Diagnosis of fetal alcohol spectrum disorder (FASD): fatty acid ethyl esters and neonatal hair analysis

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Summary. Measuring levels of fatty acid ethyl esters (FAEE) in hair has been recently shown to discriminate between adult heavy and non-drinkers. Here, we review the potential of neonatal FAEE measurement in detecting infants exposed to alcohol *in utero* by outlining current progress in the development of a neonatal hair test for the diagnosis of fetal alcohol spectrum disorder (FASD). Developing a reproducible, accurate and predictable hair test for FAEE measurements in neonatal hair may prove to be a powerful tool in the detection of *in utero* alcohol exposure which is needed for the diagnosis of FASD. Such a neonatal hair test can revolutionize current FASD diagnostic methodology by providing early diagnosis, allowing intervention and treatment at stages where the adverse effects of alcohol can still be mitigated.

Key words: fetal alcohol spectrum disorder, *in utero* alcohol exposure, neonatal hair testing, alcohol biomarkers, fatty acid ethyl esters.

Riassunto *(Diagnosi di fetal alcohol spectrum disorders: etilesteri degli acidi grassi e analisi nei capelli di neonato)*. Di recente è stato dimostrato che la determinazione quantitative di etil esteri degli acidi grassi (FAEE, fatty acid ethyl esters) nei capelli può discriminare tra adulti forti bevitori e non bevitori. In questo lavoro viene presa in considerazione la determinazione di FAEE nei capelli di neonati per riconoscere i bambini esposti ad alcol durante la gravidanza e vengono presentati i più recenti progressi in campo analitico. Lo sviluppo di un metodo affidabile per la determinazione di FAEE nei capelli del neonato potrebbe costituire un mezzo estremamente efficace per la rilevazione obiettiva di esposizione *in utero* necessaria per la diagnosi di fetal alcohol spectrum disorders (FASD). Ciò può innovare l'attuale metodologia diagnostica e rendere possibile una diagnosi precoce permettendo intervento e trattamenti adeguati in uno stadio in cui gli effetti negativi dell'alcol possono ancora essere limitati

Parole chiave: fetal alcohol spectrum disorder, gravidanza, alcol, capelli, analisi, biomarcatori.

INTRODUCTION

Prenatal alcohol exposure is associated with a wide spectrum of adverse effects known as fetal alcohol spectrum disorder (FASD). Diagnosing an infant who has been exposed to alcohol *in utero* can be an extremely difficult task since often, the effects of gestational drinking on the fetus may not be clinically evident at birth or shortly thereafter. In less apparent cases of FASD where no physical signs have manifested, (*e.g.*, the alcohol related neurodevelopmental disorder, ARND), children exposed to ethanol during pregnancy may go undetected until the adverse effects of impaired brain growth become evident [1].

Diagnosis of FASD is difficult since this requires in most cases positive confirmation of heavy maternal drinking. According to current diagnostic guidelines [2], without the distinctive pathognomonic facial features seen in fetal alcohol syndrome (FAS), confirmation of *in utero* alcohol exposure is required. Admission to gestational drinking, especially of addictive patterns, may not be the most accurate information source for *in utero* ethanol exposure [3]. Maternal self-reporting is often unreliable because of the countless stigmas associated with a pregnant mother's admission to risky behaviours [3]. Questionnaires, such as the TWEAK and T-ACE, have been developed to facilitate a physician's ability to screen their pregnant patients for problem drinking. Unfortunately, the effectiveness of these tests is dependent on frank maternal reports [3].

In attempting to find an accurate method to detect problem drinking in pregnancy, laboratory biochemical blood markers have often attempted alone or in combination to identify alcohol consumption. Immediately following ingestion, ethanol can be measured in blood, breath or urine. However, using ethanol itself or its aldehyde can only indicate recent exposure due to their relatively rapid metabolism and lack of appreciable accumulation for long periods of time. The association between gestational alcohol consumption and maternal biochemical markers such as gamma-glutamyl transferase (GGT), mean corpuscular volume (MCV), haemoglobin-associated acetylaldehyde (HAA) and carbohydrate deficient transferrin (CDT) have some potential, but many of these tests are unavailable in

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most settings [4]. Studies evaluating the effectiveness of these biochemical markers in pregnancy are still very limited and to date, no single laboratory test exists that is sufficiently reliable for the identification of heavy gestational drinking.

Unlike biochemical blood markers, hair analysis is often used as a tool for the retrospective detection of illicit and/or therapeutic drug exposure over a prolonged time period. It has been successfully described for detection of cocaine, marijuana, nicotine, opiates and amphetamine use [5]. The parent compounds and their metabolites are deposited in the cortex of the hair shaft through the blood stream [6]. Hydrophobic drugs tend to accumulate significantly more into the hair shaft and remain for the life of the hair or until cut [7].

In typical hair analysis, substances are extracted from the hair shaft and the hair extract is screened using an immunoassay. Results are then confirmed by gas chromatography/mass spectrometry (GC/MS). Hair may also be dissolved and compounds detected by radioimmunoassays (RIA) [6].

Since many drugs of abuse are retained in hair for prolonged periods of time, maternal hair analysis is a useful method for monitoring drug use in pregnancy [8]. This technique has also been performed successfully in the neonate to confirm suspected *in utero* exposures to such drugs as nicotine and cocaine [7, 9]. Neonatal hair begins to grow at approximately six to seven months of fetal life [6]. Thus, any exposures within the last trimester of pregnancy may be theoretically found in neonatal hair after birth.

Ethanol is a highly volatile compound and hence, it is not retained within the hair matrix. When looking for a possible hair biomarker for *in utero* alcohol exposure, candidates must have the capability of accumulating sufficiently within the hair shaft; ethyl glucuronide, phosphatidylethanol, cocaethylene, acetylaldehyde adducts to protein and fatty acid ethyl esters (FAEE) are the current main contenders.

Until recently, lack of neonatal biological markers for *in utero* exposure to ethanol has severely limited the ability of physicians to appropriately diagnose FASD. FAEE are products of the non-oxidative metabolism of ethanol and have been proposed as biological markers of acute and chronic exposures to alcohol in adults due to their long half-life in blood and their ability to accumulate within various biological matrices [10, 11].

The following is a review of the usefulness of FAEE in detecting infants exposed to alcohol *in utero*, outlining current progress in the development of a neonatal hair test for the diagnosis of FASD. Developing a reproducible, accurate and predictable hair test for FAEE measurements in neonatal hair may prove to be a powerful tool in the detection of *in utero* alcohol exposure leading to the subsequent diagnosis of FASD in many infants. A neonatal hair test to identify infants exposed to alcohol *in utero* can revolutionize current FASD diagnostic methodology and provide early diagnosis, allowing intervention and treatment at stages where the adverse effects of alcohol can still be mitigated.

FATTY ACID ETHYL ESTERS

FAEE are non-oxidative metabolites of ethanol. They are formed via the esterfication of ethanol with endogenous fatty acids or fatty acyl-CoA *(Figure 1)*. Two main enzymes are involved in FAEE formation: FAEE synthase and acyl-coA:ethanol O-acetyltransferase (AEAT). FAEE synthase is present in almost all human tissue with the highest levels reported in the pancreas [12, 13]. Studies have shown that AEAT activity can be several-fold higher than FAEE synthase activity in many human organs and tissues, including the heart, liver, duodenal mucosa, lung, adipose tissue and gall bladder [10].Only pancreatic activities of AEAT and FAEE synthases have been found to be comparable [14]. Fatty acids most recently incorporated into the cell are preferred as substrates for FAEE synthesis [15].

In plasma, albumin transports the majority of FAEE [16]. Fatty acids have a higher affinity for albumin and effortlessly displace FAEE. Once displaced, free FAEE are readily and extensively broken down by a variety of cellular structures in the blood, such as white blood cells. Substantial degradation also takes place in the liver and pancreas [14].

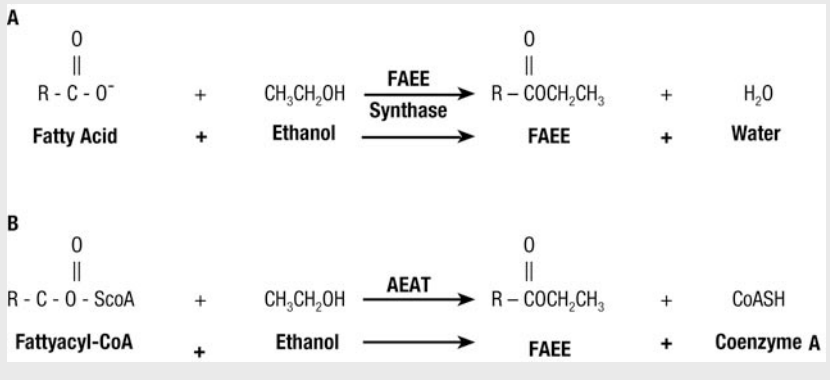

Fig. 1 | *Formation of FAEE.*
A. *FAEE synthase pathway using fatty acids as a substrate for FAEE production.*
B. *AEAT pathway using acyl-CoA as a substrate for FAEE production.*

Unlike ethanol, FAEE persist in blood for more than twenty-four hours after significant alcohol consumption and have been found to concentrate in adipose tissue quite readily (half-life = 16.5 hrs) [17, 18]. FAEE are highly hydrophobic and have been found to concentrate in organs commonly damaged by chronic alcohol abuse. Consequently, they have been implicated as mediators of alcohol-induced organ damage [19]. *In vitro*, FAEE have the ability to uncouple oxidative phosphorylation by binding to cell mitochondrial structures. Furthermore, they are able to disrupt the membrane bilayer, increase lysosomal fragility and decrease protein synthesis, showing a significant capacity to induce cell death [20].

There are some theories that FAEE may also play an important role in the development of FAS. Studies have indicated that human and mouse placentae have substantial amounts of FAEE synthase activity [21]. These same investigators also noted that mouse heart, liver, placenta and fetal tissues accumulate ample amounts of FAEE following maternal ethanol exposure. Additionally, FAEE have the ability to persist in placental tissue for up to 7 days in mouse placenta models [21]. Thus, the potentially toxic effects of FAEE may also extend into the neonate.

ANALYSIS OF FAEE IN MECONIUM

FAEE have the ability to concentrate in meconium, a matrix unique to the developing fetus which has been widely used in neonatal screening for *in utero* substance exposures [8]. The deposition of drugs in meconium begins at the 13th week of human gestation. Hence, unlike blood and urine samples, this cumulative matrix can yield a detailed history of fetal exposure in the second and third trimesters. Recent studies have been able to document significantly higher levels of FAEE in the meconium of neonates of self-reporting heavily drinking mothers when compared to non-drinking controls [22]. Ethyl palmitate (E16), ethyl oleate (E18:1), ethyl stearate (E18), and ethyl linoleate (E18:2) were the predominant FAEE detectable in the meconium of neonates exposed to excessive amounts of alcohol *in utero* [23].

These findings have documented the promise of using FAEE levels as a powerful biological marker of *in utero* ethanol exposure. One of the issues concerning meconium analysis, however, is that meconium exists only during the first three post-natal days. Consequently, diagnosis of maternal drinking may be missed thereafter. Unlike meconium, neonatal hair collection can occur up to 3 months after birth, at which point neonatal hair typically sheds. Thus, measuring FAEE in neonatal hair rather than meconium may increase the window of opportunity to confirm *in utero* alcohol exposure.

FAEE ACCUMULATION IN HAIR

Although much potential may exist for other biological hair markers to identify *in utero* alcohol exposure, presently none show as much promise and reliability as FAEE. As a result of their hydrophobic nature, FAEE have the potential to accumulate significantly into the hair and remain for the life of the hair or until it is cut [7]. Pragst and colleagues have documented increased FAEE concentrations in the hair of adult alcoholics [24, 25]. Using headspace solid-phase micro extraction (HS/SPME) and gas chromatography-mass spectrometry (GC/MS), they succeeded in developing a reliable and sensitive method for the routine analysis of myristic (E14), E16, E18, and E18:1 in adult hair. Detection limits (LOD) ranged between 0.03 and 0.13 pmol/mg of hair with an assay reproducibility between 3.5 and 16% [2, 26]. E16 and E18:1 were found in the highest concentrations, with means of 5.94 and 7.08 pmol/mg of hair, respectively. In contrast, hair taken from children and teetotallers failed to yield detectable levels of FAEE. For social drinkers, defined as an alcohol consumption of approximately 2 to 4 standard drinks per week, levels of FAEE were much lower than what was seen in alcoholic samples, with maximum E16 and E18:1 levels of 1.40 and 1.03 pmol/mg. These results have provided solid evidence that the accumulation of FAEE in hair may be dose-dependant and that the measurement of FAEE concentrations in hair can be used as biological markers for excessive alcohol consumption in adults.

USING FAEE AS BIOLOGICAL MARKERS IN NEONATAL HAIR TO DETECT *IN UTERO* ALCOHOL EXPOSURE

A case study using the method described by Pragst *et al*. has revealed that FAEE are present in the hair of neonates exposed to alcohol prenatally. Klein *et al*. have documented the presence of measurable levels of FAEE in the hair of an admitted gestational drinker and her neonate [22]. Both maternal and newborn hair were positive for FAEE, at 2.6 and 0.4 pmol/mg respectively. This case study was the first to suggest that neonatal hair analysis of FAEE may hold much promise as a potential biomarker for *in utero* alcohol exposure.

Following these preliminary evidence, our laboratory has been extensively involved in establishing a valid method for the measurement of FAEE in neonatal hair. In investigating the possibility of using FAEE levels in neonatal hair as a means to identify gestational drinking, studies involved assay development, analysis of FAEE stability within the hair matrix, animal studies and baseline establishment of FAEE in neonates born to non-drinking mothers.

Assay development

In developing an assay for the measurement of FAEE in neonatal hair, one of the main challenges was to ensure the assay was sufficiently sensitive to detect the low levels of FAEE that were expected in this type of sample. To date, we have successfully created such a an assay using solid-phase extraction (SPE) with GC/MS in chemical ionization (CI) mode. This method was modified from our meconium assay and a GC/MS assay currently implemented by Pragst *et al*. [25, 27]. Briefly, 10 to 20 mg of washed and dried, hair samples were cut into pieces of about 1 mm length and extracted overnight with a mixture of 0.5 ml of dimethylsulfoxide, 4.0 ml hexane and 100 µl of heptadecanoic as an internal

Table 1 | *LOD and LOQ for each individual FAEE included in neonatal hair assay*

FAEE	LOD (pmol/mg)	LOQ (pmol/mg)
Lauric (E12)	0.022	0.055 – 0.219
Myristic (E14)	< 0.010	0.010 – 0.049
Palmitoleic (E16:1)	0.018	0.044 – 0.177
Palmitic (E16)	< 0.009	0.009 – 0.044
Oleic (E18:1)	< 0.008	0.008 – 0.040
Stearic (E18)	< 0.008	0.008 – 0.040

standard for the quantification of FAEE. The hexane layer was then collected and SPE performed. Following a final reconstitution in 50 µl of hexane, samples were analysed for six FAEE using GC/MS/CI with isobutene as our ionizing gas. Extraction efficiencies range from 40% to 73% while the LOD ranged from 0.008 to 0.084 pmol/mg for individual esters *(Table 1)*.

SPE has been extensively used in the isolation of FAEE from matrices such as blood, tissue and meconium [19, 27, 28]. This is the first time it has been used in isolating FAEE from hair. In addition, CI is a "softer" ionization mode which causes less fragmentation of FAEE upon impact. In choosing CI rather than EI for our analysis, we have been able to develop a method with analytical limits 4-fold lower than those found in current methods [24, 25]. We have also expanded the FAEE analysis profile to six rather than four esters, choosing to include lauric (E12), E14, E16, palmitoleic (E16:1), E18, E18:1 in our FAEE analysis *(Table 2)*. There is a growing body of evidence to suggest that the cumulative analysis of all commonly occurring FAEE in biological matrices rather than the analysis of one specific species of FAEE is a more accurate way to identify neonates with suspected prenatal exposure to ethanol [29]. Thus including more FAEE in the analysis of hair may have increased the predictive value of this test to identify individuals, adults and neonates, who have been exposed to heavy levels of alcohol.

Using this assay, hair samples from three heavy drinkers (defined by a weekly alcohol consumption of more than 9 standard drinks for females and 14 standard drinks for males) and three non-drinkers were analysed. The average amount of FAEE quantified in our drinking cohort was 6.33 ± 1.03 pmol/mg while only trace amounts of FAEE were measured in the hair of our no drinking cohort, indicating that the current assay is able to discriminate between adult heavy drinkers and non-drinkers.

Stability of FAEE in the hair matrix

Recent studies in our laboratory have found traces of FAEE in hair samples taken from adult Peruvian and Chilean mummies dating back to 1000-1250 AD [30]. Quantifiable FAEE included E16, E16:1, E18, and E18:1 while the mean cumulative FAEE concentration was 0.773 ± 1.136 pmol/mg. Their presence indicates that FAEE are highly stable within the hair matrix and may be useful as long-term biological markers for alcohol exposure. Similar stability has been documented in mummy hair for cocaine and its metabolite, benzoylecognine [31, 32]. Studies have indicated that FAEE have no general tendencies to deteriorate over several days in refrigerated meconium samples [33]. Given their stability in an enzymatically active matrix such as meconium, it is not surprising that FAEE exhibit prolonged stability in an enzymatically innate matrix like hair.

Animal studies

In our attempts to establish whether FAEE accumulate in neonatal hair in a reproducible and predictable manner after chronic prenatal ethanol exposure, we used guinea pigs as an experimental animal to investigate the potential of FAEE in neonatal hair (Caprara *et al.*, *Pediatr Res*). Guinea pigs were chosen as an ideal animal model since, similar to humans and unlike rats and mice, pups are born with hair. The pharmacokinetics of ethanol in pregnant guinea pigs are well established with measured maternal ethanol blood concentrations corresponding to those shown in alcoholic mothers [34-36]. Furthermore, this model has been successfully used in studying ethanol teratogenesis [37-39]. Thus, this animal model of gestational alcohol exposure can provide needed insight into the feasibility of using FAEE in neonatal hair as potential biomarkers for *in utero* alcohol exposure.

Pregnant guinea pigs were dosed daily throughout their pregnancy with chronic maternal ethanol regimens mimicking a binge-type drinking pattern with an apparent blood-alcohol concentration (BAC) of 260 mg/dl, more then twice the legal BAC limit in Canada. It is a dosing regimen shown to cause fetal neurotoxicity in the guinea pig [40]. Dosing began on gestational day (GD) 2 through

Table 2 | *Individual FAEE molecular weights, retention times and quantification/qualifier ions*

FAEE	Molecular weight (g/mol)	Approx. retention time (min)	Ions
Lauric (E12)	228.4	7.55	***229***, 227
Myristic (E14)	256.4	8.71	***257***, 255
Palmitoleic (E16:1)	282.5	9.66	***283***, 284
Palmitic (E16)	284.5	9.75	***285***, 286
Heptadecanoic IS	298.5	10.25	***299***, 300, 297, 298
Oleic (E18:1)	310.5	10.60	***311***, 312
Stearic (E18)	312.5	10.70	***313***, 314

Quantification ions are expressed in bold italic.

GD 67 and hair samples were taken from pregnant mothers at GD 57 and 65, as well as from offspring at postnatal day (PD) 1 and 10. Isocaloric sucrose and water control animals were used as a means for comparison.

Elevated levels of FAEE were repeatedly measured in the hair of chronically dosed pregnant guinea pigs and their pups in comparison to their respective controls. Overall, ethanol treated mothers had 10-fold higher levels of FAEE compared to their controls while ethanol exposed pups had a 15-fold higher cumulative FAEE level than their sucrose and water counterparts (Caprara *et al.*, *Pediatr Res*). Our results verify for the first time that chronic exposure to alcohol leads to increased levels of FAEE in both maternal and neonatal hair in guinea pigs. The documented presence of FAEE in the hair of neonatal guinea pigs provides evidence that FAEE do have the ability to accumulate to significant concentrations in the hair matrix of neonates exposed to heavy amounts of alcohol *in utero*. These data suggest that using FAEE in neonatal hair as biomarkers for gestational alcohol exposure in humans is feasible and may be useful in identifying children exposed to alcohol during pregnancy.

The use of an experimental animal model overcomes the most critical challenge of human studies extending the use of FAEE into neonatal hair; the reliance on maternal reports of alcohol consumption and drinking schedules. By using an animal model, one can administer a highly controlled dose of ethanol and have accurate account of prenatal alcohol exposure. Such a controlled environment has allowed us to evaluate the detailed relationship between FAEE levels in hair and ethanol exposure. We have been able to shed light on the full potential of FAEE as biological hair markers for *in utero* alcohol exposure. Future studies will employ this novel model to establish the full dose-response curve between steady-state maternal alcohol blood concentrations and neonatal outcomes (*e.g.*, neurobehavioral).

Human studies: baseline establishment of FAEE in neonatal hair

Preliminary baseline studies indicate that certain FAEE can be found in the meconium and hair of neonates without daily or prenatal alcohol exposure [27]. The reason for this phenomenon is unclear but may be due to physiologic and pathologic conditions. Ethanol is a common by-product of routine physiologic metabolism in the human gut [41]. Small quantities of alcohol may also be present in medications and food additives. Furthermore, fatty acid alkyl esters, mostly ethyl and methyl esters, are naturally present in different types of olive oil [42]. Thus, it is not unreasonable to expect endogenous levels of FAEE to exist that may originate from sources other than alcohol consumption.

To account for endogenous production and accumulation of FAEE in neonatal hair, a baseline study was conducted using samples taken from non-drinking and socially drinking mothers and their neonates (Caprara *et al.*, *Ther Drug Monit)*. All drinking patterns were social. Fifty-six neonates up to 2 months of age were enrolled in the study and their hair was analysed for FAEE. Our results demonstrated that a baseline level of FAEE exists in the hair of neonates born to non-drinking mothers. No significant differences in total FAEE concentrations were found between neonates born to gestational drinkers and non-drinkers. As a group, the mean (± SEM) level of FAEE measured in neonatal hair was 0.321 ± 0.088 pmol/mg with levels ranging from 0.000 to 2.953 pmol/mg. Within the ranges of alcohol consumption seen in this study population, quantity, time and duration of gestational alcohol exposure did not have a significant effect on total FAEE concentrations found in neonatal hair. Similar baseline results were found in meconium samples taken from infants born to non-drinking mothers [27]. As shown above, data from guinea pig model also demonstrated baseline concentrations of FAEE in hair taken from sucrose and water control pups that were not exposed to ethanol *in utero*. It is evident that measurable levels of FAEE are present in humans not actively consuming alcohol. Consequently, it is critical to define such a baseline level before FAEE levels in neonatal hair can be used to accurately identify infants exposed to alcohol *in utero*.

Data collected from our baseline cohort indicated that 39% of the women in our population consumed any amount of alcohol at some point throughout gestation *(Table 3)*.

Table 3 | *Drinking patterns among women in baseline study*

Number of women with a history of alcohol use	40 (65%)
Total number of gestational drinkers	24 (39%)
Total number of drinks in pregnancy among gestational drinkers (mean±SD)	8.7±12.9
Range	0.5-56
Number of women drinking in T_1	16 (66%)*
Number of women drinking throughout pregnancy (T_1 - T_3)	5 (21%)*
Drinking by trimester:	
T_1	8 (33%)*
$T_1 + T_2$	1 (4%)*
$T_1 + T_3$	2 (8%)*
$T_2 + T_3$	3 (15%)*
T_3	5 (21%)*

** % of gestational drinkers*

No difference in FAEE concentrations in neonatal hair was seen between these gestational drinkers and those women who abstained completely from alcohol throughout their pregnancy. All of the gestational drinkers were categorized as mild, social drinkers. Our assay may not be able to discriminate between neonates unexposed to alcohol *in utero* from neonates exposed to mild levels of alcohol throughout gestation. Nonetheless, if a neonatal hair sample is analyzed and FAEE levels are found to be significantly higher than our baseline cohort (drinker and non-drinkers), this may be an indication that maternal drinking during pregnancy was at a heavier, problematic level; a consumption level whereby the toxic effects of *in utero* alcohol exposure may be much more prevalent.

In our sample population, 26% of women admitted to drinking small amounts of alcohol in the second and/or third trimester, a period of gestation when hair grows *in utero*. This constitutes the first evidence that mild, rare drinking in the second and third trimester will not lead to a significant accumulation of FAEE in neonatal hair above baseline levels.

Future challenges

The collection of drinking history using a standardized questionnaire is a major limitation to any study, as maternal admission to drinking in pregnancy is an unreliable and inaccurate method to identify *in utero* alcohol exposure [3]. We are assuming the women in our study cohort were truthful about their ethanol ingestion throughout pregnancy. However, recall bias, guilt and embarrassment may affect our data on gestational alcohol consumption.

Although a preliminary baseline for FAEE levels in neonatal hair has been established, much work is still needed to validate the use of FAEE in neonatal hair as potential biomarkers for *in utero* alcohol exposure. Using a guinea pig animal model where the chronic maternal ethanol regimen mimics the binge-type drinking pattern found in humans, we have shown that the use of a neonatal hair test to identify prenatal ethanol exposure is feasible. To properly validate such a test in humans and to determine clinical sensitivity and specificity of the assay, hair samples from confirmed heavy gestational drinkers and their neonates are critical to define a positive FAEE level screening cut-off in hair. Until such studies are conducted, expected FAEE levels in hair samples taken from this heavy drinking population will remain unknown.

When using hair analysis for any type of drug testing, there are other issues that must also be addressed. Studies have shown that for certain compounds like nicotine and cotinine, hair properties, such as colour and texture, may influence drug incorporation and distribution into hair [43]. Chemical treatments, such as hair dyes and perms can damage the structural integrity of the hair shaft and may further affect drug accumulation and detection [44]. As the use of FAEE levels in hair increases, it will be necessary to investigate such issues that may influence the incorporation of FAEE into both adult and neonatal hair.

CONCLUSIONS

The development of a reliable biomarker of alcohol consumption during pregnancy is a critical step in allowing early diagnosis of FASD. Diagnosing FASD is a difficult task, especially in cases where characteristic physical facial abnormalities have not manifested.

FAEE have shown much promise as the first useful biomarkers for *in utero* alcohol exposure and have been used successfully in meconium to identify alcohol-exposed infants. Meconium, however, is a short-lived species. If collection is not performed days after birth, meconium is lost and so is the opportunity to confirm alcohol consumption during pregnancy in cases where maternal admission is unreliable or unavailable. By extending the use of FAEE into neonatal hair, we may extend the window of opportunity to collect samples up to 3 months postpartum, thus increasing our ability to identify children exposed to alcohol *in utero*.

We have successfully developed a sensitive, specific and reliable assay for the measurement of FAEE in hair able to discriminate adult non-drinkers from heavy drinkers. FAEE are highly stable within the hair matrix and that baseline, endogenous levels of FAEE exist, independent of active alcohol exposure in both guinea pig and human neonatal hair.

Using this novel assay, we have confirmed for the first time that chronic exposure to alcohol leads to increased levels of FAEE in both maternal and neonatal hair in guinea pigs. This provides solid evidence that using FAEE in neonatal hair as biomarkers for *in utero* alcohol exposure is feasible and may be useful in identifying children exposed to alcohol during pregnancy.

Our studies have served in extending the use of FAEE into neonatal hair to objectively identify children exposed to alcohol *in utero*. Such advancement will revolutionize a physician's ability to diagnose FASD and will facilitate the early diagnosis of FASD, providing intervention at stages where the effects of prenatal alcohol exposure can be minimized and/or prevented.

Acknowledgements

Supported by grants by CIHR (NET grant and MT 1787). DC was recipient of an open studentship from The University of Toronto. G. Koren is a Senior Scientist of CIHR and holder of the Ivey Chair in Molecular Toxicology, The University of Western Ontario.

Submitted on invitation
Accepted on 9 December 2005.

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