damage the hair cuticle, making it more permeable.[3] Excluding all patients reporting hair treatments ($n$ = 10) did not change the significant decrease of EtG concentrations from T1 to T2. Also, the routines for hair washing would expectedly influence wash-out. In the present study we did not find any significant correlation between hair-washing routines and decrease in EtG concentrations. This could be due to the fact that detailed information was not provided, but could also be influenced by the fact that we did not control the quality of the hair and to which extent EtG is available for wash-out. It could be speculated that the EtG values in the patients showing the largest decrease in concentrations were dominated by sweat incorporation, which was more available for wash-out effects, but the present study could not answer this question.

To our knowledge, no previous studies have used a longitudinal design to investigate change of EtG concentrations during abstinence. Some previous studies, however, have addressed the question through other study designs.[11,14-17] Appenzeller et al[17] studied 15 patients included in an alcohol treatment program and collected one hair sample per patient 0.5–4 months after cessation of drinking. For the majority of the patients they found a match between EtG concentrations in distal segments (up to 6–8 months old) and alcohol consumption history, but the significant correlation was only reported for the segments close to the time of drinking. The authors stated that their results indicated that EtG seems to be stable in hair for several months.

The study of Agius et al[11] used 4126 hair samples to compare the EtG results for the 3 cm hair lengths to the EtG values from shorter and longer hair lengths analyzed. They found no significant difference between the results obtained for the 3 cm hair samples and the results obtained for other hair samples of lengths greater than 3 cm and up to 12 cm. With the assumption of the same alcohol consumption over the investigated period, the authors concluded that this indicated that EtG wash-out effects seem to play a minor role in the interpretation of EtG. However, this study had no data on the alcohol consumption from the subjects, and would not have recognized if the subject for instance reduced their alcohol intake the past months prior to hair sample collection in order to regain their revoked driving license or regain other privileges.

A study of Tsanaclis et al[14] concluded that normal hair hygiene might wash out EtG from hair. The authors studied two sets of samples, one set ($n$ = 102) that was segmented into three 1-cm segments and one set ($n$ = 468) of hair samples that were only analyzed for the most recent segment. The hair samples received for testing were from a very heterogeneous group and no information about alcohol consumption was available. For the segmented samples there was a significant ($p < 0.05$) reduction in EtG concentrations from the first segment to the third segment, and the authors concluded that retrospective estimation of alcohol consumption over a period of many months is less useful on routine management of alcohol use.

Luginbühl et al[15] performed an in vitro experiment investigating the influence of chlorinated swimming pool water on EtG concentrations in hair in comparison to deionized water. The study showed decreasing hair EtG concentrations both after exposure to chlorinated swimming pool and to deionized water.

The study from Meier et al[16] also found decreasing EtG concentrations when comparing segments from seven hair samples taken at different locations on the head ($n$ = 1) after a period of constant drinking. The median ratio of EtG from proximal (0–1 cm) to distal (2–3 cm) segment was 2.0 (range 1.5–2.7), which is comparable to the EtG decline in the present study. The authors suggested wash-out effects as a possible explanation of the decreasing EtG concentrations.

The lack of longitudinal design in these studies implies some limitations when studying wash-out effects of EtG in hair after cessation of drinking. However, our results from a study with a longitudinal design support the findings by Tsanaclis et al,[14] Luginbühl et al,[15] and Meier et al[16] that EtG concentrations in hair might decrease as a result of wash-out.

A weakness of the present study is the relatively low numbers of subjects that participated. In addition, the mean hair growth is subjected to several variables, and the variations in concentration from T1 to T2 may be influenced by an inhomogeneous growth rate as a result of the presence of catagen and telogen growth phase of the hair. The assumption of the 0–1 cm segment representing the last month is therefore a simplification, but reflects the real life interpretation. Also, the assumption of 1 cm per month hair growth is a well-accepted condition when interpreting EtG in hair.[4] A possible negative bias as a result of different abstinence periods and time between the two sample collections may have affected the results in the present study. Another weakness is that several factors, such as amount and time of alcohol intake, hair washing, and treatments, could not be fully controlled. EDI represents the average alcohol for the past three months and under-reporting of alcohol consumption has to be taken into consideration when interpreting the reported EDI values. EtG analysis in the present study was done after cutting hair into small pieces and not after pulverization of the hair samples. Extraction of EtG could have been optimized by pulverization technique, but we assume this would influence the change in concentrations from T1 to T2 only to a minor degree.

The strength of this study was the use of a population from a real-life clinical setting and the possibility to study the change of EtG levels about four weeks after the first hair sample. To our knowledge, this type of longitudinal study design has not been used in previous publications to study EtG concentrations in hair in an abstinent period among alcohol-dependent subjects. Analyzing two different hair segments after drinking cessation in consecutive hair samples, assumed to represent roughly the same period of alcohol intake, provides important information about the stability of EtG in hair and whether hair EtG levels in more distal hair segment could reflect the alcohol intake during that hair growth period or not. In the present study, it was not possible to analyze even more consecutive samples after one month; however, future studies should address this issue.

## 5 | CONCLUSION

We found that hair samples collected at the start and one month after alcohol abstinence showed a reduction in EtG concentrations in most of the subjects incorporated to the study, despite the fact that the segments analyzed represented roughly the same period of alcohol consumption. The study results represent a real-life clinical setting and uncontrolled factors are present. Our results support the suggestion from SoHT to interpret EtG in distal hair segments with caution. Further studies on the mechanisms of wash-out effects are needed to fully understand the limitations when analyzing different segments and the potential clinical importance of this mechanism.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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