Rivista di psichiatria 2024; 59: 52-59

Delay in the fine-tuning of locomotion in infants with meconium positive to biomarkers of alcohol exposure: a pilot study

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Summary. Introduction. Prenatal alcohol exposure causes a variety of impairments to the fetus called Fetal Alcohol Spectrum Disorders (FASD). Since it is very difficult to identify women that consume alcohol during pregnancy, different methods have been studied to evaluate alcohol exposure. Ethyl Glucuronide (EtG) and Fatty Acid Ethyl Esters (FAEEs) are commonly used to measure alcohol consumption in individuals at-risk for alcohol abuse, including pregnant women. **Materials and methods.** We conducted a study of two cohorts of 1.5 year-old infants (of mothers without a history of alcohol abuse) with or without meconium samples positive to both EtG and FAEEs and we evaluated their cognitive-behavioral development by the Griffiths Mental Developmental Scale (GMDS) method. Our protocol included 8 infants with meconium positive to alcohol metabolites (EtG and FAEEs) and 7 with meconium negative to alcohol metabolites. **Results.** None of the 8 alcohol metabolites positive meconium infants exhibited distinctive facial features and growth retardation of severe FASD, showing that other factors may contribute to the FASD onset but elevations in EtG and FAEEs in the meconium were significantly associated with disrupted neurodevelopment and adaptive functions within the first year and a half of life. Indeed, we found out that infants with meconium positive for both EtG and FAEEs, although without displaying any FASD morphological features, had a delay in the fine regulation of their own locomotory capabilities. **Conclusions.** Further analyses and larger studies are needed to estimate the right link between prenatal alcohol exposure and the different range of disorders connected but this study provides an additional step in the field of FASD in order to suggest early treatments for at-risk newborns and infants.

Key words. Alcohol, EtG, FAEE, FASD, infants, meconium, neurodevelopment.

Ritardo nella regolazione del movimento fine in neonati con meconio positivo ai biomarcatori di consumo alcolico materno durante la gravidanza: uno studio pilota.

Riassunto. Introduzione. L'esposizione prenatale all'alcol provoca una serie di problematiche al feto chiamate disturbi dello spettro alcolico fetale (FASD). Poiché è molto difficile identificare le donne che consumano alcol durante la gravidanza, sono stati studiati diversi metodi per valutare l'esposizione all'alcol. L'etilglucuronide (EtG) e gli esteri etilici degli acidi grassi (FAEE) sono comunemente usati per misurare il consumo di alcol in soggetti a rischio di abuso di alcol, comprese le donne in gravidanza. **Materiali e metodi.** Abbiamo condotto uno studio su due coorti di neonati di 1,5 anni (di madri senza storia di abuso di alcol) con o senza campioni di meconio positivi sia a EtG che a FAEE e abbiamo valutato il loro sviluppo psicomotorio mediante la Griffiths Mental Developmental Scale (GMDS). Il nostro protocollo includeva 8 neonati con meconio positivo ai metaboliti dell'alcol (EtG e FAEE) e 7 con meconio negativo ai metaboliti dell'alcol. **Risultati.** Nessuno degli 8 neonati positivi al meconio per i metaboliti dell'alcol ha mostrato caratteristiche facciali distintive e ritardo della crescita causata da FASD grave, dimostrando che altri fattori possono contribuire all'insorgenza della FASD. Tuttavia, la presenza di EtG e FAEE nel meconio è risultata significativamente associata a uno sviluppo neurologico alterato. Infatti, i bambini con meconio positivo sia per EtG che per FAEE, pur non mostrando alcuna caratteristica morfologica FASD, avevano un ritardo nella regolazione fine delle proprie capacità locomotorie. **Conclusioni.** Sono necessarie ulteriori analisi e studi più ampi per stimare il giusto legame tra l'esposizione prenatale all'alcol e la diversa gamma di disturbi connessi, ma questo studio fornisce un ulteriore passo avanti nel campo della FASD per suggerire trattamenti precoci per neonati e bambini a rischio.

Parole chiave. Alcol, EtG, FAEE, FASD, meconio, neonati, neurosviluppo.

Introduction

The identification of the teratogen effects of drinking alcohol during gestation is referred back to 1973[1], and since then, numerous research focused on the long-term effects of prenatal alcohol exposure in-

dicating the importance of abstaining from alcohol during pregnancy and while breastfeeding[2].

Prenatal alcohol exposure can in fact lead to a plethora of consequences for the fetus called Fetal Alcohol Spectrum Disorders (FASD)[3]. Fetal alcohol syndrome (FAS) is the most severe form of FASD and relies on the presence of distinct facial anomalies and other physical dysmorphologies[4] associated with behavioral, cognitive and social disruptions[5-7].

The most devastating alterations affect the developing brain fetus, resulting in neurobehavioral deficits, including overall intellectual performance, executive function, learning and memory, language, visual-spatial ability, motor function, attention, and activity levels as well as behavioral problems including adaptive dysfunction, academic difficulties, and increased rates of psychiatric disorders[8-10].

This causes, among other things, a significant social cost: the annual expenditure expected in America for the support of all those affected by FASD was estimated, in 2018, between 1.6 and 5.4 billion dollars[11]; while the cost of treating a single FASD patient is about $2 million[12]. A systematic review of published literature on the cost of FASD reported that in the United States, one case of FASD can be prevented for a cost between $20,200 and 47,615, showing that the price of a single FASD prevention would be considerably less expensive than the cost of care for a single FASD case[13].

Despite numerous guidelines suggest abstaining from consumption of alcohol during pregnancy, about 10% of women in the general population consume alcohol while pregnant, even though the European region has the highest rate of consumption (up to 25%)[14]. The estimated prevalence of FAS in the general population is 14.6 per 10,000 people. The greatest consumption of alcohol in Europe is reflected in having the highest prevalence of FAS: 2.6 times higher than the global average (Ireland, Belarus, Denmark, the United Kingdom and Russia have the world's highest rates of alcohol consumption during pregnancy)[15].

Thus, the early identification and diagnosis of affected children is crucial for the appropriate interventions[16]; this could be very challenging, because of the lack of confirmation of prenatal alcohol exposure, lack of clinical expertise, and many possible confounding variables (i.e. children not in the care of their biological mothers, maternal death, or social stigma)[8]. Even when exposure is known, several factors may affect diagnosis: it is essential to assess the maternal prenatal alcohol intake, measured by the quantity of alcohol consumed per occasion (standard drinks per drinking day), frequency of consumption (eg, daily, times per week), and timing during gestation, because different timing of significant exposure can produce different physical and neurobehavioral phenotypes[9,10].

One of the main problems, because of the possible stigmatization associated with prenatal alcohol use, is estimating the maternal prenatal intake and it has been shown that self-declarations are often not reliable[17-21].

Hereafter, the consequence of identifying women who drink during gestation becomes even greater[22,23]. To overcome these difficulties and to obtain a reliable picture of the alcohol behavior in pregnant women, specific methodologies and strategies based on the analyses of ethanol metabolites in biological matrix such as meconium and maternal urine and hair have been established[24-26]. Indeed, Ethyl Glucuronide (EtG) and Fatty Acid Ethyl Esters (FAEEs) are commonly used to measure alcohol consumption in individuals at-risk for alcohol abuse, including pregnant women[24-27]. EtG and FAEEs are products of the non-oxidative metabolism of ethanol: EtG is an ethanol metabolite forming in the body by glucuronidation following exposure to alcohol, usually from drinking alcoholic beverages; FAEEs are products of the non-oxidative metabolism of ethanol which are formed by the esterification of ethanol with endogenous free fatty acids or with acyl-CoA[28]. The esterification process is catalyzed by two enzymes: FAEEs synthetase (present in the liver and pancreas) and acyl-CoA transferase[29].

Thus, the aim and novelties of the present study were to evaluate in two cohorts of 17 months-old infants (of mothers without a history of alcohol abuse) with or without meconium samples positive to both EtG and FAEEs, the cognitive-behavioral development by the Griffiths Mental Developmental Scale (GMDS)[30] and the presence physical abnormalities, such as facial dysmorphism and growth retardation, characteristics of FASD. We predicted that prenatal alcohol exposure, according to positive EtG and FAEEs in the meconium, could affect the infant's fine movement leading to a delay in neurodevelopment.

Materials and methods

Infants' recruitment

The original sample consisted of a total of 21 infants (12 females and 11 males) in follow-up at the Department of Pediatrics of the Sapienza University Hospital "Policlinico Umberto I" of Rome, Italy. As previously shown[31], the main exclusion criteria to avoid any bias in the selection for the infants' (and mothers') enrolment included a mean age for the infants comprised between 16 and 19 months for the GDMS neurobehavioral analysis, ongoing pediatric pathologies, inflammatory, endocrine and autoimmune disorders, diagnosed cardiovascular pathologies and the use of drugs or chemicals such as antidepressants, anti-inflammatory and immunosuppressants.

Afterwards, according to the above-mentioned recruitment criteria, the protocol included 8 age-matched infants with meconium positive to alcohol metabolites (EtG and FAEEs) (6 females, 2 males; mean age in months ± SE: 17.25 ± 0.55) and 7 with meconium negative to alcohol metabolites (EtG and FAEEs) (3 females, 4 males; mean age in months ± *SE*: 17.77 ± 0.57). The meconium, collected within the first 24 to 48 hours of newborn life, was placed into a plastic tube, homogenized, and immediately stored in aliquots at -20 °C. The aliquots were sent to the Istituto Superiore di Sanità in Rome to be analyzed for EtG and FAEEs. Samples were received anonymously because they were identified by a code with a progressive number assigned by the neonatology ward.

All the information on mother-infant dyads, identified by the same code were also provided. A structured questionnaire on cigarette smoking and drugs consumption during the 3 trimesters of pregnancy was completed by the mothers. Additional information about the mothers and the newborns was obtained from hospital records. The children enrolled in the study underwent a clinical evaluation and neurodevelopmental evaluation. The clinical evaluation was carried out by pediatricians with experience in the field of genetic and/or malformation syndromes and in particular on FASD[32-34]. This allowed us to have the most accurate auxological and dysmorphological measurements possible. The administration of GDMS was instead performed by clinical psychologists with experience in the field of pathologies of neurocognitive development in children[35].

The study was approved by the Sapienza University Hospital ethical committee (Ref. 5825); an informed consent was signed by each parent of the children and all the study procedures were under the Helsinki Declaration of 1975, as revised in 1983, for human experimentation.

EtG and FAEEs determinations in neonatal meconium

Fully validated liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods were used for the determination of EtG and the seven FAEEs (palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acid ethyl esters), respectively, in the collected meconium samples[27,36-40]. The established cut-off of 2 nmol of the total amount of the seven FAEEs and of 2 nmol for EtG per gram of meconium were used to consider whether infants were exposed or not to gestational ethanol intake.

Physical features measurement and dysmorphology exams to disclose FASD

As for the clinical diagnosis, FASD may result in a combination of facial malformations, growth retardation that may involve both length and weight and neurocognitive retardation together with a confirmed history of maternal alcohol intake during pregnancy[41]. The facial clinical features of FAS include short palpebral fissures, a low nasal implant with a short nose, a long nasal philtrum, and thinning of the upper lip[39].

To search for these anomalies in our sample, each child was examined by two clinicians, in double-blind data collection. The doctors evaluated the children measured, height, weight, occipitofrontal circumference (OFC), length of the filter, interpupillary distance, intracanthal distance (ICD) and palpebral fissure length (PFL) in each child. Measurements were taken using a rigid ruler, marked in millimeters, with examiners seated directly in front of the subject.

Each subject's upper lip and philtrum conformation was assessed using the lip-philtrum guide which provides a numerical interpretation of the qualitative aspects[42]. Independent scores were assigned for the thinness of the upper lip and the prominence or flatness of the philtral ridges. Scores of 4 or 5 allowed for the diagnosis of FASD. All children enrolled in the study then received then a total dysmorphology score that allowed them to be assigned to one of three categories: established FASD, another non-alcohol-related diagnosis, or no diagnosis of FASD[43].

Neurodevelopment assessment (Griffiths Mental Developmental Scale)

For the assessment of neurocognitive development, all children in the study underwent the Griffiths Mental Developmental Scale (GMDS)[30,44,45]. The GMDS was created to assess the cognitive and motor development of children from birth to six years of age. The scale is currently in its third edition which was revised in 2015 and is regarded as the gold standard for assessing neuromotor development in children[44-46].

The extended Griffiths scale evaluates 5 different domains such as basics of learning (A), language and communication (B), hand-eye coordination (C), personal-social-emotional (D) and gross-motor skills (E)[47]. Scale A aims to explore developmental potential and discoveries that foster innate future learning. The items administered include activities such as: manipulating objects (cubes and slots), building with bricks, repeating numbers, comparing quantities, putting in series, rearranging images to tell a story, building puzzles and activities that require associating objects and remembering[30]. Scale B evaluates the child's development of language and speech and the ability to use these skills to initiate social interactions with others. The items propose activities such as: naming objects and images, defining objects according to their function, telling a story, following instructions, saying the opposite and making similarities. The level of development

and the content of the language is also assessed[48]. The C scale evaluates the early stages of the child's development with regard to visual perception, visual directionality and object manipulation. Items in this scale include activities such as following the movement of objects, grasping toys of different sizes, building cubes, stringing beads, cutting paper with scissors, copying shapes, connecting dots, and performing more complex construction activities[46]. The D scale assesses areas of a child's personal, social and emotional development. These areas are characterized as being of fundamental importance for an adequate evolution of the child's development, learning and well-being. The administered items are organized according to the child's increasing level of emotional regulation, development of empathy, and recognition of morals. Were also included items to measure the early ability to put yourself in the shoes of others and the theory of mind[30]. The E scale evaluates the early development of postural control, general body motor coordination, and visual-spatial coordination. The items administered in this scale include activities such as observing the child's movements in space, the ability to jump on two feet and on one foot, running, playing ball, maintaining balance and jumping by alternating feet. With younger children, spontaneous body movement behaviors can be noted, regardless of the pre-established order of administration[48].

This gives total raw scores for each scale and an overall raw development score. The raw score will be converted into "equivalent age" in months, "weighted score", "developmental quotient", "confidence interval", "stanine" and "percentile"[49]:

- the equivalent age measure establishes, for each subject in each subscale examined, the chronological age to which the performances presented by the subject are referred;
- the weighted score indicates the relative position of the subject in the normative sample and provides comparable measures which make it possible to immediately compare the results achieved by the same subject in different tests;
- the development quotient indicates the level of development achieved by the child in the particular area being considered;
- the confidence interval is a set of values defined as a range of plausible values, with respect to the parameter that has been measured, which gives greater reliability with respect to the result obtained;
- the stanine is a unit on a nine-level scale used to group test results;
- the percentile is a measure used in statistics to indicate the minimum value below which a given percentage of the other elements under observation falls.

It should be noted that since the age of the sample studied was less than 2 years, the general quotients obtained from the subscales were used[50].

Data analysis

According to methods previously described[51], neurobehavioral data were evaluated by two-way analysis of variance (ANOVA) (positive/negative meconium and sex effect). Since no effects of sex were disclosed during the statistical investigation (except for body weight) this main factor was pooled out from the description of the results. Post hoc comparisons were analyzed by Tukey's HSD test. The Spearman Nonparametric test was utilized in infants to correlate the FASD features with the infants' age, BMI, weight and height. Data normal distribution was also analyzed. All data were analyzed by JASP (the University of Amsterdam, version 0.14.1 for the Mac).

Results

Physical and dysmorphological evaluations

Table 1 shows the body weight, height, BMI and head circumference of the infants characterized by meconium positive of not to ethanol metabolites. As shown by ANOVA no differences between groups were found. Furthermore, according to the data obtained from the statistical analysis, there were no statistically significant differences between the two groups of infants for the physical and dysmorphological parameters characterizing FASD. Indeed, OFC, ICD, PFL, philtrum, vermilion, honey stick and epicanthus were comparable between the two groups (data not shown). Furthermore, no Spearmen correlations were found between FASD features and body weight, height, BMI and head circumference. None of the 8 children who tested positive and then exposed to alcohol during intrauterine life had facial abnormalities attributable to severe FASD[52]. Growth percentile measurements were within the normal age ranges in all infants.

Table 1. Body weight, height, BMI and head circumference of the infants characterized by meconium positive of not to ethanol metabolites (± SE).

	Infants with Positive Meconium	Infants with Negative Meconium
Height in cm	77.67 ± 1.58	79.60 ± 1.13
Weight in kg	9.91 ± 0.30	10.42 ± 0.61
Head circumference in cm	46.25 ± 0.28	47.18 ± 0.75
BMI	16.46 ± 0.43	16.39 ± 0.64

Neurodevelopment assessment (Griffiths Mental Developmental Scale)

In order to evaluate the effects in terms of psychomotor skills in the children enrolled in the study the GMDS test was used. Figure 1 shows the developmental quotients of the main Griffiths subscales and the total developmental quotients expressed as the sum of the 5 main subscales. In particular, no significant differences induced by gestational alcohol exposure were revealed by ANOVA for the GMDS domains dealing with learning, language and communication, hand-eye coordination, and personal-social-emotional. However, the outcomes regarding the general motor coordination (figure 2) disclosed a delay in the fine regulation of locomotion expressed as equivalent age, developmental quotient, and percentile [F(1,13)=8.97, 9.98, 11.90; ps<0.05; respectively].

Discussion

To the best of our knowledge, this is the first study to demonstrate a significant delay in general motor coordination expressed as fine locomotion in a year and a half-infants with meconium positive to ethanol metabolites as EtG and FAEEs but without displaying any morphological feature of severe FASD. Notably, EtG and FAEEs in the meconium are biomarkers with high specificity for prenatal alcohol exposure in infants as previously mentioned.

According to the results of the present study, elevations in EtG and FAEEs in the meconium were significantly associated with a lower degree of neurodevelopment and adaptive functions within the first year and a half of life. None of the 8 alcohol metabolites positive meconium infants exhibited distinctive facial features and growth retardation of FAS or partial FAS. These findings suggest that other factors may contribute to the FAS onset such as paroxysmal alcohol drinking by the mother, genetic and epigenetics, unappropriated nutrition by the mother, and concomitant presence of both other substance abuse and gestational associated pathologies[53-56]. Indeed, animal model studies clearly showed that the supplementation in the diet of natural antioxidants may counteract the damage induced by alcohol abuse[57-64].

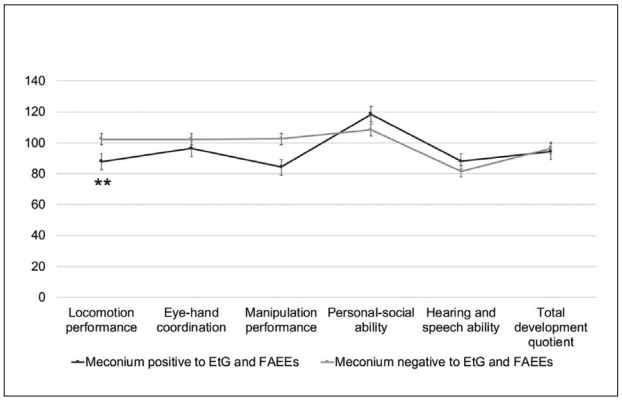

Figure 1. Mean development quotients of the Griffiths Mental Developmental Scales. Data are expressed as standard error means. Asterisks indicate significant differences between the positive/negative meconium groups (p<0.01).

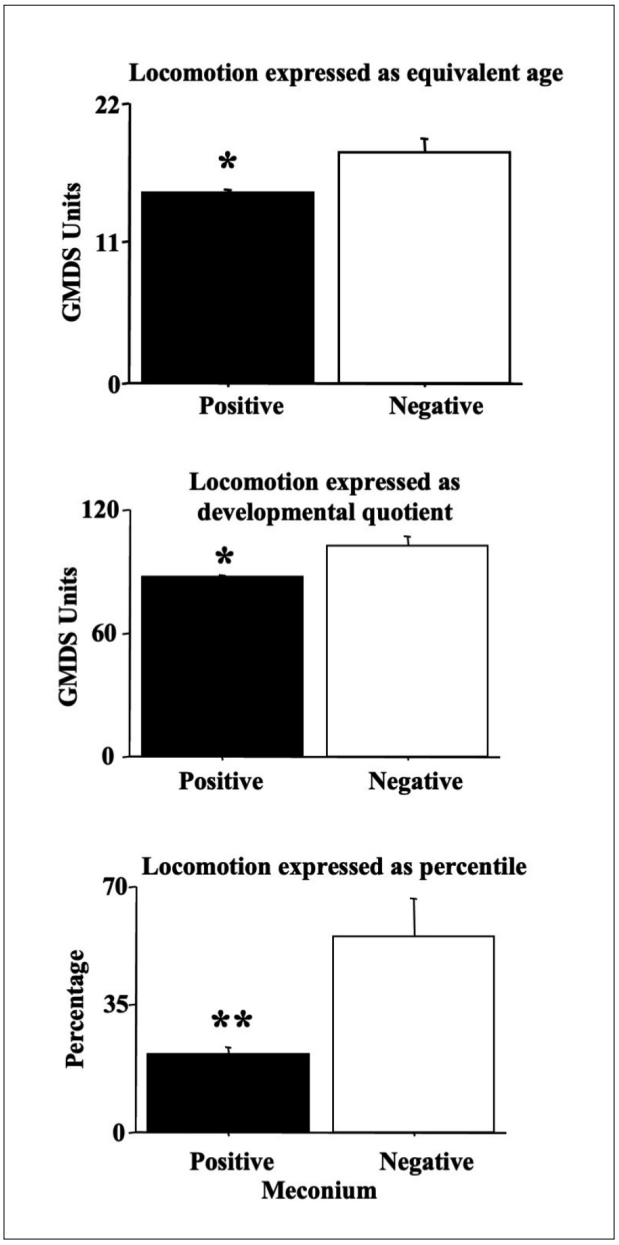

Figure 2. Outcomes of the general motor coordination expressed as equivalent age, developmental quotient, and percentile. The error bars indicate pooled standard error means (SEM) derived from appropriate error mean square in the ANOVA. The asterisks (*** p<0.01; * p<0.05) indicate post-hoc differences between groups.

Nonetheless, the recruited alcohol-exposed infants of the present investigation may only show subtle fetal alcohol effects at birth but manifest neurodevelopmental abnormalities at a later time[16,65].

Our report showed a delay in fine locomotor skills as assessed by GMDS in infants with meconium positive for FAEEs and EtG. Indeed, the group not exposed to alcohol had scores above the medium range, with an average quotient of 102.3 in locomotion ability, while the group of children exposed to alcohol was placed in the low range, with a mean score of 87.7 (SD= 2.4). Motor function impairment has been observed in children with FAS, but also in children exposed to alcohol in utero who did not meet FAS criteria[66]. A previous study showed that high and frequent alcohol consumption during pregnancy was related to abnormal walking and balance, and grasping and motor coordination at 1 year of age[67]. In another study investigating the performance of children whose mothers abused alcohol and drugs heavily during pregnancy, it was shown that the developmental performance of preschool children exposed to alcohol and drugs prenatally was, on average, substantially lower than expected for age regardless of the study group[68]. However, it should be noted that to evaluate the accuracy of motor assessment and cognition tools several factors should be taken into account such as diagnostic accuracy of motor assessment tools and subtests, accuracy of alternate cut-offs and accuracy of multiple subtests versus total scores[66].

In other studies investigating the relationship between meconium FAEEs and neurodevelopment in infants by using the Bayley's Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) scores it was found that increased FAEEs were significantly associated with poorer mental and psychomotor development during the first 2 years of age[69,70].

At the same time, neurodevelopmental delays with cognitive retardation compared with the general population and behavioral alterations were found in children exposed to alcohol during pregnancy with positive meconium for EtG or FAEEs[71]. Indeed, it was found that children with greater concentrations of FAEEs in the meconium were associated with lower Wechsler Intelligence Scales for Children-Fourth Edition Verbal Comprehension Index, Working Memory Index, and Full-Scale IQ scores[72]. Similarly, primary-school-age children with the meconium positive for EtG: i) allocated fewer attentional resources than controls to the go/no-go task; ii) had reduced IQ; and iii) and displayed ADHD-related behavior[70,73,74]. Furthermore, in a recent study, it was found that higher concentrations of FAEEs in the meconium are potential markers for children at risk for aggressive and delinquent behaviors related to the effects of prenatal alcohol exposure[75].

This study has clearly some limitations. The number of the recruited infants was small (but this depends also on the relatively restricted inclusion/exclusion criteria of the study and because the outcomes of this investigation derive from a single University hospital), so some biases could have happened. Besides, the absence of longer clinical info from additional follow-ups could be considered a study limit.

Conclusions

In conclusion, this study clearly discloses that infants with meconium positive for both EtG and FAEEs, although without displaying any FASD morphological features, had a delay in the fine regulation of their own locomotory capabilities. Furthermore, these findings once again emphasize: i) the importance, for women during before and during pregnancy, but also for the father[76], of carrying on the gestation in the healthiest mode possible, avoiding any kind of superfluous stress, which involves smoking, the intake of alcohol, and obviously, the consumption of any kind of drugs and ii) the importance of postpartum intensive hospital controls and monitoring in newborns and infants when FASD is questioned[77]. Of course, further analyses are needed, however, this investigation provides an additional step in the field of FASD in order to suggest early treatments for at-risk newborns and infants. These data could be of interest to experts involved in the study of prenatal alcohol exposure-associated disorders too.

Conflict of interests: the authors have no conflict of interests to declare.

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