ETHYLGLUCURONIDE AND ETHYLSULFATE IN MECONIUM TO ASSESS GESTATIONAL ETHANOL EXPOSURE: PRELIMINARY RESULTS IN TWO MEDITERRANEAN COHORTS

Simona Pichini[1], Luca Morini[2], Emilia Marchei[1], Ilaria Palmi[1], Maria Concetta Rotolo[1], Federica Vagnarelli[3], Oscar Garcia-Algar[4], Oriol Vall[4], Piergiorgio Zuccaro[1]

[1]Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanitá, V.le Regina Elena 299, 00161 Roma, Italy; [2]Department of Legal Medicine and Public Health, University of Pavia, Via Forlanini 12, Pavia, 27100 Pavia, Italy; [3]Arcispedale Santa Maria Nuova, Reggio Emilia, Italy; [4]Unitat de Recerca Infància i Entorn (URIE), Paediatric Service, Hospital del Mar, Barcelona, Spain

ABSTRACT

Background
In recent years, fatty acid ethyl esters (FAEEs) in meconium emerged as reliable, direct biological markers for establishing gestational ethanol exposure. Among the minor nonoxidative products of ethanol metabolism, there are ethyl glucuronide (EtG) and ethyl sulfate (EtS).

Objectives
The aim of the study was to analyse meconium specimens from two different Mediterranean cohorts to check for the presence of EtG and EtS, and to investigate the eventual correlation between meconium FAEEs and these two metabolites and their possible application as direct biomarkers of gestational ethanol exposure.

Methods
FAEEs, EtG and EtS were quantified by liquid chromatography tandem mass spectrometry in meconium samples obtained from the Neonatal Intensive Care Unit of Arcispedale Santa Maria Nuova, Reggio Emilia, Italy (N= 96) and from the Pediatric Service of the Hospital del Mar in Barcelona, Spain (N=81).

Results
EtG was present in more than 80% meconium samples while EtS only in 50% specimens Although the samples from Spain and Italy originated from similar socio-demographic cohort, EtG values in the Barcelona samples (median value: 101.5 ng/g) were statistically higher than those from Reggio Emilia ones (median value: 15.6 ng/g). In the Barcelona cohort, EtG values could differentiate between samples with FAEEs below and those equal or above 2 nmol/g - the cut-off used to differentiate heavy maternal ethanol consumption during pregnancy from occasional or no use.

Conclusion
For the first time the presence of EtG and EtS in meconium has been proven, with EtG concentration likely to discriminate heavy maternal ethanol consumption during pregnancy disclosed by FAEEs concentration in this matrix. Further investigations are needed to verify the use of these two ethanol metabolites as alternative biomarkers of chronic *in utero* exposure to ethanol.

Keywords: *Meconium, ethylglucuronide, ethylsulfate, FAEEs, gestational ethanol consumption*

In recent years, fatty acid ethyl esters (FAEEs) in meconium emerged as reliable, direct biological markers for establishing gestational ethanol exposure.[1-5] After maternal ingestion, ethanol readily crosses the placenta and within minutes reaches levels in the fetus and amniotic fluid that are nearly equivalent to maternal blood ethanol levels.[6] Among the minor nonoxidative

products of ethanol metabolism, there are ethyl-β-glucuronide (EtG) and ethyl sulfate (EtS). EtG is formed by the ethanol conjugation with glucuronic acid and EtS by the transfer of a sulfuric group from 3'-phosphoadenosine-5'-phosphosulfate to ethanol accomplished by the mitochondrial UDP-glucuronosyltransferase and sulfotransferases, respectively.[7] The order of magnitude of glucuronation is similar to that of sulfation, and it is in the range of 0.02-0.06% total ethanol intake.[7,8]

EtG and EtS have been regarded as high-specificity mid-term (1-80 h) markers of ethanol intake in conventional biological fluids such as urine or blood.[9] The maximum concentration of EtG and EtS in blood are reached 2-3.5 h after ethanol peak and are detectable up to 4-8 h after ethanol elimination.[10,11] Kidney failure however may lead to a significant delay in the excretion time.[12] In urine, EtG is detectable from 1 hour after ethanol intake and up to 22.5-31.5 h.[13] EtS elimination profile in urine is of the same order as that of EtG. Its time window of detection is 16-27 h.[14] Hair testing for EtG has been applied to assess long-term chronic ethanol consumption.[15] However, neither EtG nor EtS have been ever investigated in meconium as possible biomarkers of prenatal chronic exposure to maternal ethanol.

Meconium is the first fecal matter passed by a neonate. Its formation starts between the 12th and 16th week of gestation and usually accumulates in fetal bowel until birth and it is passed by the neonate one-five days after birth. For this reason, compared to urine, meconium analysis extends the window of detection of drug use to approximately the last 20 weeks of gestation, being more informative than urine for the detection of drug exposure in pregnancy.[16,17]

Recently we developed and validated a sensitive and selective analytical method based on LC-MS/MS for the determination of EtG and EtS in meconium.[18] The results presented for EtG and EtS analysis in specimens from two different Mediterranean study cohorts were also analysed for the seven FAEEs (palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acid ethyl esters). These were used to differentiate heavy maternal alcohol consumption during pregnancy from occasional use or no use at all.[1,5] Finally, we propose suggestions for further investigations.

POPULATION AND METHODS

Meconium samples were obtained from the Neonatal Intensive Care Unit of Arcispedale Santa Maria Nuova, Reggio Emilia, Italy (N= 96) and from the Pediatric Service of the Hospital del Mar in Barcelona, Spain (N=81). The mother-infant dyads from Reggio Emilia (a small town in Northern Italy) and Barcelona (the second most important Spanish city) recruited for this study were similar in maternal sociodemographic and ethnic characteristics and in newborns clinical parameters and somatometry (Table 1). Both groups of samples were collected in the same period of time, aliquoted and stored in the same conditions, and analyzed in subsequent batches, which contained samples from the two hospitals. A structured questionnaire for gestational ethanol consumption was submitted to pregnant women. Although the most recent Spanish National Survey on Drug abuse reported daily ethanol intake in 47.3 and 54.7% of women aged 15-34 and 35-64 respectively[19], and the Italian survey in 5.1 and 21% of women from similar age groups[20], more than 95% of the mothers in both study groups declared not to drink at all during pregnancy.

General information on consumption of tobacco and drugs of abuse could be obtained from medical records. The study was approved by the Institutional Ethical Committees of both Hospitals, conducted in accordance with the Declaration of Helsinki and signed informed consent was obtained from the parents of the newborns. Meconium, collected within the first 24 h, was immediately stored in different aliquots at –20°C until analysis. An aliquot of meconium was analysed for EtG and EtS by a validated liquid-chromatography tandem mass spectrometry method.[18] A second aliquot was analysed with another liquid-chromatography tandem mass spectrometry method for palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acid ethyl esters.[21] Mann Whitney U-test for non parametric distribution was used to compare EtG and EtS values obtained in the two cohorts. Mann-Whitney U-test was carried out using the statistical package SPSS 2001 for Windows, version 12 (SPSS Inc., Chicago, IL, USA). P-values < 0.05 were considered to be statistically significant.

TABLE 1 Sociodemographic characteristics and newborns clinical parameters and somatometry in the two study cohorts.

	Reggio Emilia study cohort (N=96)	Barcelona study cohort (N=81)	P value
Parental sociodemographics			
Mother's nationality (% immigrants)	43.0%	39.7%	0.582
Mother's social class (%)			
Skilled (Manual & non manual)	76.5%	80.3%	0.783
Unskilled	23.5%	19.7%	
Father's social class (%)			
Skilled (Manual & non manual)	83.4%	85.4%	0.462
Unskilled	16.6%	14.6%	
Maternal features			
Maternal age (years), mean (SD)	28.4 (6.1)	28.3 (5.95)	0.670
Smoking during pregnancy (%)	23.4%	23.8%	1.000
Self-reported use of drugs of abuse (%)	0.0%	0.0%	1.000
Self-reported use of alcohol (%)	3.5 %	4.8%	0.586
Pregnancy outcomes			
N. deliveries, mean (SD)	0.8 (1.1)	0.8 (1.0)	0.861
Gestation weeks, mean (SD)	38.7 (1.4)	38.5 (1.4)	0.847
Preterm births (%) (Weeks < 37)	2.7%	2.6%	1.000
Infant somatometry			
Infant sex (% males)	49.2%	48.9%	0.971
Birth weight (g), mean (SD)	3149.6(457.1)	3123.4(445.9)	0.767

TABLE 2 Distribution of EtG and EtS concentration in meconium samples from two different city cohorts.

	REGGIO EMILIA samples (N=96) %	BARCELONA samples (N=81) %
Ethylglucuronide (ng/g)		
EtG = ND*	18.5	5.0
EtG≠ND*	81.5	95.0
5-100	93.5	52.6
101-200	5.2	18.4
201-400	1.3	7.9
>400	0	21.1
Ethylsulfate (ng/g)		
EtS=ND	53.1	48.1
EtS≠ND*	46.9	51.9
1-15	95.3	90.5
16-30	2.4	4.7
31-40	0	0
>40	2.3	4.8

ND: under the limits of quantification (5 and 1 ng/g Etg and EtS, respectively) of the analytical method used to quantify EtG and EtS.

RESULTS

EtG was present in 81% meconium samples from Reggio Emilia with concentrations between 5.0 and 796.2 ng/g, while in Barcelona 95% samples showed EtG with concentrations between 6.8 and 6309.3 ng/g (Table 2). With respect to EtS, it was found in 46% and 52% Reggio Emilia and Barcelona specimens, respectively, with concentrations lower than EtG by one order of magnitude (1 to 65.2 ng/g in Reggio Emilia samples and 1.1 to 437.5 ng/g in Barcelona samples).

In both study cohorts, the EtG and EtS values were not associated with self-reported maternal drinking during pregnancy (in both groups more than 95% women declared not to drink at all during pregnancy). EtG values were then arbitrarily divided in four concentration categories (Table 2). It should be noted that while 18.5% Reggio Emilia samples did not show measurable values of ETG and the majority of the positive samples (93.5.%) presented EtG values between 1 and 100 ng/g, in Barcelona only 5% samples did not have measurable values EtG and the other 95% specimens presented values within three orders of magnitude (Table 2). Median values of EtG in Barcelona samples (101.5 ng/g) were statistically higher than those from Reggio Emilia (15.6 ng/g). In both cohorts, EtS could be measured in about 50% samples, with the majority of values between 1 and 15 ng/g (Table 2). In the absence of maternal drinking admission in pregnancy - the two groups of meconium samples were examined for the presence of seven FAEEs (palmitic; palmitoleic; stearic; oleic; linoleic; linolenic and arachidonic acid ethyl esters). A liquid-chromatography tandem mass spectrometry methodology and categorized using the internationally established cut-off of 2 nmol/ g total amount of seven FAEEs, to differentiate between heavy maternal ethanol consumption during pregnancy and occasional or no use (FAEEs < 2 nmol/ g).[5,7,21,22]

Values of FAEEs ≥ 2 nmol/ g were found in 8% of the Italian samples (ranging from 2.8 to 3.5 nmol/g) and 42% of the Spanish samples (ranging from 2.2 to 324.7 nmol/g). Whereas in the Barcelona cohort EtG and EtS median values were different in the samples with total FAEEs below 2 nmol/ g from those with FAEEs equal or above 2 nmol/ g, this was not the case for the Reggio Emilia cohort (Table 3). Moreover, when comparing Barcelona and Reggio Emilia samples within both FAEEs below or equal and above 2 nmol/g values of EtG were still statistically different in the two city cohorts. Conversely, EtS values were similar when comparing all the samples, as well as, the subgroups of specimens with FAEEs lower or above 2 nmol/g for both of the cities cohorts (Table 3). Finally, there was no correlation between EtG or EtS concentrations and the total amount or each of the seven FAEEs in the meconium samples from either cohort.

TABLE 3 EtG and EtS concentration in meconium samples from two different city cohorts. Subgroups: FAEEs < and ≥ 2 nmol/mg.

Meconium Samples		EtG (ng/g)	EtS (ng/g)
Reggio Emilia (n=96)	Median value	15.6	0.0
Barcelona (n=81)	Median value	101.5	1.4
	P value	0.0001	0.07
Reggio Emilia FAEEs < 2 nmol/g (n=87)	Median value	14.3	0.0
Reggio Emilia FAEE s > 2 nmol/g (n=8)	Median value	17.6	1.1
	P value	0.344	0.799
Barcelona FAEEs <2 nmol/g (n=47)	Median value	45.5	0.0
Barcelona FAEEs >2 nmol/g (n=34)	Median value	152.3	2.1
	P value	0.0002	0.009

DISCUSSION AND CONCLUSION

Results from this study demonstrate the presence of a broad concentration range for EtG and a narrower one fromEtS in meconium samples from two different Mediterranean study cohorts. No direct correlation was observed between these two possible new biomarkes of intrauterine exposure to ethanol and the traditional biomarkers, FAEEs, but this could be due to the different mechanism of formation of these metabolites, as already suggested by Pragst et al.[23] in a comparison study of the same analytes in the hair matrix from adults. Furthermore, these two groups of molecules show different chemical-physical characteristics. While EtG and EtS are polar, water soluble and stable compounds, FAEEs are lipophilic molecules, which can be degraded by fetal esterases. Finally, whereas FAEEs formation in the fetus after transplacental passage of maternal ethanol is well-known[24], it is not clear if EtG and EtS cross the placenta, or if similar to the FAEE formation, the fetus has the enzymatic capability of producing them subsequent to ethanol transport across the placenta.

Nonetheless, in the Barcelona study cohort, both EtG and EtS could discriminate between samples which tested negative and those which tested positive for prenatal ethanol exposure, as evidenced by cumulative meconium FAEEs $<$ and $\geq$ 2 nmol/g. In the Reggio Emilia samples, this discrimination was not observed probably due to the fact that samples with FAEEs equal or above this cut-off were a minority and with FAEE's concentration range narrower than was measured in the Barcelona samples.

In any case, these preliminary results leave several unanswered questions. First of all, FAEEs and EtG and/or EtS seem to respond differently to ethanol consumption. While It seems that glucuronides are always produced when body comes in contact with ethanol, FAEEs are not. Is this because the latter are formed by fetus when ethanol concentration is above a certain values, or they are formed by fetus and then degraded by fetal esterases or during samples storage? Is formation/degradation due to different development of metabolic fetal biotransformations? Some authors[25] have demonstrated that EtG was measurable in a majority of urine samples collected from adults without a known source of ethanol use or exposure, and EtG generation by intestinal bacteria has been demonstrated and may result in ethanol absorbtion and metabolism to EtG. Even if this last claim is true for adults, this is not the case of the fetus, whose bowel is sterile.

It is well established that FAEEs found in meconium are produced in the fetus rather than being transported across the placenta.[24] What about EtG and EtS? Are they produced by the mothers and cross placenta? If yes, what percentage of them? Otherwise, is the fetus able to produce EtG and EtS from maternal ethanol? The two cohorts we have examined seem to be very different: Barcelona meconium samples show always higher levels of FAEEs and EtG with respect to Reggio Emilia samples. Is this because of a different habit of ethanol consumption, as supported by the above-reported results of National Surveys on Drug Abuse, or a different maternal/fetal enzymatic ability to metabolize ethanol?

Finally, the scientific community established FAEEs cut-off in meconium to differentiate heavy maternal ethanol consumption during pregnancy from occasional or no use. Is it possible to establish EtG and EtS cut-off levels? All these questions require answers, therefore further investigations are necessary before proposing the use of EtG and EtS as alternative biomarkers of chronic *in utero* exposure to ethanol.

Corresponding Author: simona.pichini@iss.it

REFERENCES

1. Chan D, Bar-Oz B, Pellerin B, Paciorek C, Klein J, Kapur B, Farine D, Koren G. Population baseline of meconium fatty acid ethyl esters among infants of nondrinking women in Jerusalem and Toronto. Ther Drug Monit 2003; 25: 271-278.
2. Bearer CF, Jacobson JL, Jacobson SW, Barr D, Croxford J, Molteno CD, Viljoen D, Marais AS, Chiodo LM, Cwik AS. Validation of a new biomarker of fetal exposure to ethanol. J Pediatr. 2003; 143: 463-469.
3. Moore C, Jones J, Lewis D, Buchi K. Prevalence of fatty acid ethyl esters in meconium specimens. Clin. Chem 2003; 49: 133-136.
4. Ostrea EM, Hernandez JD, Bielawski DM, Kan JM, Leonardo GM, Abela MB, Church MW,

Hannigan JH, Vanisse JJ, Ager JW, Sokol RJ. Fatty acid ethyl esters in meconium: are they biomarkers of fetal ethanol exposure and effect? Ethanol Clin. Exp. Res 2006; 30:1152-1159.
5. Pichini S, Pellegrini M, Gareri J, Koren J, García-Algar O, Vall O, Pacifici R, Zuccaro P, Marchei E. Alarming Prevalence of Fetal Ethanol Exposure in a Mediterranean City. Ther Drug Monit 2008; 30: 249-254.
6. Burd L, Roberts D, Olson M, et al. Ethanol and the placenta: a review. J Matern Fetal Neonatal Med 2007; 20: 361-375.
7. Politi L, Leone F, Morini L, Polettini A. Bioanalytical procedures for determination of conjugates or fatty acid esters of ethanol as markers of ethanol consumption: a review.Anal Biochem 2007; 368: 1-16.
8. Helander A, Beck O. Ethyl sulfate: a metabolite of ethanol in humans and potential biomarker of acute alcohol intake. J Anal Toxicol 2005; 29: 270-4.
9. Politi L, Morini L, Groppi A, Poloni V, Pozzi F, Polettini A. Direct determination of the ethanol metabolites ethyl glucuronide and ethyl sulfate in urine by liquid chromatography/electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom 2005; 19:321-1331.
10. Schmitt G, Aderjan R, Keller T, Wu M. Ethyl glucuronide: an unusual ethanol metabolite in humans. Synthesis, analytical data and determination in serum and urine. J Anal Toxicol 1995; 19: 91-4.
11. Halter CC, Dresen S, Auwaerter V, Wurst FM, Weinmann W. Kinetics in serum and urinary excretion of ethyl sulfate and ethyl glucuronide after medium dose ethanol intake. Int J Legal Med 2008; 122: 123-8.
12. Høiseth G, Morini L, Polettini A, Christophersen A, Mørland J. Blood kinetics of ethyl glucuronide and ethyl sulphate in heavy drinkers during alcohol detoxification, Forensic Sci Int 2009, (Article in press).
13. Dahl H, Stephanson N, Beck O, Helander A. Comparison of urinary excretion characteristics of etanol and ethyl glucuronide. J Anal Toxicol 2002; 26: 201-4.
14. Helander A, Beck O. Mass spectrometric identification of ethyl sulfate as an ethanol metabolite in humans. Clin Chem 2004; 50: 936-7.
15. Politi L, Morini L, Leone F, Polettini A. Ethyl glucuronide in hair: Is it a reliable marker of chronic high levels of ethanol consumption? Addiction 2006; 101: 1408–1412.
16. Pichini S, Altieri I, Zuccaro P, Pacifici R. Meconium as a matrix. Clin Pharmacokinet 1996; 31: 81.
17. Bar-Oz B, Klein J, Karaskov T, Koren G. Comparison of meconium and neonatal hair analysis for detection of gestational exposure to drugs of abuse. Arch Dis Child Fetal Neonatal Ed (2003); 88:F98-F100.
18. Morini L, Marchei E, Pellegrini M, Groppi A, Stramesi C, Vagnarelli F, Garcia-Algar O, Pacifici R, Pichini S. Liquid chromatography with tandem mass spectrometric detection for the measurement of ethyl glucuronide and ethyl sulfate in meconium: new biomarkers of gestational ethanol exposure? Ther Drug Monit 2008; 30:725-32.
19. http://www.pnsd.msc.es/Categoria2/publica/pdf/oed-2004.pdf (Accessed April 2009).
20. http://www.istat.it/salastampa/comunicati/non_calendario/20080417_00/testointegrale20080417.pdf (Accessed April 2009).
21. Pichini S, Pellegrini M, Gareri J, Koren J, Garcia-Algar O, Vall O, Vagnarelli F, Zuccaro P, Marchei E. Liquid chromatography-tandem mass spectrometry for fatty acid ethyl esters in meconium: a assessment of prenatal exposure to alcohol in two European cohorts. J Pharm Biomed Anal 2008; 48: 927-933.
22. Chan D, Klein J, Karaskov T, Koren G. Fetal exposure to alcohol as evidenced by fatty acid ethyl esters in meconium in the absence of maternal drinking history in pregnancy. Ther Drug Monit.2004; 26:474-481.
23. Pragst F, Yegles M. Determination of fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) in hair: a promising way for retrospective detection of ethanol abuse during pregnancy? Ther Drug Monit 2008; 30: 255-263.
24. Cook JD. Biochemical markers of ethanol use in pregnant women: review. Clin Biochem 2003; 36: 9-19.
25. Rosano TG, Lin J. Ethyl glucuronide excretion in humans following oral administration of and dermal exposure to ethanol. J Anal Toxicol 2008; 32: 594-600.