Critical Review

Biomarkers for the Detection of Prenatal Alcohol Exposure: A Review

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Alcohol exposure during pregnancy can cause adverse effects to the fetus, because it interferes with fetal development, leading to later physical and mental impairment. The most common clinical tool to determine fetal alcohol exposure is maternal self-reporting. However, a more objective and useful method is based on the use of biomarkers in biological specimens alone or in combination with maternal self-reporting. This review reports on clinically relevant biomarkers for detection of prenatal alcohol exposure (PAE). A systematic search was performed to ensure a proper overview in existing literature. Studies were selected to give an overview on clinically relevant neonatal and maternal biomarkers. The direct biomarkers fatty acid ethyl esters (FAEEs), ethyl glucuronide (EtG), ethyl sulfate, and phosphatidylethanol (PEth) were found to be the most appropriate biomarkers in relation to detection of PAE. To review each biomarker in a clinical context, we have compared the advantages and disadvantages of each biomarker, in relation to its window of detectability, ease of collection, and the ease and cost of analysis of each biomarker. The biomarkers PEth, FAEEs, and EtG were found to be applicable for detection of even low levels of alcohol exposure. Meconium is an accessible matrix for determination of FAEEs and EtG, and blood an accessible matrix for determination of PEth.

Key Words: Prenatal Alcohol Exposure, Neonatal Screening, Prenatal Development, Fetal Alcohol Spectrum Disorder.

ALCOHOL INTERFERES WITH fetal development (Goodlett and Horn, 2001) and prenatal alcohol exposure (PAE) can lead to adverse effects on the fetus (Mukherjee et al., 2006). Alcohol crosses the placenta, causing fetal blood to reach a blood alcohol concentration resembling that of the mother (Guo et al., 1994) and causes, in high doses, neuroteratogenic effects by its interactions with molecular regulators of brain development (Goodlett et al., 2005; Riley and McGee, 2005). The general recommendation is to avoid ingestion of alcohol during pregnancy and breastfeeding (Christoffersen and Soothill, 2003; Riley and McGee, 2005; Russell and Skinner, 1988). If the alcohol intake is extensive, it may lead to fetal alcohol syndrome (FAS) (Stratton et al., 1996). FAS is diagnosed by (i) facial abnormalities such as short palpebral fissures and abnormalities in the premaxillary zone, (ii) growth retardation, including at least one of the following characteristics: low birthweight, decelerating weight over time not due to nutritional deficiencies, or disproportionally low weight to height, and (iii) neurodevelopmental abnormalities, including at least one of the following: decreased cranial circumference at birth, structural abnormalities of the brain, or neurological signs, as relevant for age (Stratton et al., 1996).

Long-term consequences of fetal alcohol exposure include mental disorders and behavioral problems (Christoffersen and Soothill, 2003; Streissguth et al., 2004). Early identification of prenatally exposed infants is more likely to avoid these later effects (Kahlberg and Gelo, 2011; Nordberg et al., 1993). It is difficult to estimate the prevalence of PAE, as most of the exposed children are never diagnosed. Elliott and colleagues (2008) found the median age of diagnosis was 3.3 years, 6.5% were diagnosed at birth and 63% by 5 years of age. Early identification involves identification of women at risk, better training of health personnel, and development of biomarkers for alcohol exposure (Fig. 1).

The relationship between low to moderate levels of PAE and adverse outcome is ambiguous (Flak et al., 2014; Patra et al., 2011), and Henderson and colleagues (2007) argue that inaccurate data on alcohol intake may produce these conflicting results. Diagnosis often relies on maternal self-report, which is considered the clinical standard in detection of PAE. However, some pregnant and nursing mothers underreport their drinking behavior (Zelner et al., 2012), as drinking during pregnancy is considered socially unacceptable (Bearer et al., 2003; Joya et al., 2012), stressing the importance of a variety of diagnostic tools as objective

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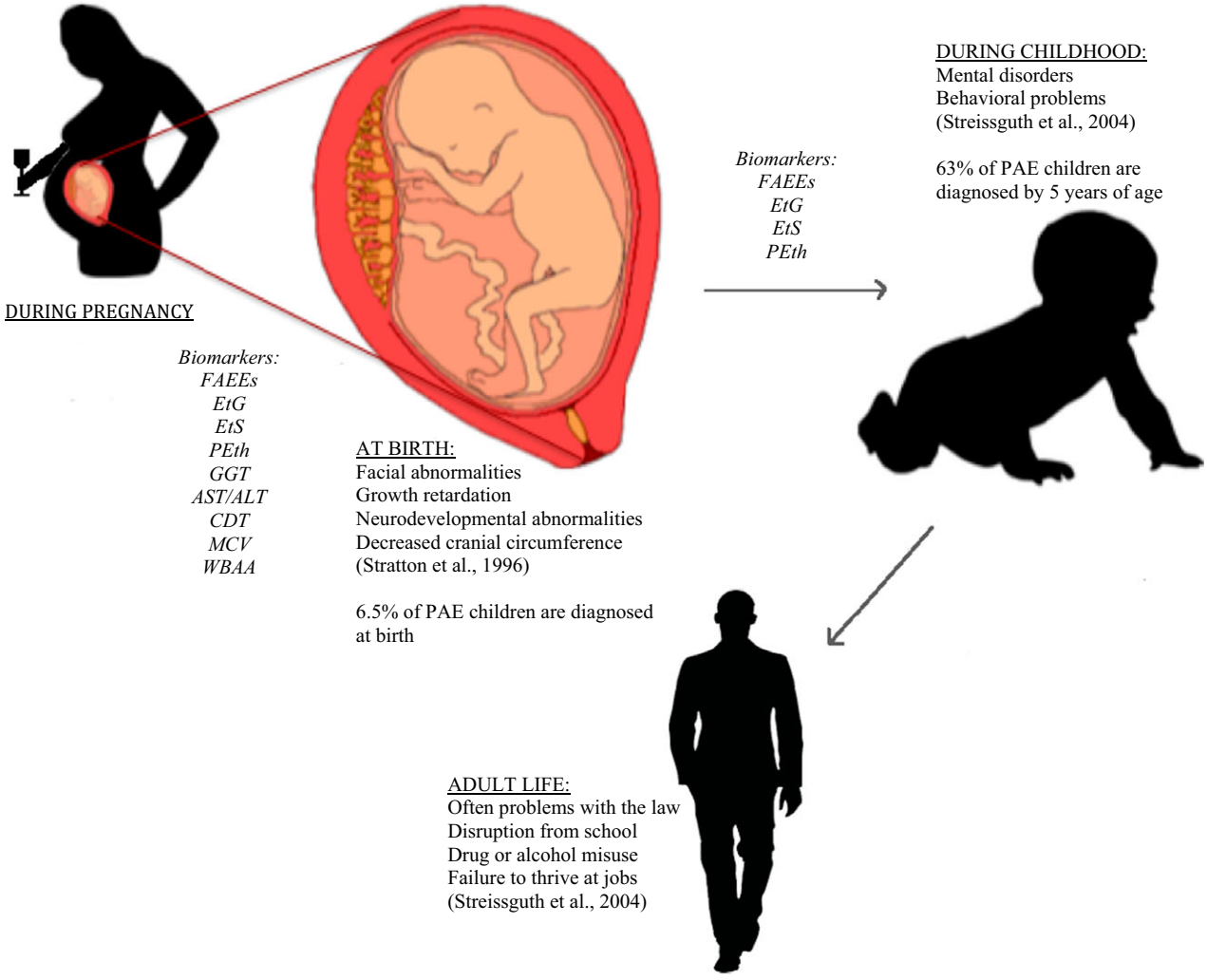

Fig. 1. The biomarkers found in the alcohol-consuming mother and the neonate, and consequences of prenatal alcohol exposure (PAE). Only 6.5% of PAE children are diagnosed at birth. Outcome that might be found at birth: facial abnormalities, growth retardation, neurodevelopmental abnormalities, and decreased cranial circumference. 63% of PAE children are diagnosed at the age of 5 years. Outcomes that might lead to the discovery at this age are mental disorders and behavioral problems, often regarding school-life. The frequency of PAE-exposed individuals that are diagnosed in adult life, or remain undiagnosed, is unknown. Outcome seen at this point is frequent problems with the law, disruption from school, drug or alcohol misuse, and failure to thrive at jobs.

biomarkers to detect prenatal exposure to alcohol. However, identifying reliable biomarkers has proven to be difficult. Derauf and colleagues (2003) found conflicting results in their study, as fatty acid ethyl esters (FAEEs) were found in the meconium of infants whose mothers denied alcohol intake, yet these biomarkers were not found in the meconium of infants whose mothers admitted drinking alcohol in the third trimester of pregnancy (Derauf et al., 2003). A Swedish study (Comasco et al., 2012) used the Alcohol Use Disorders Identification Test (AUDIT) for maternal self-report and 2 blood biomarkers carbohydrate-deficient transferrin (CDT) and phosphatidylethanol (PEth) for use of alcohol; the AUDIT suggested a significant number of women continued using alcohol during pregnancy, while the biomarkers indicated only modest drinking levels. Another study on Swedish women (Wurst et al., 2008) combined the AUDIT with ethyl glucuronide (EtG) and FAEEs in hair and concluded that the combined use of AUDIT and biomarkers identified more potential alcohol consumers than the AUDIT questionnaire alone. A more recent meta-analysis compared maternal self-report versus meconium testing and found the prevalence estimates of PAE 4 times higher when using meconium testing compared to prevalence using maternal self-report (Lange et al., 2014). However, instruments to obtain self-reported data varied across studies. Using focus group interviews, Muggli and colleagues (2015) investigated which determinants would enable women to provide accurate data on alcohol consumption. They found that categories that facilitate accurate data provision such as clear guidelines in serving size and drink choices, ability to record drinking, understanding of purpose of question, and high level of confidentiality increased the participants' ability and willingness to be realistic on their alcohol consumption habits (Muggli et al., 2015). Based on these studies, biomarkers can play an

important additional role in the diagnosis of infants at risk of developing fetal alcohol spectrum disorder (FASD), which consists of various permanent birth defects that result from PAE but not severe enough to cause FAS, but still resulting in permanent damage to the fetus (Roozen et al., 2016). A study in a Midwestern U.S. community estimated the prevalence among first grade students with FASD to 2.4 to 4.8% (May et al., 2014). As of today, it is not possible to treat FASD, which makes it a lifelong condition. Preventive initiatives, early identification, activation, and mental stimulation are crucial to achieve the best possible conditions for the affected child (Bearer et al., 1999; Grant et al., 2004; Kahlberg and Gelo, 2011; Stoler and Holmes, 2004).

Biomarkers of the Alcohol Metabolism

Alcohol is metabolized and generates distinct biomarkers. Approximately 95% of the alcohol is metabolized via the oxidative pathway in the liver, and the rest via the nonoxidative pathway in the pancreas, liver, brain, heart, and other organs (Laposata et al., 2000). Residual metabolites are stored in various tissues. Distinct biomarkers resulting from alcohol include the production of FAEEs, EtG, ethyl sulfate (EtS), and PEth (Joya et al., 2012). These biomarkers are known as direct, as they are derived directly from the metabolism of the ethanol (EtOH) molecule, still containing 2 carbon atoms. Furthermore, there are indirect biomarkers, which are measurable in the body due to the toxic effect of alcohol on organ systems or its biochemistry. These include the hepatic membrane glycoprotein enzyme gamma-glutamyl transpeptidase (GGT), the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the glycoprotein CDT, mean corpuscular volume (MCV) of the erythrocytes, and whole-blood-associated aldehyde (WBAA), which are metabolites of the oxidative alcohol metabolism (Joya et al., 2012).

This review focuses on the direct biomarkers as they are important and frequently applied clinical biomarkers in the context of revealing PAE. Table 1 provides an overview of the biomarkers presented in this review, including their respective clinical properties.

FATTY ACID ETHYL ESTERS

FAEEs are nonoxidative EtOH metabolites, formed by the conjugation of EtOH to endogenous free fatty acids and acyl-CoA fatty acids. FAEE formation is most often catalyzed by cytosolic FAEE synthases that are found ubiquitously throughout the body. The synthases uses EtOH and free fatty acids as their substrates or microsomal acyl-CoA/EtOH *O*-acyl-transferase, which uses EtOH and acyl-CoA as its substrates. Thus, the variability in concentrations of FAEEs in different organs, tissues, and cells following EtOH exposure is a result of differential expression of enzymes involved in the production of FAEEs, differences in fatty acid substrate availability, tissue composition, etc. (Zelner et al., 2013). The FAEEs comprise a group of more than 20 different fatty acid derivatives with E12:0, E14:0, E16:0, E16:1, E18:0, E18:1, E18:2, E18:3, E20:4 being the most predominant in meconium and hair (Table 1) (Bearer et al., 2005; Brien et al., 2006; Cabarcos et al., 2015; Caprara et al., 2005; Chan et al., 2004; Kulaga et al., 2006, 2009, 2010). FAEEs remain in serum for at least 24 hours after alcohol intake and are subsequently stored in various tissues, both maternal and neonatal (Joya et al., 2012). FAEEs can therefore be detected for a long time after alcohol is eliminated from the bloodstream.

Several methods for the analysis of neonatal matrices for FAEEs have been developed, using headspace-solid phase microextraction (HS-SPME) and SPME coupled with gas chromatography-mass spectrometry (GC-MS) or liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Table 1) (Cabarcos et al., 2015; Joya et al., 2012). As meconium is accumulated in the fetal gut from around the 20th week of gestation and until birth, it is potentially a good matrix to detect chronic alcohol exposure throughout most of the pregnancy. However, meconium is only available for the first 0 to 3 days of the life of the neonate. Several studies have reported on the investigation of FAEEs in meconium, finding it a useful biomarker in detection of PAE with a cutoff value at 0.5 pg/mg, which gives a high sensitivity and specificity (Bakdash et al., 2010; Cabarcos et al., 2015; Gauthier et al., 2015; Goecke et al., 2014; Himes et al., 2015; Hutson et al., 2010; Joya et al., 2012; Min et al., 2015; Moore et al., 2003; Pichini et al., 2008). Yang and colleagues (2015b) reported that the concentrations of FAEEs increase linearly with the dose only in babies born to mothers who reported >3 drinks/wk. These results indicate that the correlation between PAE and FAEE concentrations in meconium is nonlinear, with a threshold probably at 3 drinks/wk. As meconium is accumulated in the fetal gut from around the 20th week of gestation and until birth, it is potentially a good matrix to detect chronic alcohol exposure throughout most of the pregnancy. In addition, Chan and colleagues (2003) found that FAEEs in meconium are associated with maternal consumption of olive oil, and therefore, the diet can be the cause of false positive if FAEEs in meconium are used as the only biomarker for identifying PAE. Furthermore, Himes and colleagues (2014) reported on a postcollection instability among FAEEs in meconium and recommended that the meconium be frozen immediately after sampling to permit accurate quantification of FAEEs. Kulaga and colleagues (2009, 2010) investigated the use of hair samples for the determination of FAEEs using HS-SPME, followed by detection and quantification by GC-MS, finding conformity with self-reported alcohol use. Hair sampling is an alternative matrix, which provides a larger detection window than that of meconium, as neonatal hair is available for 1 to 3 months after birth. In utero, neonatal hair grows during the third trimester. Hair can also be sampled from the mother, where the proximal 6 cm of hair represent the last 6 months of exposure to various substances. In a recent

Table 1. Biomarkers for the Detection of Prenatal Alcohol Exposure. Overview of Maternal and Neonatal Bio-Specimens, the Time Span in Which Certain Biomarkers are Detectable, the Ease by Which the Bio-Specimens can be Collected, and Extraction and Assay Methods for the Determination of Biomarkers in Bio-Specimens

Bio-specimen	Window of detectability	Ease of collection	Biomarkers	Extraction/assay methods presented in this review	Ease/cost of analysis
Meconium	Reflects the latest months of exposure in fetal life	(i) Noninvasive procedure, (ii) easy to obtain and store, (iii) relatively narrow window	FAEEs	SPE/GC-MS; LOD = 50 pg/mg, LOQ N.A. (Moore et al., 2003). HS-SPME/GC-MS; LOD = 15 pg/mg, LOQ = 50 pg/mg (Bakdash et al., 2010). HS-SPME/GC-MS; LOD/LOQ N.A. (Goecke et al., 2014). Liquid–liquid and SPE/GC-FID; LOD/LOQ N.A. (Hutson et al., 2010). Liquid–liquid and SPE/LC-MS/MS (MRM); LOD = 0.04 to 0.07 nmol/g, LOQ = 0.12 to 0.20 nmol/g (Pichini et al., 2008). SPE/LC-MS/MS (SRM); LOD = 0.04 to 0.07 nmol/g, LOQ = 0.14 to 0.20 nmol/g (Morini et al., 2010). SPE/LC-MS/MS (MRM); LOD N.A., LOQ = 0.045 to 0.219 nmol/g (Himes et al., 2014, 2015)	Reproducible and sensitive analytical methods. Easy to validate and use. Analysis relatively inexpensive. Uses D_5-FAEEs or ethyl heptadecanoate as IS (commercially available)
			EtG	Vortex-assisted liquid–liquid/LC-MS/MS (MRM); LOD = 10 ng/g, LOQ = 30 ng/g (Bakdash et al., 2010). Liquid–liquid and SPE/HILIC-MS/MS (MRM); LOD N.A., LOQ = 20 pg/mg (Tarcomnicu et al., 2010)	Reproducible and sensitive method. Easy to validate and use. Analysis relatively inexpensive. D_5-EtG as IS (commercially available)
			EtG and EtS	SPE/LC-MS/MS (MRM); EtG: LOD = 2 pmol/g, LOQ = 8 pmol/g; EtS: LOD = 7 pmol/g, LOQ = 22 pmol/g (Morini et al., 2010). SPE/LC-MS/MS (MRM); EtG: LOD N.A., LOQ = 23 pmol/g; EtS: LOD N.A., LOQ = 20 pmol/g (Himes et al., 2014, 2015)	Reproducible and sensitive method. Easy to validate and use. Analysis relatively inexpensive. Uses D_5-EtG and D_5-EtS as IS (commercially available)
Hair	Reflects exposure over time, as substances are stored as the hair grows.	(i) Noninvasive sampling procedure, (ii) easy to obtain and store, (iii) requires that the test person has hair	FAEEs	HS-SPME/GC-MS; LOD/LOQ N.A. (Kulaga et al., 2010)	See meconium FAEEs
			EtG	Liquid–liquid and SPE/HILIC-MS/MS (MRM); LOD N.A., LOQ = 20 pg/mg (Tarcomnicu et al., 2010). Liquid/UHPLC-MS/MS (MRM); LOD = 3 pg/mg; LOQ = 7 pg/mg (Joya et al., 2016a,b)	See meconium EtG
Urine	Reflects recent exposure. Depends, however, on the frequency of urination	(i) Relatively difficult to obtain and store, (ii) requires cooperation with the test person or parents	EtG and EtS	No derivatization. Extract redissolved after being concentrated/LC-MS/MS (MRM); EtG: LOD = 52 ng/ml, LOQ = 152 ng/ml; EtS: LOD = 50 ng/ml, LOQ = 110 ng/ml (Weinmann et al., 2004; Wurst et al., 2008)	Matrix effect from the sample is a challenge. Requires the use of a triple-quadrupole MS and special MS expertise. Highly sensitive method. Uses D_5-EtG and D_5-EtS as IS. Analysis relatively inexpensive

Continued.

Table 1. (Continued)

Bio-specimen	Window of detectability	Ease of collection	Biomarkers	Extraction/assay methods presented in this review	Ease/cost of analysis
Placenta	Reflects months of exposure	(i) Easy to obtain and store, (ii) quite narrow time window for collection	FAEEs	Liquid–SPE/GC-MS; LOD = 6.0 to 8.7 μg/ml, LOQ = 8.2 to 37.0 μg/ml (Gauthier et al., 2015)	Relatively simple preparation procedure using pentadecanoic acid ethyl ester as IS. Reproducible and sensitive method. Inexpensive
			EtG and EtS	Vortex-assisted liquid–liquid/ LC-MS/MS (MRM); EtG: LOD = 13 pmol/g, LOQ = 22 pmol/g; EtS: LOD = 23 pmol/g, LOQ = 40 pmol/g (Morini et al., 2011)	See meconium EtG and EtS
Nails	Reflects exposure over time, as substances are stored as the nails grows. Sample size is small	Easy to obtain and store	EtG	Sonication-assisted liquid/LC-MS/MS (MRM); LOD = 3 pg/mg, LOQ = 10 pg/mg (Morini et al., 2013)	See meconium EtG
Neonatal blood	Reflects recent exposure, as the substances are constantly eliminated from the bloodstream	Invasive procedure but can be collected by routine sampling	PEth	Vortex-assisted liquid–liquid/ LC-MS/MS (MRM); LOD/LOQ N.A. (Kwak et al., 2012). N.A./LC-MS/MS; LOD = 2 ng/ml, LOQ = 8 ng/ml (Baldwin et al., 2015)	Reproducible and highly sensitive method. Phosphatidylpropanol as IS. Relatively easy and inexpensive sample preparation and analysis
Maternal blood	Reflects recent exposure, as the substances are constantly eliminated from the bloodstream	Invasive procedure but can be collected by routine sampling	PEth	Vortex-assisted liquid–liquid/ LC-MS/MS (MRM); LOD/LOQ N.A. (Kwak et al., 2012; Yang et al., 2015a)	See neonatal blood PEth
Fetal tissue (i.e., umbilical cord)	Will reflect months of exposure	(i) Easy to obtain and store, (ii) quite narrow time window for collection	EtG and EtS	Vortex-assisted liquid–liquid/ LC-MS/MS (MRM); EtG: LOD = 13 pmol/g, LOQ = 22 pmol/g; EtS: LOD = 23 pmol/g, LOQ = 40 pmol/g (Morini et al., 2011)	See meconium EtS and EtG

EtG, ethyl glucuronide; EtS, ethyl sulfate; FAEEs, fatty acid ethyl esters; FID, flame ionization detection; GC-MS, gas chromatography-mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; UHPLC, ultra-high performance liquid chromatography; HS-SPME, headspace-solid phase microextraction; IS, internal standard; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; N.A., not available; PEth, phosphatidylethanol; SPE, solid phase extraction; SRM, selected reaction monitoring.

study, Gauthier and colleagues (2015) investigated the ability of placental FAEEs to predict maternal drinking during pregnancy and found that placental FAEEs are promising biomarkers to accurately determine maternal alcohol use in pregnancies and PAE.

ETHYL GLUCURONIDE AND ETHYL SULFATE

EtG is a minor nonoxidative EtOH conjugate, which is formed by the EtOH conjugation with glucuronic acid mediated by UDP-glucuronyl transferases. EtG is a very sensitive biomarker of alcohol exposure, and maximum concentrations of EtG in serum are reached 3 to 5 hours after consumption of EtOH. EtG is detectable 4 to 8 hours after EtOH elimination as shown in adults (Halter et al., 2008; Joya et al., 2012). Similarly, in urine, EtG is detectable after 1 hour and up to 5 days after EtOH intake (Wurst et al., 2005). EtG is, however, only detectable in bio-specimens if alcohol has been consumed. The specificity and sensitivity of EtG seem to exceed those of all other known EtOH biomarkers, although EtG has a relatively low incorporation rate in hair due to its acidic properties with a pKa of 3.21 (Cabarcos et al., 2015). The determination of EtG in hair therefore requires very sensitive analytical techniques such as LC-MS/MS. The concentration of EtG is, however, not influenced by pigmentation (Appenzeller et al., 2007). A cutoff value for EtG in hair of 30 pg/mg has been suggested for an excessive consumption of alcohol. A cutoff above 7 pg/mg has been suggested for moderate alcohol consumption whereas a cutoff lower than 7 pg/mg has been suggested as a negative result, although occasional consumption of alcohol is possible (Cabarcos et al., 2015; Liniger et al., 2010). The sensitivity of EtG increases when its determination is combined with FAEEs. However, Himes and colleagues (2015) investigated

agreement between self-reported PAE and the meconium alcohol biomarkers FAEEs, EtS, and EtG for identifying PAE and found that EtG at a cutoff value of 30 ng/g in meconium after 19 weeks of gestation was a better biomarker for identifying maternal alcohol consumption than currently used FAEEs at a cutoff at 2 nmol/g.

EtS, another minor nonoxidative conjugate, is formed by the transfer of a sulfuric group from 3′-phosphoadenosine-5′-phosphosulfate to EtOH. This process is mediated by mitochondrial sulfotransferases. The maximum concentration of EtS in serum is reached 2.5 to 4 hours after consumption of EtOH and is, like EtG, detectable 4 to 8 hours after EtOH elimination (Halter et al., 2008; Joya et al., 2012). In urine, the detection period for EtS is up to 30 hours after EtOH consumption (Kissack et al., 2008). Although not much research has been done on EtS compared to EtG, they are both considered as useful urinary biomarkers for recent EtOH exposure (Table 1).

Like FAEEs, EtG and EtS are detectable in various maternal and neonatal tissues. Several studies have reported on the detection of EtG and EtS in meconium (Bakdash et al., 2010; Cabarcos et al., 2015; Joya et al., 2012; Morini et al., 2010, 2011; Tarcomnicu et al., 2010; Wurst et al., 2008), and it has been proven that EtG and EtS in meconium are more stable at room temperature than FAEEs (Himes et al., 2014). In a study by Tarcomnicu and colleagues (2010), in which a new hydrophilic interaction liquid chromatography (HILIC)–MS/MS method was used (Table 1), with a cutoff value of 20 pg/mg, it was found that EtG in hair is a useful biomarker for alcohol intake, although the samples in the study were obtained from nonpregnant individuals. These results are in accordance with those obtained by Wurst and colleagues (2008), who found that EtG in hair samples from pregnant women is a reliable biomarker, which is able to identify more potential consumers of alcohol in combination with AUDIT questionnaire, than the use of AUDIT questionnaire alone. Also Joya and colleagues (2016a) found EtG in maternal hair to be a useful marker for identifying exposed newborns by comparing this biomarker to EtG in meconium. Using the combination of EtG in meconium (cutoff 30 ng/g) and a median of EtG > 11 pg/mg in maternal hair during the second and third trimesters of pregnancy, prenatal EtOH exposure could be predicted with a sensitivity of 85.7% and specificity of 73.7% (Joya and colleagues, 2016b). These results are in accordance with those of Himes and colleagues (2015) described above and clearly indicate that EtG in meconium is an excellent biomarker for identifying PAE during pregnancy. Gutierrez and colleagues (2015) compared EtG (cutoff 8 pg/mg) in maternal hair to, for example, PEth (cutoff > 8 ng/ml) in maternal blood, and EtG and EtS in maternal urine with cutoffs at 38.7 and 7.2 ng/ml, respectively, and found that the sensitivity of hair-ethyl glucuronide (hEtG) was comparable with these biomarkers. On the other hand, the specificity of hEtG was lower (86%) compared to these other biomarkers (98 to 100%) for the determination of PAE. However, the validity of hEtG was improved in women with less frequent shampooing and those who did not use chemical hair treatments and dying, suggesting that hEtG alone is not a sufficient biomarker to identify PAE, but might be a useful biomarker together with other maternal biomarkers. Morini and colleagues (2011) detected EtG and EtS in placental tissue and fetal tissue from pregnancies terminated at 12 weeks of gestation. The placentas and fetal tissue samples were deproteinized and directly injected into an LC–MS/MS system, confirming the possibility of testing for both metabolites to evaluate alcohol use in mothers at the beginning of their pregnancy.

Highly sensitive GC-MS methods for the detection and quantification of EtG have been developed (Matlow et al., 2013; Sharma et al., 2015; Wurst et al., 2008). However, the drawback using GC-MS for the determination of EtG is the requirement of a derivatization step. Consequently, LC-MS/MS is a method of choice for the investigation of EtG and EtS in bio-specimens, such as tissue and hair samples (Table 1). The quantification of EtG and EtS in urine samples requires, however, special LC-MS/MS equipment and expertise due to, for example, matrix effect (ion suppression) of the sample in order to maintain a high resolution and sensitivity (Weinmann et al., 2004; Wurst et al., 2008). The sample preparation of urine samples is, on the other hand, very straightforward (Table 1). A solid phase extraction (SPE) step in the sample preparation of urine samples has been used in the analysis of other urinary metabolites to increase resolution and sensitivity by high-performance liquid chromatography (HPLC) and LC-MS/MS (Radko et al., 2013) but has also been used in sample preparation for the analysis of EtG and EtS in meconium and placenta (Table 1); thus, an SPE step could perhaps help to develop a more simple LC-MS/MS method for the analysis of EtG and EtS in urine samples.

PHOSPHATIDYLETHANOL

PEth is formed from EtOH and phosphatidylcholine in cell membranes, a reaction, which is catalyzed by phospholipase D. Normally phosphatidylcholine reacts with water, but has a higher affinity for EtOH. PEth has a long half-life and is measurable up to 6 weeks after alcohol intake (Gunnarsson et al., 1998; Joya et al., 2012; Varga et al., 2000), and it has been shown that women with blood PEth concentrations more than 4 nM had a higher risk ratio of spontaneous abortions in first trimester (Yang et al., 2015a).

LC-MS/MS is used for detection of PEth in blood (Table 1). Kwak and colleagues (2012) detected PEth in blood samples from pregnant women 4 to 6 weeks after low-to-moderate alcohol ingestion, and in a study from 2014 (Kwak et al., 2014), they were able to quantify PEth blood levels 3 to 4 weeks after ingestion. In addition, the analysis of neonatal dried blood specimens for PEth has been investigated and validated by Baldwin and colleagues (2015) and Bakhireva and colleagues (2013, 2014, 2016), proving this biomarker's ability to identify PAE during the last 3 to 4

weeks of pregnancy, and also advantageous in terms of collection, storage, and minimal invasiveness to the child.

DETECTION OF BIOMARKERS IN BIO-SPECIMENS

A prerequisite for the determination of biomarkers for PAE is the collection of bio-specimens. In Table 1, different samples from maternal and neonatal origin for the determination of various biomarkers are presented.

Maternal Tissue

It is natural to test the mother for alcohol biomarkers in order to reveal PAE. For that purpose, different bio-specimens have been investigated. Urine is routinely sampled and allows a relatively large sample size. The analytical methods of choice for the analysis of EtG and EtS in urine, as well as in other bio-specimens, are LC-MS/MS, due to high sensitivity and specificity. However, in urine, matrix effect from the sample is a challenge, and the use of, for example, a triple-quadrupole MS along with special MS expertise is needed. Still, it is a highly sensitive and inexpensive method, which uses D_5-EtG and D_5-EtS as internal standard (IS), with limit of detection (LOD) and limit of quantification (LOQ) for EtG and EtS in the ng/ml range (Table 1). Other methods for the determination of EtG that are not based on mass spectrometric detection, and therefore provide less selectivity and sensitivity, have been published. Among these methods are immunoassays such as ELISA (Zimmer et al., 2002) and enzyme immunoassay (EIA) (Böttcher et al., 2008; Turfus et al., 2013). ELISA, using polyclonal antibodies with affinity to EtG and other glucuronide conjugates, appears however, to have limited value for screening for EtG in urine, due to cross-reactivities with other glucuronide conjugates (Zimmer et al., 2002). In contrast, the developed EIA method employs a monoclonal antibody with a high specificity and affinity to EtG and has successfully been used for qualitative and semiquantitative measurement of EtG in urine, using a cutoff between 0.1 and 0.5 μg/ml (Böttcher et al., 2008; Turfus et al., 2013). If greater sensitivity is demanded, an LC-MS/MS analysis could be performed directly, but the result should then be supplemented with detection of related compounds, such as EtS. Consequently, LC-MS/MS is also the method of choice for the determination EtG and EtS in urine samples.

Biomarkers from the metabolization of alcohol can also be measured in blood samples (Joya et al., 2012; Kwak et al., 2012, 2014; Yang et al., 2015a). Sampling of blood is invasive and painful, and some markers of the alcohol metabolism are available for only a short period of time. However, blood is routinely sampled for other purposes, which makes it an obvious possibility for alcohol testing. The analytical method for detecting PEth in blood samples is a reproducible and highly sensitive method that uses phosphatidyl propanol as IS. It is relatively easy and inexpensive (Table 1).

Finally, FAEEs in maternal hair can be used to determine the timing of exposure during pregnancy (Kulaga et al., 2010) as substances are stored in the hair as it grows. The sampling procedure is noninvasive, and the material is easy to collect and store, even for a longer time. However, the level of substances may be affected by hair treatment like shampoo and hair dye. The same applies to the use of nails, but this material does often not allow a large sample size, if any, as most people routinely cut their nails.

Neonatal Tissue

One of the first biological matrices applied was blood. Analysis of neonatal blood is limited to identifying recent exposure. The procedure is invasive, but blood can be obtained from the umbilical cord. Blood is routinely sampled, which makes the sample easy to obtain.

Meconium is one of the most applied biological medias for sampling, and studies have been able to detect FAEEs, EtG, and EtS in meconium (Table 1) (Cabarcos et al., 2015; Joya et al., 2012). One of the advantages of meconium is that it is easy to obtain and store, and sampling is noninvasive. However, the time span for sampling is limited to a few days after birth and requires cooperation with the parents, as many parents are discharged within hours after birth, and the meconium needs to be scraped off the diaper (Kummer et al., 2016). A major problem is that meconium is only formed in the last few months of pregnancy; thus, detection of biomarkers only reflects PAE in this time window. However, teratogen effects on the developing brain were discovered in all 3 trimesters of the pregnancy (Goodlett et al., 2005; Riley and McGee, 2005).

The analytical methods for analyzing for FAEEs, EtG, and EtS in meconium are reproducible and sensitive. The methods are easy to validate and use, and analysis is relatively inexpensive (Table 1). The studies on FAEEs use D_5-FAEEs or ethyl heptadecanoate as IS, the studies on EtG use D_5-EtG as IS, and the studies on EtS use D_5-EtS as IS.

Urine can be sampled from the neonate as well. However, urine sampling from neonates is not simple, as it requires special equipment and the urine volume is quite small. Furthermore, it requires parental cooperation, as the parents must collect the sample from the diaper. However, substances like EtG persist in urine for longer than they do in blood, which makes the time span for sampling longer.

A few studies have used placental tissue for sampling and detection of FAEEs (Gauthier et al., 2015). This tissue is simple to collect and store, but the window of opportunity for collection is narrow. This may be compensated by the fact that healthcare professionals often are present at the time when the placenta arrives. The analysis of FAEEs in placenta is a relatively simple preparation procedure, using pentadecanoic acid ethyl ester as IS. The method is reproducible and sensitive and relatively inexpensive. The analytical methods for analyzing EtG and EtS in placenta are like the analytical methods for meconium: reproducible,

sensitive, easy to validate and use, and the analysis is relatively inexpensive (Table 1). One study, from Morini and colleagues (2011), investigates the content of EtG and EtS in fetal tissue. The type of fetal tissue is not specified, and therefore, it is not possible to transfer these results directly to the use of umbilical cord, which is the type of neonatal tissue material we are able to harvest from living individuals.

More recent studies have used hair as a biological medium for sampling and determination of FAEEs and EtG (Table 1) (Cabarcos et al., 2015; Joya et al., 2012). Hair collection is a noninvasive procedure; however, not all children are born with hair, and in cases where neonatal hair is present, it can be a problem to collect a sample which is large enough to detect eventual biomarkers. The analysis of FAEEs and EtG in hair involves some of the same analytical methods as meconium analysis.

Use of Indirect Biomarkers

Direct biomarkers are specific and indicative of alcohol consumption, and therefore, recent research focuses mainly on the use of direct biomarkers to identify alcohol consumption during pregnancy (Cabarcos et al., 2015; Joya et al., 2012); hence, literature on the use of indirect biomarkers in this context is relatively old. Indirect biomarkers are products reflecting permanent organ damage and thus are very unspecific as their formation in the body can be affected by many physiological and pathological states other than alcohol consumption. Consequently, the studies on indirect biomarkers reflecting alcohol intake during pregnancy have generally shown a lower sensitivity than that of the direct biomarkers (Joya et al., 2012).

OUTCOME MEASURES

Early diagnosis leads to earlier identification of FASD and of possible intervention against detrimental conditions, allowing interventions regarding medical support, social support, and even foster and/or family care (Streissguth et al., 2004). This would provide the child with better conditions for integration in society and prevent later complications.

Very few studies have shown a direct link between the occurrence of biomarkers in pregnant women or neonates and the neurodevelopment of the neonates. Peterson and colleagues (2008) investigated the relationship between FAEEs in meconium and neurodevelopmental measures in infants at 6.5 months, 1 year and 2 years of age, according to specific developmental index scores, and found that increasing concentrations of FAEEs were linked to poorer mental and psychomotor development. A study by Min and colleagues (2015) investigated the amounts of FAEEs in meconium linked with IQ as a measure of poor cognitive development in children at ages 9, 11, and 15 years and found that increasing amounts of FAEEs at birth were associated with lower IQ in later childhood.

However, another study showed no link between the biomarkers PEth and CDT in pregnant women and neuropsychological development in the infant (Comasco et al., 2012). High levels of biomarkers (FAEE and EtG) may lead to low birthweight (Bana et al., 2014; Patra et al., 2011), as also demonstrated in guinea pigs by Brien and colleagues (2006) who found an association between meconium FAEE concentrations in guinea pigs and body weight and brain weight.

STRENGTHS AND LIMITATIONS OF THIS REVIEW

The review was supported by a systematic review of existing literature. We have chosen specific databases that cover the scope of this review, whereby the risk of selection bias has been minimized. An overview over the specific databases used and of the literature searches is outlined in supporting information along with a complete list of research studies relevant for the subject of this review. By performing a search in Web of Science using the "all databases" function, we have attempted to minimize the possibility of omission of other relevant literature.

CONCLUSION AND PERSPECTIVES

As of today, the reference standard in detecting alcohol fetal exposure is maternal self-reporting. An alternative way to assist in the diagnosis of children exposed to different levels of alcohol during their fetal life is the use of biomarkers. Biomarkers for such exposure will be a useful adjunct for research as well as for clinical practice. A number of different biomarkers have been suggested for this purpose. The most frequently applied biomarkers for detection of PAE are PEth, FAEEs, and EtG. Among these biomarkers, FAEEs in meconium and hair are presently the most used tools to estimate the incidence of PAE, as it has high sensitivity and specificity. Like FAEEs, EtG and EtS are detectable in various maternal and neonatal tissues. Even though only limited amount of data exist for EtG and EtS compared to FAEEs, recent studies have shown that EtG in meconium in combination with maternal hair is also a useful biomarker for prediction of PAE with high sensitivity and specificity. EtG and EtS are also both considered as useful urinary biomarkers for recent EtOH exposure. However, further studies of a combination of markers may be advantageous (Bana et al., 2014). FAEEs and EtG biomarkers have, in numerous studies, clearly demonstrated that they are applicable alone and in combination and are therefore obvious biomarkers to use for prediction of PAE. Studies on PEth in blood samples also indicate that this biomarker is applicable for detection of PAE, although the number of studies on this biomarker is few. However, PEth is clearly a biomarker that needs more attention in the future. In combination with a panel of other biomarkers such as FAEEs and EtG, it may be possible to predict PAE with very high sensitivity and specificity, thus providing

more insight into the prevalence of PAE. Besides being good predictors of PAE, FAAEs, EtG, and PEth also have in common that they can all be determined by various LC-MS/MS techniques with high sensitivity and selectivity, although the extraction and sample preparation is dependent on biomarkers and bio-specimens (Table 1). LC-MS/MS is therefore the most obvious analytical technique for the analysis of the most important biomarkers of PAE regardless of the bio-specimen, although other analytical techniques such as GC-MS and EIA have been used for the determination of these biomarkers. Meconium has been advocated as the most accessible matrix for detection of FAEE, EtG, and EtS and thus determination of PAE, but may be difficult to obtain within the required time, as opposed to blood and urine. Blood sampling is an invasive and painful procedure, but may be collected from routine samples at birth. Urine can be hard to collect, and the procedures for analyzing urine samples require, at present, special LC-MS/MS instrumentation and MS expertise, which, however, may be solved by developing a more appropriate urine sample preparation procedure involving, for example, a SPE step. Recently, hair has been used as a matrix and both FAEEs and EtG have been investigated in hair as a matrix. Biomarkers in hair contain the ability to measure exposure over time, as the metabolites are stored in the hair as it grows. However, studies conducted on neonatal hair samples are limited. A population study with a bio-bank of neonatal samples could be eminently suited for revealing the optimal biomarker or combination of biomarkers.

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AUTHORSHIP

All authors have read this manuscript and agreed to the submission for publication.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Data extracted from the studies on FAEEs.

Appendix S2. Data extracted from the studies on EtG and EtS.

Appendix S3. Data extracted from the studies on other biomarkers.

Appendix S4. Methods used in this review.