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Italian Guidelines for the diagnosis and treatment of Fetal Alcohol Spectrum Disorders: detecting alcohol drinking during pregnancy

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Summary. Fetal Alcohol Spectrum Disorders (FASD) is an encompassing term used to describe a range of afflictions brought about by the consumption of alcohol during gestation. The detrimental effects primarily manifest in the central nervous system, growth, and distinctive facial features. Given that there are no known treatments for FASD, the meticulous screening for this condition in the earliest stages of pregnancy bears immense significance, ensuring the avoidance of the grievous consequences stemming from exposure to alcohol in utero. Screening measures for FASD encompass the assessment of alcohol biomarkers such as Phosphatidylethanol (PEth) in the maternal bloodstream, Fatty Acid Ethyl Esters (FAEEs) in the meconium, and Ethyl Glucuronide (EtG) in the meconium, maternal urine and hair. In particular, urinary EtG is highly sensitive and could be routinely used in pregnant women for detecting also occasional drinking. Questionnaire evaluations including AUDIT-C, T-ACE, and TWEAK, alongside a detailed Food Diary method to identify alcohol misuse and high-risk pregnancies, are also available. However, these questionnaires might provide an inadequate reflection of alcohol consumption in women due to their inclination to dissemble to comply with prevailing sociocultural expectations. Hence, this comprehensive review advocates for the indispensable integration of alcohol biomarkers detection in the course of pregnancy monitoring, as it constitutes a valuable tool for facilitating FASD screening.

Key words. Alcohol, EtG, EtS, FAEE, FASD, PEth.

Linee guida italiane per la diagnosi e il trattamento dei disturbi dello spettro feto-alcolico: rilevamento del consumo di alcol durante la gravidanza.

Riassunto. Il disturbo dello spettro feto-alcolico (FASD) è un termine onnicomprensivo utilizzato per descrivere una serie di disturbi causati dal consumo di alcol durante la gestazione. Gli effetti dannosi si manifestano principalmente nel sistema nervoso centrale, nella crescita e nelle caratteristiche facciali distintive. Dato che non esistono trattamenti noti per il FASD, lo screening meticoloso per questa condizione nelle prime fasi della gravidanza ha un significato immenso, limitando le gravi conseguenze derivanti dall'esposizione all'alcol in utero. Le misure di screening per la FASD comprendono la valutazione dei biomarcatori dell'alcol come il fosfatidiletanolo (PEth) nel flusso sanguigno materno, gli esteri etilici degli acidi grassi (FAEE) nel meconio e l'etilglucuronide (EtG) nel meconio, nelle urine materne e nei capelli. In particolare, l'EtG urinario è altamente sensibile e potrebbe essere utilizzato di routine nelle donne in gravidanza per rilevare anche il consumo occasionale. Sono inoltre disponibili valutazioni tramite questionari tra cui AUDIT-C, T-ACE e TWEAK, insieme a un diario alimentare per identificare l'abuso di alcol e le gravidanze ad alto rischio. Tuttavia, questi questionari potrebbero fornire un quadro inadeguato del consumo di alcol nelle donne a causa della loro inclinazione a mentire per conformarsi alle aspettative socioculturali prevalenti. Pertanto, questo lavoro evidenzia l'indispensabile integrazione del rilevamento dei biomarcatori dell'alcol nel corso del monitoraggio della gravidanza, poiché costituisce uno strumento prezioso per facilitare la scoperta precoce di un eventuale FASD.

Parole chiave. Alcol, EtG, EtS, FAEE, FASD, PEth.

Introduction

Alcohol abuse represents a risk in everyday life that may induce damage to all the organ systems, but especially affects the liver, pancreas, brain, heart, and immune system, impairing also sleeping and causing brain damage and severe cognitive issues like dementia or Wernicke-Korsakoff syndrome[1-10].

About 10% of women in the general population consume alcohol during pregnancy[11] and there is a higher risk of alcohol consumption during pregnancy in women of lower socio-economic status, women belonging to marginalized communities or women with substance use disorders[12-17].

It has been widely demonstrated that alcohol consumption during pregnancy is related to negative pregnancy and neonatal outcomes, such as spontaneous abortion, stillbirth, pre-term birth, intrauterine growth retardation and fetal alcohol spectrum disorders (FASD), a group of conditions caused by maternal alcohol consumption during pregnancy[18-25]. Low levels of prenatal alcohol exposure can still be noxious for the fetus's development and increase the risk for FASD[26,27].

Screening for prenatal alcohol exposure is a relevant tool for FASD prevention and it allows to reduce the cost and service burden in healthcare globally[28].

Within the body, ethanol is distributed from the blood into all tissues and fluids in proportion to their relative water content and quickly reaches equilibrium with the concentration of ethanol in plasma[29-32]. However, some factors influence alcohol drinking, including ethnicity, culture, religion, and family environment, while individual characteristics, such as genetic polymorphisms, can affect alcohol absorption and metabolism[33,34]. Ethanol and its teratogenic metabolite acetaldehyde can easily cross the placental barrier and compromise the development of the fetal nervous system[35-37], thus it is strongly recommended that women totally abstain from alcohol consumption during pregnancy and nursing[38-40].

The assessment of a dose-response link between the levels of prenatal alcohol exposure and the effect on fetal and neonatal outcomes remains a problem. Even if binge drinking is considered less concerning than chronic heavy alcohol consumption, adverse effects on fetus development can still occur especially during critical phases of organogenesis[41,42]. Moreover, alcohol metabolism is slower in the fetus than the mother and so in fetal blood, there could be a higher concentration of alcohol for a longer period[9,43-47].

The brain has a high susceptibility to prenatal alcohol exposure. A wide number of studies has described the effects of alcohol exposure on behavior, cognition and mental health[48,49]. In individuals with prenatal alcohol exposure, it has been found the presence of neuroanatomical changes, such as grey matter reduction in specific brain regions and a global decrease in white matter volume[50-52].

It is difficult to assess the prevalence of FASD; some studies estimated 0.2 to 7 individuals with FASD in every 1000 live births in specific areas of the United States[53,54]. FASD may be associated with neuropsychological disorders[55-57], attention deficit disorder[58,59], physical abnormalities[60,61] and behavioral problems. FASD is irreversible and there is no treatment for it, but it can be completely prevented with abstention from alcohol consumption during pregnancy and when trying to conceive[62-65].

FASD evaluation requires a multidisciplinary team, including an audiologist, cardiologist, neurologist, psychiatrist, psychotherapist, ophthalmologist, and many others. The diagnosis of the disorder begins with the estimation of prenatal alcohol exposure by documenting the mother's reported levels of alcohol consumption up to three months before pregnancy[66]. The analysis during gestation of biomarkers of alcohol drinking and the administration of alcohol screening questionnaires to the pregnant women are used for this purpose. The risk of FASD is related either to the amount of alcohol used and the frequency of alcohol consumption. The severity of the symptoms is related to the phase of fetal development and to the gestational age in which alcohol consumption occurs[67,68].

Materials and methods

Biomarkers of alcohol consumption

Conventional alcohol biomarkers are mainly measured in blood matrix, both on serum and plasma, but other matrices, such as urine and keratin matrix, which have gained importance in recent years, especially for ethylglucuronide (EtG), are also considered[29-32,69,70]. It should be noted that not all the biomarkers available for alcohol abuse detection are suitable for being used during pregnancy[71].

In this work we take to count only direct biomarkers, namely that compounds created from alcohol metabolism. Direct biomarkers are: Phosphatidyl Ethanol (PEth), Fatty Acid Ethyl Esters (FAEEs), Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS). Indirect biomarkers, such as the hepatic enzyme gamma-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the glycoprotein CDT, mean corpuscular volume (MCV) of the erythrocytes, are not ideal in the contest of pregnancy where a tempestive assessment of alcohol misuse is needed. MCV in example, takes 6-8 weeks of heavy drinking (more than 40 grams of alcohol/day) to significantly increase. Enzymes like AST, ALT and GGT need as well high levels of alcohol drinking to elevate, have a low level of sensitivity and a low positive predictive value. CDT levels tend to be higher in women than men, independently of their history of drinking. Furthermore, pregnancy was found to be correlated with increased CDT levels, regardless of alcohol consumption[72,73] and for this reason CDT will be not discussed in this work.

Phosphatidylethanol (PEth)

PEth is not a single molecule, but a class of ethanol derivatives in the form of phospholipids. These

are obtained through a trans-phosphatidylation reaction of phosphatidylcholine present in cell membranes and catalyzed by phospholipase D, which, in the presence of ethanol, selectively catalyzes the PEth-forming reaction[74]. Among the different molecular forms, there is a predominant homolog chosen as an analytical target: 16:0/18:1 PEth, which will be referred to from here on simply as PEth. For the detection of this biomarker of alcohol use and abuse, dried blood spot testing (DBS) is used. The spot containing about 50μL of sample is placed in a vial and submerged with 250μL of a 2:4:0.2 solution of water-isopropanol-ammonium acetate 2mM solution 0.01% formic acid; in this way, the phosphate group of phosphatidylethanol is protonated, making it extractable into the organic phase, and basic molecules in the blood are deprotonated. Subsequently, the internal standard (PEth-D5) and the extraction solvent, usually hexane, are added. The vials are then mixed on vortex shaker for a few seconds, and left for 30 min in sonicator. Next, the vials are mixed again for 10 minutes on multimixer, with the aim of maximizing the amount of analyte passing into solution. At the end of agitation, the paper spot is removed from the vial with a special hooked instrument, and the organic phase is recovered through a Pasteur pipette. The organic phase is separated and brought to dryness under nitrogen flow, and then resuspended with 50μL of acetonitrile and transferred to a conical-bottomed glass vial, capped with a pierceable autosampler cap, for analysis in UHPLC-MS/MS. PEth-DBS is a stable method, especially when stored at lower temperatures[75].

Fatty Acid Ethyl Esters (FAEEs)

In the fetal circulation, ethanol can be metabolized into FAEEs, which accumulate in meconium and thus can be used as a biomarker of fetal alcohol exposure[76].

For the analysis of FAEEs, an optimized and validated method previously described by Hutson et al.[77] could be headspace-solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS). Ethyl myristate (E14), ethyl palmitate (E16), ethyl linoleate (E18:2), ethyl oleate (E18:1) and ethyl stearate (E18) were analyzed, then 25μL of their internal standards (1 μg/mL in n-heptane) were taken and placed in a 10 mL SPME headspace vial. N-heptane was completely evaporated at 40 °C in a stream of nitrogen and 50 mg meconium and 1 ml phosphate buffer (0.1 M, pH 7.6) were added. The mixture was shaken for 30 minutes and then 500 mg NaCl was added. Finally, the vials were capped with screw caps. Ethyl myristate was then excluded from the analyses as it was observed that it was also found in the meconium of infants with abstinent mothers. On the other hand, all samples with a sum of concentrations of the other four esters (E16, E18, E18:1, E18:2) greater than or equal to 100 ng/g were considered analytically positive.

Ethyl Glucuronide (EtG) and Ethyl Sulphate (EtS)

EtG and EtS are metabolites of ethanol and are used as biomarkers of alcohol consumption. EtG can be detected in a variety of biological matrices; in this work, methods on urine, hair and infant meconium are described. EtS accumulates in hair after chronic alcohol consumption and its detection can be used as a biomarker for alcohol consumption[78]. The main reason for using EtG as an alcohol biomarker is its long detection time for recent drinking, if compared to breath or blood ethanol test. Blood is not usually used as a matrix for the detection of EtG because it would involve shorter detection times due to its lower concentrations in blood[79].

Urinary EtG is studied by an enzyme immunoassay using specific antibodies that can detect EtG without creating cross-reactivity with other glucuronic compounds[30,31]. The test is based on competition between glucose-6-phosphate dehydrogenase (G6PDH)-conjugated drug and free drug in the urine sample for a fixed number of antibody-specific binding sites. In the absence of a free drug in the sample, the antibody binds to the drug conjugated with G6PDH causing a decrease in enzyme activity. This phenomenon creates a direct relationship between drug concentration in urine and enzyme activity. The active enzyme converts NAD to NADH producing an alteration in absorbance, which can be measured by spectrophotometric examination at 340 nm. In this way, we obtain qualitative and semiquantitative detection of urinary EtG, which is considered positive when concentrations are greater than or equal to 100 ng/mL[70,80].

In the hair, EtG can be detected by gas chromatography (GC) or liquid chromatography (LC), coupled with mass spectrometry (MS). Before analysis, the hair is treated with organic solvents, usually methanol and dichloromethane, to remove interfering substances, and is chopped. Approximately 50mg of hair is then taken and brought into 2mL of deionized water, to which 15μL of EtG-D5 deuterated standard is added. The samples are placed in the oven at 60° overnight, and the following day the extraction is done in SPE columns.

The column is first washed with 2 mL methanol and 2 mL deionized water, then the sample is loaded and slowly flowed. The SPE column is washed with 2 mL of 0.1 mM ammonia solution in water with a slight vacuum, followed by washing with 2 mL of methanol under a high vacuum, after which elution of the species of interest is made with 2 mL of a 99:1 solution of methanol and formic acid. The collected

eluate is brought to dryness under nitrogen flow and derivatized with 30µL N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), then the sample is ready for analysis. The mass analyzers were used in selected reaction monitoring mode and m/z 405 fragments (m/z 410 for EtG-D5 internal standard) was used as the precursor ion, while m/z 405 → 359 and m/z 410 → 359 transitions were set for quantification and m/z 405 → 331 and m/z 405 → 287 for qualification/confirmation[81]. Depending on which chromatographic separation technique is used and the instrument conditions applied, the EtG peak comes out at different retention times. Under normal conditions, its retention time is between 7 and 12 minutes and the limit of detection is between 5 and 6 pg/mg.

EtG accumulates in the intestine (in meconium) of the fetus from the 20th week of gestation until birth. Meconium from the newborn is taken (about 1 g) in the first 2 to 24 hours after delivery and stored frozen for analysis; some studies have confirmed alcohol exposure with values of EtG present in meconium as low as 10 ng/g, up to higher values of 112 ng/g for higher exposure[82]. The analysis starts by taking about 200 mg of meconium, stirring for 10 seconds, and adding about 0.5 mL of methanol. After stirring again, 1 mL of hexane is added and centrifuged. Next, 1 mL of acetonitrile is added, and after centrifuging again, SPE is performed. The column is conditioned with hexane and EtG elution is done with about 1 mL of methanol. At this point, the sample is dried under nitrogen flow, imaged with water and analyzed by LC-MS/MS[83].

Alcohol screening questionnaires

Table 1 shows the questions and hallmarks of the Alcohol Use Disorders Identification Test – Consumption (AUDIT-C), of the Tolerance-Worried-Eye opener-Amnesia-(K)Cut down (TWEAK test) and of the T-ACE/TACER-3, all aiming at disclose putative alcohol drinking during pregnancy[84-86].

The AUDIT-C has three short questions that estimate alcohol consumption in a standard, meaningful and non-judgmental manner. The total score from these questions provides an indication of the risks to the woman's health and can be used to guide conver-

Table 1. TWEAK, T-ACE/TACER-3 and AUDIT-C description.

<	TWEAK	T-ACE	AUDIT-C
Questions	• (T) Tolerance hold: How many drinks can you hold? Alternatively, (T) Tolerance high: How many drinks does it take to make you feel high? • (W) Worried: Have close friends or relatives worried or complained about your drinking in the past year? • (E) Eye Opener: Do you sometimes take a drink in the morning when you get up? • (A) Amnesia: Has a friend or family member ever told you about things you said or did while you were drinking that you could not remember? • (K) Cut down: Do you sometimes feel the need to cut down on your drinking?	• (T) Tolerance: How many drinks does it take to make you feel high? • (A) Annoyed: Have people annoyed you by criticizing your drinking? • (C) Cut down: Have you ever felt you ought to cut down on your drinking? • (E) Eye opener: Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover?	• How often do you have a drink containing alcohol? a: never b: monthly or less c: 2-4 times at month d: 2-3 times a week e: 4 or more times a week • How many drinks containing alcohol do you have on a typical day when you are drinking? a: 1 or 2 b: 3 or 4 c: 5 or 6 d: 7 to 9 e: 10 or more • How often do you have six or more drinks on one occasion? a: never b: less than monthly c: monthly d: weekly e: daily or almost daily
Score	A positive response to question T (T-high: 3 or more; T-hold: 5 or more) or W yields 2 points each; an affirmative reply to question E, A, or K scores 1 point each. A total score of ≥2 points indicates a positive outcome for pregnancy risk drinking.	Replying more than 2 drinks to question T scores 2 points; a positive response to question A, C, or E scores 1 point, each. A total score of ≥2 points indicates a positive outcome for pregnancy risk drinking (≥3 for TACER-3).	In pregnant women, a score of 3 or more is considered to indicate hazardous or harmful alcohol use.

sations about alcohol and pregnancy. AUDIT-C has been validated for use with pregnant women[87]. The AUDIT-C is a shortened version[88] of the 10-item AUDIT tool developed in a WHO collaborative project[84] and has been extensively used and studied since. The score for each question is from 0 to 4 in a five-point scale. The score of the single items summed makes the total score. For women, a cumulative score of 3 or more may indicate alcohol misuse. The AUDIT questions/scores and a chart illustrating the approximate number of standard drinks in different alcoholic beverages are available online at https://www.drugabuse.gov/sites/default/files/files/AUDIT.pdf. Information about the sensitivity and specificity of the test can be found at: https://pubs.niaaa.nih.gov/publications/arh25-3/204-209.htm.

The TWEAK test was originally used to screen for harmful drinking and to diagnose alcoholism or heavy drinking in different populations[89]. In 1994 the TWEAK was then used as a screening tool for periconceptional risk drinking among obstetric outpatients; the aim was to target risky drinkers and to ameliorate the outcome of their pregnancy by reducing alcohol intake[85]. Questions and scoring of the test are shown in table 1; further information about the test is available at the following address: https://pubs.niaaa.nih.gov/publications/arh25-3/204-209.htm.

The T-ACE is a screening test for at-risk drinking based on the CAGE questionnaire, but modified to be used in obstetric-gynecologic practices[90]. It was developed by an obstetrician who observed that a question about Tolerance was better accepted by patients than the Guilt question from the CAGE. The shortness of the test (takes less than 1 minute to ask) and the fact that any score above 0 is considered positive (0/1 easy scoring) make it an attractive instrument to identify potential pregnant risk drinkers. More recently an updated version of the test (TACER-3) has been proposed and validated[86,91]. Questions and scoring of the test are shown in Table 1; further information about the test is available at the following address: https://pubs.niaaa.nih.gov/publications/arh25-3/204-209.htm.

A food diary[30] was also used to measure dietary intake over the pregnancy period, based on the University Hospital Umberto I Rome Guidelines. The aim was to assess the intake of a variety of nutrients and alcohol. The food diary questions related to the eating habits during breakfast, lunch and dinner are as follows:

- What do you usually eat for breakfast, lunch and dinner during working days?
- What do you eat habitually for breakfast, lunch and dinner on days that differ from normality, such as the weekend?

It was also asked what kind of favorite beverage she assumes during breakfast, lunch and dinner as follows:

- With working meals what are your favorite beverages?
- With weekend meals what are your favorite beverages?

If the women assume alcohol (positive score), the investigators ask the specification of drink size for different types of alcoholic beverages. In general, it is recommended to include questions about drinking in the context of a diet diary, always to avoid social stigma[92].

Results and discussion

Table 2 summarizes the detection window, clinical application and different cut-offs of the five metabolites discussed in the previous chapter. Accuracy in analysis is important because detecting the fairest concentration of the metabolite within the biological

Table 2. Detection window and cut-offs of alcohol biomarkers, and their potential clinical application in the screening and prevention of FASD.

Analysis	Detection window	Cut-off	Clinical application
PEth on blood	Up to 2-4 weeks	< 200 ng/mL	Heavy, moderate and occasional alcohol consumption[93]
FAEEs on meconium	Third trimester of pregnancy	2 nmol/g total amount of 7 FAEEs	Fetal cumulative alcohol exposure[94]
Urinary EtG	Up to 80 hours	< 100 ng/mL	Alcohol intake[30,31,70]
EtG in the hair	Some months, depending on the length of the hair	< 6 pg/mg *limit of detection* 6-20 pg/mg low consumption 20-30 pg/mg medium consumption >30 pg/mg high consumption	Chronic alcohol abuse[69,81]
EtG in the meconium	From the 20th week of gestation	< 30 ng/g	Fetal alcohol exposure[95]

matrix makes it possible to discriminate moderate alcohol use from more persistent use.

Since there is no treatment for FASD, the screening of this condition in the earliest week of gestation has a great importance in order to prevent fetus damage.

Among the metabolites discussed in this article, blood PEth and EtG proved to be high reliable. Furthermore, the detection of EtG in urine has higher sensitivity than in other matrices making this test more reliable compared to the other methodologies. Indeed, in two Italian studies based on urinary EtG on pregnant women data showed percentage values of alcohol consumption comprised between 34.28 and 25.6[30,70]. By contrast, in other Italian studies on pregnant women based on EtG in the hair or meconium data showed much more lower values of alcohol drinking[94,96].

Collection of maternal urine samples is most likely to be done during the third month of pregnancy when maternal screening begins. The optimization of techniques such as GC-MS/MS allows many toxicology laboratories worldwide to perform analyses for EtG easily and effectively.

The global prevalence of FASD is 0.2-7.7%, with a higher prevalence of 2-5% in Europe and North America[60,96], underscoring the need for increased diagnosis and treatment. However, different diagnostic systems and disagreements over criteria have slowed progress in the diagnosis and management of the disorder. Furthermore, by measuring the number of pregnant women drinking alcohol throughout the analysis of ethanol metabolites as the EtG in the urine, hair or meconium data showed high variability of consumption with values ranging from 2/3% up to values superior than 30%[30,31,70,94,96,97], with the highest percentages when the EtG was measured in the urine of the pregnant women. Nonetheless, there is evidence that a combination of biomarkers, or combining biomarkers with self-report, increases diagnostic accuracy[98].

It should be mentioned, however, that not all women who drink during pregnancy give birth to a child with Fetal Alcohol Syndrome; this implies a greater need for both pre- and postnatal testing.

Conclusions

This review aims to emphasize the imperative of incorporating the examination of alcohol consumption in pregnant women into primary care systems. By implementing strategies such as screening for alcohol use in women of childbearing age, referring those who exhibit alcohol-related disorders to rehabilitation programs, raising awareness about the subject to promote health maintenance, we could potentially witness a decline in the number of cases of FASD.

However, the utilization of the T-ACE and TWEAK questionnaires alone may be considered insufficient and less than completely reliable, as women might not always be inclined to disclose the full truth regarding their alcohol consumption. In contrast, the analysis of various biological samples, in particular urinary EtG for ethanol metabolites, presents an opportunity to definitively confirm or rule out alcohol consumption through tests that can be carried out by any toxicology laboratory. These analyses additionally permit non-invasive sampling techniques and offer long-term assessment potential, as exemplified by hair analysis. Moreover, in cases where these markers indicate alcohol consumption, it is worthwhile to extend the analysis to include the biological matrices of the newborn, thereby providing an initial foundation for the diagnosis of FASD.

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