Patterns and Prevalence of Alcohol Consumption in Pregnancy Using Infant Biomarkers

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From research conducted in the Princess Royal Maternity, Glasgow

Abstract

Ethanol is an intoxicant widely available within the UK which has implications for the behaviour and capabilities of persons who have consumed even modest amounts, as well as significant longer-term health issues. Although the sale and consumption of alcohol is subject to legislative restrictions, consumption remains widespread in the UK.

Ethanol is recognised as being teratogenic to the developing fetus; while awareness of the potential adverse consequences of alcohol consumption in pregnancy is increasing, a significant number of pregnant women continue to consume alcohol despite national advice to the contrary.

This thesis explores the known effects of *in utero* alcohol exposure upon the unborn child, examines methods of detecting alcohol consumption in pregnancy and describes the pattern and prevalence of prenatal alcohol consumption in the West of Scotland as measured by ethanol biomarkers in the newborn and by confidential postnatal maternal interview. The thesis also explores the potential use of routinely stored newborn blood spot cards for retrospective ascertainment of prenatal alcohol exposure (PAE).

A total of 840 mothers consented to participate in an anonymised study of randomly selected participants, 92.5% of those approached. 827 mothers completed a confidential postnatal health questionnaire, including details of alcohol consumption in pregnancy and were asked to retain their infant's first stool (meconium) for analysis for alcohol biomarkers. Additionally, if mothers agreed and the baby's heel bled sufficiently, an extra blood spot card was obtained on day five, coincident with routine newborn blood spot screening. In total 740 meconium samples and 668 dried blood spot cards were returned, of which 712 meconium samples and 502 dried blood spots cards were analysable. 463 recruited mothers had previously delivered a baby and 17.8% of all participants had smoked in pregnancy. 46.4% of mothers declared some alcohol consumption in pregnancy, the majority of whom did not drink after realising that they were pregnant. 114 (13.8%) mothers declared some alcohol consumption after 20 weeks' gestation; in only three cases did this include five or more units of

alcohol. Mothers who declared alcohol consumption in later pregnancy were more likely to be aged > 35 years and to identify as white British ($p < 0.05$).

Fatty acid ethyl esters (FAEEs) were identified in all meconium samples with a concentration ≥600 ng/g in 39.6%. There was no relationship between maternal age, body mass index, socioeconomic status as determined by postcode of residence, parity or ethnicity and the likelihood of the infant's meconium being either negative or positive for FAEEs, as defined by a concentration ≥600 ng/g. Mothers tended to have been less likely to smoke during pregnancy within the group whose infant's meconium was positive for FAEEs (14 *vs* 20%, p=0.071). Correlation between FAEEs in meconium and self-reported alcohol consumption in pregnancy was poor; of the eight mothers that reported drinking at least three units of alcohol on any one occasion beyond 20 weeks of gestation, only three had an infant whose meconium was positive for FAEEs.

Ethylglucuronide (EtG) was detected in 41.1% of meconium samples and the concentration was ≥30 ng/g in 14.5%. There was no relationship between maternal age, body mass index, socioeconomic status as determined by postcode of residence or parity and the likelihood of the infant's meconium being either negative or positive for EtG, as defined by a concentration ≥30 ng/g. Infants whose meconium sample was positive for EtG were less likely to have a mother who identified as white British (71.3 *vs* 81.8%, p=0.028). When infant meconium was positive for EtG the mother tended to have been less likely to have smoked in pregnancy (13.6 *vs* 18.5%); this difference was not significant. Maternal self-report of any alcohol consumption in later pregnancy did not predict an infant meconium EtG concentration of ≥30 ng/g. When meconium samples positive for both FAEEs (≥600 ng/g) and EtG (≥30 ng/g) (n=51) were considered, there was a weakly positive correlation between the two biomarkers (Pearson's coefficient= 0.283, p=0.044).

Phosphatidylethanol (PEth) was detectable in 262 (52%) dried blood spot samples, with concentrations ranging from 2.4 to 3991.6 ng/ml. When the infant blood spot card contained ≥8 ng/ml PEth, the mother was more likely to self-identify as white British (86.6 *vs* 79.1%, p=0.028) and to have smoked during pregnancy

(p=0.047). Mothers of infants whose blood spot card contained ≥ 20 ng/ml PEth were younger (p=0.023), and their BMI was greater (p=0.038). They were also more likely to have smoked in pregnancy (p=0.21). There was no correlation between PEth measured in dried blood spot cards and either FAEEs or EtG measured in meconium.

Compared to self-report of alcohol consumption in pregnancy, the sensitivity of the individual biomarkers ranged from 11.6% for EtG ≥30 ng/g to 47.8% for a PEth concentration of ≥8 ng/ml, and specificities ranged from 57.1% (PEth ≥8 ng/ml) to 84.8% (EtG ≥30 ng/g). Using a combination of total FAEEs ≥600 ng/g and EtG ≥30 ng/g had a very high specificity for alcohol consumption either ever in pregnancy or only after 20 weeks' gestation, but a very low sensitivity of 6.7 and 5.3% respectively. A combination of FAEEs <600 ng/g and EtG <30 ng/g had a positive predictive value of 87% for no alcohol consumption after 20 weeks of gestation.

It is concluded that one in seven mothers delivering in the west of Scotland has consumed alcohol in pregnancy beyond twenty weeks' gestation. Reported amounts of alcohol consumed are likely underestimated.

This study indicates that routine use of meconium biomarkers for detection of alcohol consumption in pregnancy is not currently justified. Similarly, retrospective testing of stored dried blood spot samples taken on day five of life cannot be recommended as a measure of assessing PAE. Further work is required to explore relationships between alcohol biomarkers in meconium and longer term neurodevelopmental outcomes, as well as the role of measuring PEth in the newborn.

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Abbreviations

ADH	Alcohol Dehydrogenase
ADHD	Attention deficit hyperactivity disorder
APGAR	Appearance, pulse, grimace, activity, respiration
ASD	Autistic spectrum disorders
AUDIT	Alcohol use disorders information test
BMI	Body mass index
CAGE	Alcohol screen (cut down, annoyed, guilty, eye-opener)
CDC	Centre for Disease Control and Prevention
CHI	Community health index
CI	Confidence Intervals
CO	Carbon monoxide
CYP2E1	Cytochrome P450 2E1
DBS	Dried blood spot cards
DEPCAT	Carstairs deprivation category
EtG	Ethyl glucuronide
ELISA	Enzyme linked immunosorbent assay
FAEEs	Fatty acid ethyl esters
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
FRAMES	Brief intervention for risky alcohol use.
GGT	Gamma-glutamyl transferase
IQ	Intelligence quotient
Km	Michaelis constant (measure of affinity)
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOQ	Limit of quantification
MAST	Michigan alcohol screening test
MEOS	Microsomal ethanol oxidizing system
NPV	Negative predictive value
MRI	Magnetic resonance imaging
OFC	Occipital-frontal circumference
PAE	Prenatal alcohol exposure

PEth	Phosphatidylethanol
PNBS	Pregnancy and NewBorn Screening
PPM	Parts per million
PPV	Positive predictive value
PRM	Princess Royal Maternity
R&D	Research and Development
REC	Research Ethics Committee
SBR	Scottish birth record
SD	Standard deviation
SGA	Small for gestational age
SIGN	Scottish Intercollegiate Guidelines Network
SIMD	Scottish Index of Multiple Deprivation
SVD	Spontaneous vertex delivery
TACE	Alcohol screen (Tolerance, Annoyed, Cut-down, Eye-opener)
TLFB	Timeline follow back
TWEAK	Alcohol screen (Tolerance, Worries, Eye-opener, Amnesia, Kut-down)
UK	United Kingdom
USA	United States of America
USNIAAA	United States National Institute on Alcohol Abuse and Alcoholism
USDTL	United States drug testing laboratories
YCC	Yorkhill Children's Charity

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Except where otherwise stated, all of the work described in this thesis was my own.

1.1 Introduction

Presented in this thesis is a narrative of the consequences of prenatal alcohol exposure (PAE) including a brief description of fetal alcohol spectrum disorders (FASD). The importance of early diagnosis of FASD is emphasised, including potential for alcohol biomarkers as screening tests. Discussion is undertaken of how the prevalence each of PAE and FASD have and can be established, highlighting potential challenges with this. Consideration of demographic factors potentially affecting variation in alcohol consumption will highlight that focused interventions may be beneficial in reducing PAE. A brief description of the metabolism and teratogenicity of alcohol is presented to understand the mechanisms of harm, as well as metabolites which can potentially be utilised as alcohol biomarkers. A literature review of infant alcohol biomarkers focuses on establishing appropriate diagnostic cut-off values and combinations of biomarkers used for the assessment of PAE in media including meconium and blood spot cards.

Ethanol is an intoxicant widely available within the UK, with implications for the behaviour and capabilities of persons who have consumed even modest amounts, as well as significant longer-term health issues. Although the sale and consumption of alcohol is subject to legislative restrictions, consumption remains widespread in the UK, with 80% of adults in the UK reporting consuming alcohol at any time, and 57% reporting having consumed alcohol within the week prior to interview(1, 2).

Drinking alcohol in pregnancy can cause a wide range of neurodevelopmental and other harms to the embryo/fetus and is a leading, but preventable, cause of birth defects with lifelong implications(3). PAE (>5 drinks per week) is also associated with stillbirth(4). The effects of PAE manifest as FASD, often associated with other co-morbidities including congenital heart disease(5-7).

Appropriate prevention responses are therefore a public health priority. Pregnancy offers a unique time in a woman's life to engage with healthcare services and for public health interventions which can impact future pregnancies, care for the index child and the woman's future health. Screening for alcohol consumption in pregnancy is recommended by the World Health Organisation but it can be difficult for midwives to identify women at risk and many women under-report their alcohol consumption(8-10). A study in Glasgow which tested samples of

meconium for alcohol biomarkers in a high risk population found at least a five-fold higher prevalence of drinking in pregnancy than was self-reported(11). Preventive interventions at national level include health promotion and minimum pricing. It is important to have a reliable measure of PAE to be able to assess the success of current and future government and healthcare interventions.

Biomarkers of alcohol exposure in pregnancy for both mother and infant have been identified. A reliable alcohol biomarker would enable accurate measure of PAE both for individuals and at population level.

This thesis describes a research study exploring the use of biomarkers of PAE in the infant population within a Glasgow population in order to measure the prevalence and also the pattern of PAE. It aims to answer two main questions:

1) are there sociodemographic factors within the West of Scotland which are associated with a greater prevalence of PAE, therefore facilitating targeting of public health interventions and/or targeted screening for PAE?
2) is there potential to measure retrospectively PEth in newborn blood spot cards as a biomarker for PAE?

1.2 Epidemiology of PAE

1.2.1 Prevalence – UK and internationally

UK prevalence

A European cross-sectional study found self-reported prevalence of PAE highest in the UK (28.5%)(12). A systematic review of original quantitative studies that reported the prevalence in the general population of the respective country's alcohol use during pregnancy from 1984 to 2014 concluded that the UK had the fourth highest prevalence of PAE worldwide at 41.3%; 95% confidence intervals (CI) 32.9-39.0%(13).

Comparison with other countries

In Toronto one in 10 of the population reports consuming alcohol during pregnancy, with one in five drinking while breast feeding(14). There has been a recent rise in reported alcohol consumption in the last 30 days among pregnant

women in the USA, from 9.2% in 2011 to 11.3% in 2018 (15, 16). It is estimated that world-wide consumption of alcohol during pregnancy is around 9.8% but ranging from 0.2-25.2%, with Europe having the highest prevalence (13). There is international variance in the quantification of alcohol with a "unit" being 8 (UK) or 14 (US) g of pure alcohol, which must be taken into account when comparing studies(17).

1.2.2 National guidance

In 2016 the United Kingdom's Chief Medical Officer advised the safest approach to drinking alcohol in pregnancy is not drinking at all; this replaced previous advice of avoidance of alcohol in pregnancy(18). All women in Scotland should be asked about their alcohol intake during their initial pregnancy assessment to allow alcohol brief intervention or referral to addictions support(9).

1.3 Consequences of PAE

A brief summary of the metabolism of ethanol, the handling of alcohol by the fetus and subsequent teratogenicity is described as well as how metabolites may be utilised as biomarkers.

1.3.1 Alcohol Metabolism

Ethanol (C_2H_6O) is a hydrocarbon consisting of one ethyl group (CH3-CH2-) and a hydroxyl group (-OH). The ethanol molecule is hydrophilic and crosses cell membranes by diffusion. Its metabolism is a complex process dependent on movement between fluid compartments within the body and various enzymes with variable efficiency. The blood concentration of ethanol is determined by the rates of absorption, metabolism and excretion(19). Peak concentration is affected by the rate of consumption, strength of the drink, and factors affecting gastric emptying and absorption as well as the volume of distribution. Metabolism of ethanol is by several oxidative and non-oxidative pathways, with production of metabolites which have potentially toxic effects on cell function(20).

20% of ethanol is absorbed in the stomach with the remaining 80% absorbed in the intestine(19). Alcohol is mainly metabolised in the liver but also in a number of extra-hepatic tissues such as the brain(19). Genetic enzymatic variation can lead

to variation in both handling of alcohol and alcohol effects upon individuals(19). Most of the ethanol directly absorbed from the stomach undergoes first pass metabolism by ADH in the gastric mucosa. Variations in gastric ADH related to ethnicity and gender exist; the isoenzyme σADH is present in low levels in the Asian population compared to the Caucasian population and has lesser activity in females(21). First pass metabolism also accounts for the fact that blood alcohol concentration is less when alcohol is consumed orally compared to being injected intravenously. Gastric ADH function reduces in women following chronic alcohol use; the resultant lesser first pass metabolism is thought to contribute to the higher incidence of sequelae of chronic alcohol consumption in women(21). Normal gut bacteria also demonstrate ADH activity; resultant accumulation of acetaldehyde within the gut mucosa may explain gastric symptoms associated with chronic alcohol intake(21). Increased gastric emptying by drugs such as aspirin, H_2 receptors or food consumption, or direct administration of ethanol into the duodenum lead to higher peaks in blood ethanol concentration by bypassing first pass metabolism(21, 22).

Hepatocytes take up oxygen from surrounding blood, which causes localised hypoxia and so hepatocytes surrounding veins are the first to be impacted by ethanol(19). It is plausible that this diversion of oxygen from other tissues may adversely affect a developing fetus. There are three hepatic pathways for metabolism of ethanol occurring in varying intracellular locations: in the cytosol (alcohol dehydrogenase - ADH), endoplasmic reticulum, (microsomal ethanol oxidizing system - MEOS), and the peroxisomes (catalase). All of these pathways produce a toxic molecule called acetaldehyde. Acetaldehyde is then converted in the presence of aldehyde dehydrogenase to acetate.

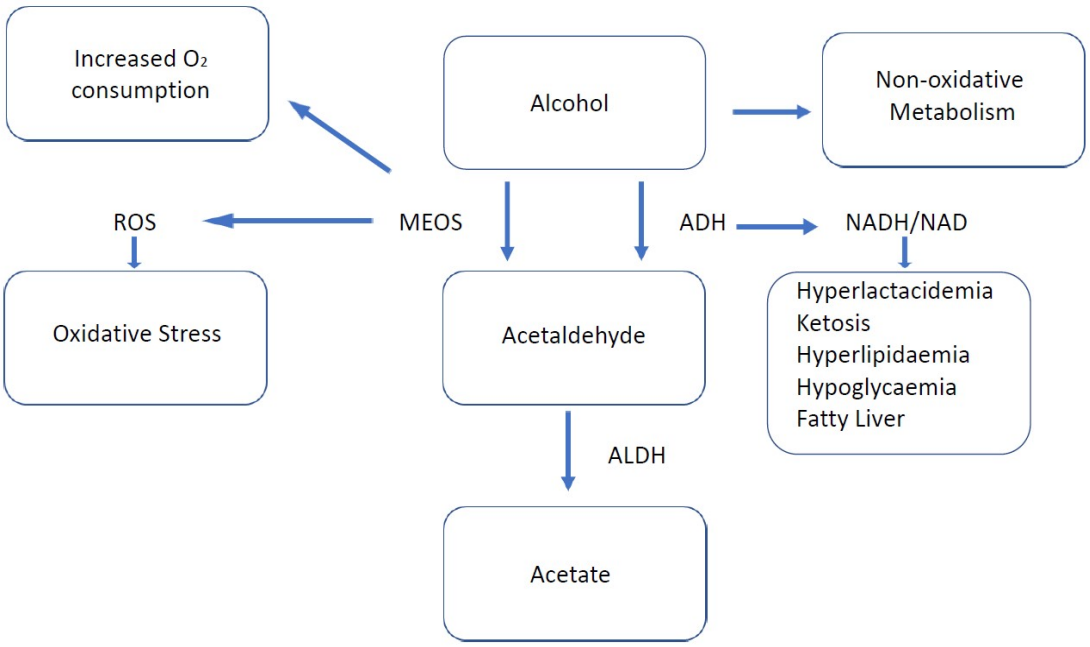

Figure 1.1 Pictogram of the metabolism of alcohol to acetate in the liver(21) (ADH - alcohol dehydrogenase, MEOS - Microsomal ethanol oxidizing system, ROS- reactive oxygen species)

ADH is responsible for most ethanol metabolism, both in the stomach and liver. Oxidation of ethanol to acetaldehyde and then to acetate by ADH involves transfer of a hydrogen molecule to nicotinamide adenosine dinucleotide (NAD), which becomes NADH. The ratio of NADH/NAD influences the concentration of α-glycerophosphate which affects triglyceride deposition within the liver. A higher ratio of NADH/NAD reduces the functional ability of the kidneys to excrete uric acid leading to hyperuricaemia. These cumulative changes contribute to the effects of liver disease subsequent to alcohol consumption(21).

ADH has several forms, classified based on the expression of seven genes(21). ADH class I is the most important and absence of this variant is associated with an increased likelihood of FASD. By contrast, mothers with more efficient ADH have a reduced incidence of children affected by FASD(23). The risk of FASD within different ethnic groups varies, being greater in Afro Caribbean women compared to those of Caucasian descent(24). This risk appears to be independent of the volume and pattern of consumption of ethanol.

The microsomal ethanol oxidizing system (MEOS), involving cytochrome P-450, presents an alternative oxidative pathway for ethanol and is important in the presence of high concentrations of ethanol(20). Chronic alcohol consumption does not result in increased ADH activity but induces CYP2E1 (of cytochrome-P450)(20, 21). Induction of CYP2E1 also leads to the production of other hepatotoxic agents and the formation of free oxygen radicals. The oxidative stress caused by this process contributes to alcoholic liver disease. MEOS may also lead to localised production of acetaldehyde, causing damage at the point of production(20).

Chronic exposure to alcohol is associated with increased malignancy in the gastrointestinal and respiratory systems(21). This may be explained, at least in part, by increased cytochrome P-450 activating carcinogens but there is also a strong link between alcohol consumption and cigarette smoking(21). Acetaldehyde is a highly toxic substance. In the liver, acetaldehyde can bind with proteins to produce acetaldehyde-protein complexes which lead to the production of antibodies(19, 21). Acetaldehyde also binds to proteins on erythrocyte membranes leading to enlarged erythrocytes(21).

There are two non-oxidative metabolic pathways for ethanol. The first produces fatty acid ethyl esters (FAEEs) from fatty acids and ethanol. FAEEs persist in tissues and play a role in tissue damage(21). The second pathway is the reaction between ethanol and phospholipid (phosphorus containing lipid) to phosphatidylethanol (PEth) in the presence of the phospholipase D, which has a high Km (Michaelis constant, affinity for substrate) for ethanol(19, 21). I will discuss these alcohol biomarkers in the literature review.

In summary, variation in an individual's ability to metabolise alcohol means that the effects of the consumption of alcohol are individualised, with no one cut-off defining a safe alcohol intake.

1.3.2 Alcohol distribution and metabolism in pregnancy

Body water volume of distribution for ethanol is lower in women than in men and is further affected by the reservoir of amniotic fluid and the fetal circulation

during pregnancy(20). Multiple factors affect kinetics of ethanol in pregnancy, including gestation, genetics and placental perfusion(25).

Placental function and ethanol metabolism

The main three enzymes involved in the metabolism of ethanol; CYP2E1, ADH and ALDH are found in placental tissue in varying concentrations and affinities. The placenta can therefore metabolise some ethanol. CYP2E1 has the highest affinity of the placental enzymes involved in alcohol metabolism and plays a larger role in placental metabolism; activity of CYP2E1 appears to be directly related to PAE(26). When ADH has been detected in placenta, it has been class II ADH, with lower affinity and reduced metabolic rate and therefore likely not to play a prominent role in alcohol metabolism. ALDH in the placenta also has a lower affinity and lower activity than that found in liver tissue. Thus, the placenta metabolises alcohol at a much slower rate than other maternal and fetal tissues(26).

Fetal absorption of ethanol

Ethanol in the blood stream of a pregnant mother readily crosses the placenta into the fetal circulation via the umbilical cord(25). Due to the highly vascular nature of the placenta and the hydrophilic nature of the molecule, ethanol crosses by rapid diffusion, and can be detected in the fetus within one minute of ethanol presence in the maternal blood stream. By one to two hours following maternal consumption, ethanol concentration in the fetal blood stream reaches equilibrium with the mother's blood concentration of ethanol(20, 27). The amount of ethanol crossing the placenta is affected by both peak maternal blood alcohol concentration and placental perfusion(26).

Having reached the fetus, ethanol is metabolised to several substances which include potential biomarkers including FAEE, ethylglucuronide (EtG) and PEth but excretion is still largely dependent on maternal metabolism and excretion(24). Transfer of ethanol to the fetus varies throughout pregnancy with amniotic fluid varying in volume in proportion to the fetus throughout gestation(20). Amniotic fluid makes up a larger proportion of the uterine contents during the first trimester, reducing to less than a quarter of the contents of the uterine cavity in later pregnancy. Once ethanol is in the amniotic fluid it is held in a closed

reservoir, to be diffused or reabsorbed by the fetus(28). Absorption of ethanol through swallowing occurs from around 11 weeks of gestation; in the last two trimesters, fetal swallowing increases with resultant increased exposure to ethanol in the amniotic fluid(20). Ethanol can also be absorbed from the amniotic fluid via the intra-membranous pathway, across a very thin amniotic surface into the fetal vasculature, at a rate of 200 to 500mls per day(28).

Fetal excretion of ethanol

Up to approximately 20 weeks of gestation alcohol permeates across the fetal skin into the amniotic fluid, but it is also excreted through lung fluid and by excretion of urine into the amniotic fluid cavity. Between 20 to 24 weeks of gestation when the skin becomes a more impermeable structure movement of alcohol between amniotic fluid and the fetus decreases(20, 28).

The rate of excretion of alcohol from the fetal compartment (based on reduced metabolism and dependency of diffusion across the placenta) is approximately 3-4% of the maternal rate of excretion of ethanol(28). Since diffusion across the placenta is bidirectional, the fetus can excrete alcohol back to the maternal circulation, but this requires a reduction in the maternal blood alcohol concentration and is affected by ongoing maternal alcohol consumption(24).

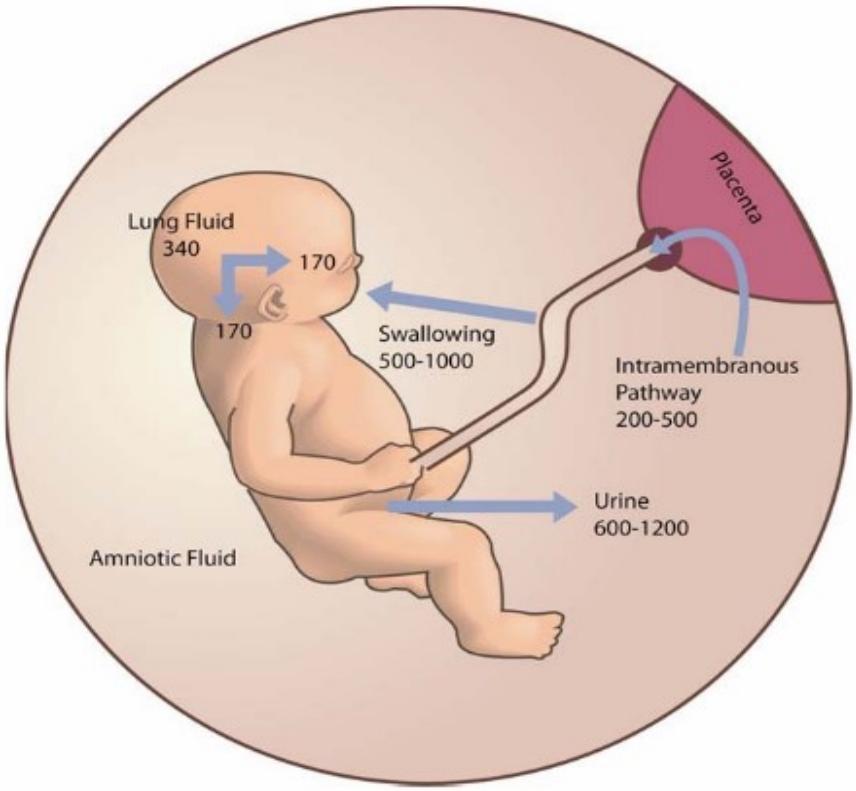

FIGURE 3. *Fluid flow rates in the fetal compartment are depicted by the schematic diagram of all known pathways for fluid and solute entry into and elimination from the fetus/amniotic fluid near term. The numbers represent volume flows in ml/day. The asterisk symbol denotes recirculating pathways. The double arrow represents the proportions of pulmonary excretions that are swallowed (50%) and those that enter the amniotic fluid (50%).*

1.2 Diagram of the fetal circulation and the flow rates of amniotic fluid in ml/day demonstrating re-circulating pathways(28). Reproduced with permission.

Fetal metabolism of alcohol

As noted above, alcohol is cycled through the fetal pulmonary and urinary fluid until it is metabolised by the fetus or placenta or removed back into maternal circulation. There are several fetal pathways for metabolism of alcohol, and these vary between fetuses(28).

Most fetal alcohol metabolism occurs in the liver. The liver is however not fully formed in the first trimester and indeed does not have adult capacity until one year of age. Enzyme function increases throughout gestation; fetal liver, as well as lung and kidney tissue, has functioning ADH at two months of gestation but enzyme activity is only 3-4% of adult hepatic function. CYP2E1 has been found in

fetal liver tissue from 16 weeks onwards and has a functional capacity of 10-30% compared to adult levels of CYP2E1(23). As the fetus has little ability to undertake oxidative metabolism of alcohol, metabolism of ethanol depends more on non-oxidative pathways than in adults(20). FAEE synthesis has been demonstrated in placental and embryological tissues of mice, indicating that the necessary enzymes are present early in pregnancy(20).

CYP2E1 is present in fetal brain tissue between 7-16 weeks of gestation(20). CYP2E1 can produce oxidative free radicals and acetaldehyde within the brain tissue which may cause direct neurotoxic effects on the developing central nervous system(20). Incomplete metabolism of alcohol in fetal tissue may also lead to the presence of toxic chemicals with teratogenic effects(21).

1.3.3 Teratogenicity

A wide range of teratogenic effects of alcohol have been described(29). The mechanisms of harm are multiple, not fully understood and do not appear to be directly dose related(29). Teratogenic effects may be caused by alcohol metabolites crossing from the maternal circulation (*e.g.* acetaldehyde) or by end products of metabolic pathways in the fetus (*e.g.* FAEEs)(29). Although ethanol levels in the fetus correlate with maternal levels, acetaldehyde levels do not. Despite the placenta providing some protective barrier of oxygenation prior to diffusion, maternal acetaldehyde crosses the placenta, where it can accumulate in the fetus, including the brain(30).

The organ most affected by alcohol exposure in the embryonic period is the brain. This is probably related to the high level of neurogenesis underway, including differentiation of cells and the migration of neurones(31). Other confounding factors such as nutritional deficiencies and smoking also undoubtedly play a role and the timing of alcohol exposure during pregnancy is associated with differing teratogenic effects(4). At a cellular level, the teratogenicity of ethanol and its metabolites is due to a combination of its effects on cell development, neurogenesis, tropic signalling, cell survival and cell death. Apoptosis of the neural crest cells brought about by oxidative stress is considered a plausible explanation of the typical fetal alcohol syndrome (FAS) facies(29, 32).

Ethanol can induce prostaglandin in the fetus, inhibiting growth and resulting in lower birthweight. Oxidative stress also has an adverse effect on the enzymes directly involved with chromatin structure, potentially leading to epigenetic alterations(33).

Teratogenic effects on the central nervous system

Microcephaly is the commonest structural brain defect identified, but microscopic brain changes are also becoming apparent and better understood with improved imaging, including both structural and functional magnetic resonance imaging (MRI). Much of our understanding of the teratogenic effects of alcohol comes from rodent studies; while rodent brain development is similar to human brain development, the rat is born at an earlier stage, with rodent gestation reflecting second trimester brain development in humans and the early neonatal period of a rodent reflecting the last trimester of human pregnancy(34). Rodents exposed to ethanol on day seven of gestation may develop abnormal union of left and right midbrain including the corpus callosum, with facial features including abnormally closely placed nostrils and long narrow upper lip(34). Day eight exposure to ethanol affects the hindbrain and cranial neural crest cells as well as cranial nerves, and larger ventricles can also be identified. Thus, when ethanol exposure occurs at these critical early stages of embryogenesis, the affected child will present phenotypically with the full clinical picture of FAS whereas exposure later in pregnancy may result in few, if any phenotypic effects(29, 35).

A reduction in volume in the corpus callosum is seen in PAE brains, with correlation between the macro and micro structural changes and the severity of FASD signs and symptoms, including the degree of facial dysmorphology. In general, reduction in corpus callosum size is associated with decreased executive functioning and impairment of motor skills(35).

The cerebral cortex of PAE brains shows aberrant formation of the gyri and sulci. Neurone migration into the cerebral cortex is impaired. Structural imaging identifies atrophy of the frontal cortex and functional imaging identifies deficits in frontal, parietal and temporal lobe functioning(35). Subcortical changes include

the hippocampus, thalamus and globus pallidus with a strong correlation between structural and functional deficits(35).

The cerebellar volume is smaller in PAE affected brains with displacement of the anterior vermis and posterior regions. Defects in the cerebellum tend to correlate with severity of FASD symptoms, and can present as behavioural difficulties(35). Small neuroimaging studies of 12 children with FAS and fetal alcohol effects (FAE) have demonstrated reduced overall levels of serotonin. Having reviewed animal models it is speculated this may be due to the mechanism of serotonin binding, apoptosis of serotonergic neurons or a combination of both factors(36, 37).

Understanding of the effects of ethanol at a cellular level may allow future therapies to be considered and implemented(35). Some animal studies have investigated whether controlled supplementation of retinoic acid may reduce the effects of alcohol on neural cell migration, but this requires caution as high retinoic acid levels can in themselves be teratogenic(38).

Twin pregnancies and PAE

Monozygotic PAE twins had similar phenotypical presentation and IQ scores, compared to just over 60% of dizygotic twins suggesting a genetic predisposition to the effects of PAE(39, 40) Subsequent work has highlighted some genetic mutations predisposing to alcohol induced cerebellar damage, including the homozygous mutation of the nNOS gene(41).

Potential contributors/confounders

Tobacco, cannabis, and cocaine have well described negative effects upon the fetus including vascular effects, both in the uterus/placenta and in the fetus (arterial occlusion). It has been postulated that impaired uterine blood flow could enhance the teratogenicity of ethanol(42).

Nutritional deficiency may also contribute to the adverse effects of PAE and has become a focus of research exploring potential dietary supplementation to reduce or prevent the teratogenic effects of ethanol(43). Although there is speculation that it is the overall reduction in nutritional intake seen alongside alcohol consumption which is important, studies are focusing on specific nutritional

replacements including vitamin A, choline and folic acid, as well as the role of zinc in neuronal development in the fetus(44). There is evidence from the mice model that zinc deficiency plays a role in the pathogenesis of FASD(45).

‘Safe’ amounts of alcohol in pregnancy

There has been a drive in research to identify ‘safe’ levels of alcohol intake in pregnancy to be able to guide expectant mothers. A number of systematic reviews have considered the evidence to date, with variable outcome measures of alcohol harm including FASD, poor fetal growth and childhood behaviour. A systemic review in 2007 of low-to moderate alcohol intake in pregnancy found no evidence of harm but was unable to draw conclusions due to weak evidence(46). In 2013 a review highlighted binge drinking was common in mothers whose child had been diagnosed with FASD and that binge drinking at least twice a week was more associated with the phenotype of FASD(47). A further systematic review in 2016 of low to moderate alcohol intake (32g or four UK units per week) and its effect on infants also did not identify specific harms but found that low to moderate alcohol intake may be associated with infants being born small for gestational age. This review therefore recommended a precautionary measure of abstaining from alcohol during pregnancy(48). Flak *et al.* in 2014 found that low level alcohol consumption (less than daily intake) may affect child behaviour, including social engagement, affect and conduct(49). The findings from this review suggested that alcohol harm may occur with less than one drink per day, resulting in a recommendation that mothers abstain from alcohol through pregnancy(49).

As discussed above the teratogenic effects of alcohol on the fetus are multifactorial including the mother’s ability to metabolise alcohol, fetal genetics, timing or exposure and nutrition; thus infant phenotype does not directly correlate with individual maternal alcohol intake(41-45). Safe levels of alcohol intake are therefore likely to be individualised to mother and fetus. Currently the advice from the United Kingdom Chief Medical Officers is to abstain from alcohol throughout pregnancy(18).

1.3.4 Description of FASD/FAS

PAE places children at risk of FASD, including its most severe presentation, FAS. The diagnosis of FAS rests on a triad of criteria including poor growth, specific facial features, and central nervous system abnormality in the context of a positive history of PAE. As the wider diagnosis of FASD includes conditions related to PAE which do not meet the full diagnostic criteria for FAS, the prevalence of FASD is considerably greater than the prevalence of FAS. It has been estimated that one in 67 children prenatally exposed to alcohol will develop FAS but many more will have alcohol related morbidities which do not meet the diagnostic criteria for FAS(13). The latter children are commonly undiagnosed(50).

FASD is associated with a wide range of comorbidities and its costs are seen in the healthcare, justice, and education systems(51, 52). Earlier diagnosis of FASD (before age six years) results in a reduction in secondary complications and adverse life outcomes as does stability in childhood, including fewer placements and absence of witnessing violence in the household(7, 8, 49, 53). Focused interventions may also lead to better outcomes. There is increased likelihood of a sibling having FASD, making the case for family screening and intervention when a positive diagnosis is made(54, 55).

FASD is often under or misdiagnosed, as the behavioural phenotype overlaps with other conditions particularly autistic spectrum disorders (ASD) and activity-deficit hyperactivity disorder (ADHD)(50, 56, 57). Guidelines exist for distinguishing the different presentations, noting that that children with FASD present a very specific profile(57). Healthcare professionals report a lack of knowledge of FASD which provides a challenge to ensuring accurate and timely diagnosis, furthermore children may have multiple diagnoses; such as ASD in addition to FASD complicating a diagnosis(58). The importance of skilled psychology assessment in addition to general awareness cannot be overemphasised.

This study described in this thesis concerns the prevalence of PAE, *i.e.*, the number of children who are born at risk of FASD because of having been prenatally exposed to the potentially teratogenic effects of alcohol. These children will be referred to as infants who have been ‘alcohol exposed’.

1.3.5 Prevalence of FASD

A number of population studies have been undertaken to establish the prevalence of FASD as well as FAS. Globally, the estimated prevalence of FASD is 7.7 per 1000 in the general population, with the highest prevalence in the European region at 19.8 per 1000(59). McQuire noted in her thesis that in the UK, one study published in 2017 estimated that 3.3% (95% CI 2.0% - 4.9%) of children in the general population may have FASD, based on the assumption that one in 13 children with PAE will develop FASD(59, 60). A study in the UK applied a novel FASD algorithm to pre-collected data and estimated an incidence of FASD of between 6-17%, but the true prevalence of FASD is not known(61). It has been estimated that 80% of FASD is never diagnosed and that 7% is misdiagnosed as other neurodevelopmental conditions(62). Worldwide it is been estimated that 1.5 in 1000 infants are born with the full stigmata of FAS(13).

Caution should be applied in comparing reports of the prevalence of FASD as there is variability between studies, including diagnostic guidelines. Current FASD guidelines include Astley's 4-digit code, Canadian Guidelines and the more recently published Scottish Intercollegiate Guidelines Network (SIGN) guidance(56, 63, 64). It is good practice for the diagnostic criteria to be documented in studies reporting prevalence of FASD.

Study design also varies, and may include clinic-based studies, passive surveillance, and active case attainment. Clinic-based studies will only pick up those that engage with services and will, by definition, report higher rates. Passive studies use previously collected data such as birth records and can capture large populations but are dependent on accurate databases(62, 65). Active case attainment utilises population screening to seek out positive cases and is not dependent on either existing databases or case reporting as for surveillance studies. Active case attainment is expensive and labour-intensive but is likely to be the most accurate means of calculating prevalence. It can be used to capture a focused population group, *e.g.*, school pupils(62).

In Scotland, it is acknowledged that under-reporting of FASD occurs due to lack of knowledge and misdiagnosis as well as poor access to prenatal alcohol history(58,

64). During a 60-month period (2010-2015) active surveillance carried out by the Scottish Paediatric Surveillance Unit reported 41 cases of FAS, giving a prevalence of 0.19/1000 live births(64, 66, 67). There was a reported association with maternal smoking and drug misuse(67). A further active reporting study is underway by the British Paediatric Surveillance Unit Team (Royal College of Paediatric and Child Health)(68). By comparison, a UK study estimating FASD prevalence in the UK identified that a diagnosis of FASD was more common in unplanned pregnancies and children of lower socioeconomic background(61).

Prevalence of a condition may depend upon the population selected; special education, looked after and accommodated children and prison populations have all been recognised as having higher prevalence of FAS/FASD compared to the general population. The prevalence of FASD is between 30.5 and 52% in children in foster care and between 9.8 and 23.3% in prison settings(69). An estimated 12-23% of individuals entering the juvenile justice system have FASD, largely previously undiagnosed(70-72). This high prevalence may explain at least in part common impulsive behaviours and difficulty comprehending consequences which Is described as part of FASD phenotype.

1.3.6 Cost of FASD

The financial cost of FASD to the population includes healthcare, education and judicial services and is difficult to estimate(52, 71). Individuals with FASD have increased hospital admissions, increased comorbid conditions such as congenital heart disease, low birth weight, sensory-neuro hearing loss, alongside a range of abnormal neurological findings in keeping with their diagnosis(5). Extra education support is often required, and there is potential loss of earning through the challenges of employment in the presence of disability. Secondary health complications and suicide lead to premature death(52, 54, 73). Taking into account all these factors, it has been estimated that the lifetime cost of FASD is $1.5 million per individual(52, 74).

1.4 Ascertaining PAE

A number of studies have been undertaken to ascertain the prevalence of alcohol intake in the pregnant population, utilising both maternal self-report and alcohol biomarkers. There are however concerns in regard to the accuracy of these methods, and thus the accuracy of reported prevalence of PAE. The importance of documenting the prevalence of alcohol consumption in pregnancy includes being able to measure the effectiveness of public health interventions and make preparation for health and education requirements of children with FASD. The Scottish Maternal and Infant Study in 2017 reported that younger and more deprived mothers were more likely to report alcohol avoidance pre-pregnancy(75). 45% of mothers reported not drinking in pregnancy of whom 14% never drank and 31% stopped drinking in pregnancy(75). 54% of mothers reported continuing to drink in pregnancy, although 25% cut down the amount of alcohol taken. 88% of all mothers who reported drinking alcohol stated that they had stopped drinking alcohol when they found out they were pregnant. The survey response was however only 30%, with potential for selection bias; younger and more deprived women were underrepresented in this survey(75).

A further study in Scotland carried out cross sectional assessment of maternal intake using a 7-day retrospective diary(76). It also found that alcohol consumption fell on recognition of pregnancy and that the pattern of drinking remained biased towards the weekend. The most socioeconomically deprived women drank less than their less deprived counterparts but the patterns in drinking also varied with deprivation. Women from more deprived areas tended to drink less alcohol but more consistently and women from more affluent areas reported exceeding recommended limits for alcohol consumption more often. These findings are not in keeping with alcohol harm being greater in poorer areas(76). The introduction of minimum alcohol pricing in Scotland has seen a reduction in the amount of alcohol purchased per household, particularly in poorer families; ascertaining the true impact of this on PAE is difficult without accurate assessment of PAE(77).

A population based observational study of 235 mother infant dyads born at the

Princess Royal Maternity in Glasgow measured two different alcohol biomarkers, FAEEs and EtG, in meconium. This pilot study, carried out by a different researcher from the supervisor's team, provided feasibility for the main study described in this thesis, concluded that, based on EtG concentration of ≥30 ng/g in meconium, 15% of pregnancies were exposed to alcohol, despite only 3% of mothers reporting alcohol consumption(78). In this study, mothers from more affluent areas tended to be more likely to have infants whose meconium contained significant amounts of FAEEs, but the difference was not significant(78).

1.4.1 Maternal self-report

PAE screening by self-report appears to be more accurate soon after delivery compared to later in the infant's life(79). Retrospective diaries have been demonstrated to identify higher alcohol consumption(76). However, under-reporting is a major concern in alcohol screening, with recall bias and worries around stigma all being cited(76). Women commonly underestimate the volume of alcohol they are pouring and mothers are reported to underestimate by two thirds the amount of spirits they perceive themselves to be drinking(76, 80). There is some evidence that self-report is more accurate when not collected by a healthcare provider(76).

There are a range of validated screening tools for alcohol intake; some screening only for risky drinking and others seeking to establish more accurate attainment of alcohol intake. AUDIT-C (Alcohol use disorder Identification test) and TWEAK (tolerance, worry about drinking, eyeopener, amnesia and cut-back) are screening tests which identify at risk drinking(81). Timeline follow back is a retrospective diary which documents the amount of alcohol over a period of time, providing an accurate report of alcohol intake including variation, e.g., weekdays *vs* weekends, pregnancy *vs* pre-pregnancy(79, 82). The traditional timeline follow back is 14 days, however this can be modified to focus on a particular period. As women may change their drinking behaviour throughout pregnancy, a timeline follow back method is able more accurately to establish drinking patterns throughout pregnancy(79). This is important when validating alcohol biomarkers formed from 19-20 weeks of pregnancy onwards. Ascertainment of accurate cut-off values for alcohol biomarkers is made difficult by inaccurate self-report and little

differentiation between descriptions of “low” or “moderate” alcohol consumption through pregnancy.

Lange’s systemic literature review of studies between 1999 and 2005 summarised 10 population studies of the use of FAEEs in meconium compared to maternal self-report for the assessment of PAE(83). Nine of the included studies utilised a cut off ≥600 ng/g; prevalence of positive meconium samples ranged between 2 and 45%. There was wide variance from the maternal self-report, which was 0.7 to 43 times less likely than a positive meconium sample. The studies included a range of methods of maternal self-report, including medical records and questionnaires with likely variation in accuracy, but it is reasonable to conclude that PAE is consistently underreported to healthcare professionals(83).

1.4.2 Sociodemographic patterns of PAE

A number of studies have focused on sociodemographic factors in an attempt to identify high risk groups of pregnant women. Drinking in pregnancy has been associated with deprivation and therefore assumed to contribute to health inequalities associated with poverty(84). However, as noted, a recent pilot study of alcohol biomarkers in meconium in Glasgow noted a trend towards more PAE for newborns of more affluent pregnant women, as did a Scottish government survey study of recently delivered mothers in 2017(75, 78). In the latter study younger women were more likely to report avoiding alcohol. Across two Scottish health boards, more affluent women were more likely to drink alcohol, although as already discussed, the pattern of alcohol consumption varied with socioeconomic status(76). A pan European cross-sectional study identified higher education and smoking as risk factors for PAE(12). A study performed in the United States Centre for Disease Control (USCDC) identified that mothers >35 years of age as well as mothers who had attained higher education level, were in employment and were unmarried were more likely to consume alcohol in pregnancy(85). A 2015 study in a Canadian population found that smoking either before or after pregnancy increased the likelihood of alcohol use in pregnancy(86). Confirmation of sociodemographic predictors of PAE could allow focused public health intervention and early screening of potentially affected infants.

1.4.3 Alcohol biomarkers

Since there is no diagnostic test for FASD and maternal self-report of PAE is unreliable, measuring alcohol biomarker may be useful in the identification of PAE(83). While work has been undertaken in the adult population assessing markers of alcohol exposure including mean cell volume, gamma-glutamyl transferase (GGT) and carbohydrate-deficient transferrin, caution must be exercised in the interpretation of biochemical changes associated with pregnancy(87). A study of the prevalence of alcohol exposure in early pregnancy based on antenatal booking bloods in a Northern England population demonstrated elevated GGT in 4.2% of blood tests analysed, suggesting early pregnancy alcohol exposure(88).

Among the range of other biomarkers, measurement of FAEEs in infant meconium is reported to be the most promising medium for detection of alcohol biomarkers, with studies reporting correlation with neurodevelopmental outcomes(89-91). Newly delivered mothers have demonstrated willingness to take part in studies of infant meconium as a potential screening tool for PAE, as demonstrated by Abernethy *et al.*(78). Infant biomarkers may be of use for both individual and population screening, enabling targeted developmental follow up as well as measurement of public health outcomes and could potentially be complementary to self-report. Carbon monoxide (CO) measurement is currently used in Scotland as an adjunct to self-report for smoking screening in pregnancy. In determining PAE, there is potential benefit to the child of early diagnosis of FASD, and for family interventions, both sibling screening and removal of risk for future pregnancies(7, 54, 92). Validated cut-off values for infant alcohol biomarkers are important and so a literature review focusing on infant biomarkers was undertaken in order to establish cut-off values for both FAEEs and EtG measured in meconium.

1.4.4 The ethics of screening

Pregnancy is a unique time when the ethical focus of care changes from one to two lives; screening may be undertaken during pregnancy and/or in the newborn period. Pregnancy and newborn screening aim to detect treatable conditions to effect better outcome for either or both mother and baby. The primary purpose of screening is that it offers benefit to the person being screened(93, 94). The UK

national screening committee has a very specific list of criteria which must be met before a screening test is approved; the screening test should provide more benefit than harm and should be affordable. For tests of alcohol screening in pregnancy and infancy to translate from research to a national screening process, the issues should be focused on reliability of testing, changes to outcomes for the child, cost to NHS and ethical issues surrounding the use of tissue for screening. A screening test should have high sensitivity and specificity for the condition(95).

Informed consent is required for participation in screening and mothers consenting to screening of their infant must understand that some tests (including testing for alcohol biomarkers in meconium) are actually testing both infant and mother. The use of meconium as a screening tool for PAE is non-invasive and therefore relatively easy, with no need to consider discomfort to the patient, but limitations in terms of both the window in pregnancy which will be screened and the accuracy of alcohol biomarkers must be considered(96).

A positive meconium screen for PAE would have both positive and negative connotations. On the one hand, a result would be useful in allowing alcohol interventions for the mother and potentially earlier assessment with heightened awareness of a potential diagnosis of FASD for the infant. However, there are negative social consequences for the mother of positive alcohol screening, and more recently a legal case has been bought against a mother for alcohol related harm(97). To be useful, national screening of maternal and/or infant biomarkers for PAE would need a high uptake and be supported by potential developmental interventions for the infant. Women have suggested they would support screening when coupled with such follow up(95, 98). However, while a positive screen may be of benefit to the infant it may not be of interest for the mother and therefore generate a conflict of interest in acting for the patient's best interest(94). At the time of carrying out this research project the infrastructure required to carry out developmental screening on a potentially large, identified population of at-risk infants was not present. The new SIGN guidelines have however increased awareness of FASD in Scotland, and highlighted the importance of accurate measure of the prevalence of PAE in order to be able to allocate resources in the future(64, 84).

Newborn blood spot cards are used for screening within the UK as part of national screening for treatable conditions, with the conditions screened for recommended by the UK national screening committee(93, 99). In the UK informed written parental consent which includes storage for future research is required for newborn screening(100). Retrospective testing of blood spot cards is undertaken with parental consent for certain conditions, including congenital cytomegalovirus infection for those children presenting with attributable signs and symptoms. However, it is noted that in some countries, concerns regarding potential misuse of residual newborn blood spot cards have led to the destruction of these cards(100). If an alcohol biomarker can reliably be detected retrospectively in stored newborn blood spot cards, this might have potential for helping in the diagnosis of children presenting with signs of FASD, particularly if no PAE history is available. As noted earlier, FASD is over-represented in accommodated children whose mothers may not be available to discuss alcohol behaviours in pregnancy. This would raise issues of consent for testing, which would need to be given by the parent or legal guardian. A number of research papers have reported the usefulness of measuring PEth in dried blood spot cards as a reflection of PAE for surveillance purposes; to date there have been no studies from the UK(101-103).

1.4.5 Measurement of alcohol biomarkers in tissues

As previously discussed, ethanol is rapidly metabolised in the mother and therefore direct assay of ethanol, either in blood or urine cannot be utilised as a measure of fetal exposure(27). Biomarkers which have been utilised in the adult population to monitor compliance to an alcohol-free lifestyle such as FAEEs and EtG may also be measured in the infant. Biomarkers from the fetus/newborn have potential not only to provide an absolute measure of PAE but also to reflect the effects of PAE upon the fetus. Confounding factors in fetal exposure, including maternal and placental metabolism of alcohol and placental diffusion, mean that maternal alcohol consumption may not directly correlate with fetal exposure. Media from the fetal compartment which have been considered for the analysis of biomarkers to assess PAE including placenta, amniotic fluid, meconium and neonatal hair and blood. Currently no fetal compartment sample is routinely screened for PAE in the United Kingdom. Indeed, none of these media is routinely

collected, with the exception of a blood sample on day 5 of life for the purpose of newborn blood spot screening.

Placental tissue is able to metabolise ethanol independently resulting in accumulation of FAEEs(26). Ethanol affects placental and umbilical cord blood flow with oxidative stress within the placental tissue a possible mechanism of harm(26). A study of placental FAEEs in combination with maternal self-report (AUDIT+) suggested PAE in one third of preterm infants but the authors concluded that measuring FAEEs in infant meconium was likely to be a more accurate measure of ethanol effects on the infant(104). Collecting placentas for such a large population as our study was impractical, as was collection of amniotic fluid. Neonatal hair is not a reliable marker of PAE(105). In a study of 99 mother-infant dyads both maternal and infant hair as well as meconium were analysed for FAEEs and EtG; all samples of hair tested negative for both biomarkers compared to an 82.2% positivity rate for EtG in meconium(105). Coupled with the difficulties in obtaining the 30–50 mg hair required for analysis for FAEE or EtG, neonatal hair sampling was not considered appropriate for this study.

The majority of data in regard to alcohol biomarkers in the newborn relate to meconium, the first stool passed by the infant. Meconium is formed from epithelial tissue, bile and hair and consists of 60-80% water(106). It accumulates from 16-20 weeks' gestation onwards and increases exponentially in weight throughout the pregnancy. Meconium is passed by the infant within 72 hours of birth, generally in the first 24 hours. It is considered a waste product and is not routinely collected. Three quarters of the baby's meconium is attributed to last eight weeks of pregnancy, but analysis of any meconium passed after birth will potentially reflect alcohol exposure from 16-20 weeks of gestation. Meconium should be sterile, although rarely it can be contaminated by bacteria. The two main alcohol biomarkers explored to date in meconium include FAEEs and EtG(106).

In a systematic review of objective measures of PAE published in 2016, McQuire *et al*. noted a high risk of bias and selective outcome reporting. Studies including three different cut off values of total FAEEs of 600 ng/g, 1000 ng/g and 10,000 ng/g and utilising combinations of 4, 6 and 9 FAEEs respectively were

examined(107). A total concentration of 4 FAEEs in meconium of ≥600 ng/g demonstrated the highest sensitivity for PAE (100%), but with very variable specificity (13 – 98%). However, while McQuire found FAEE in meconium to be one of the most promising biomarkers of PAE, there was insufficient evidence to support the use of objective measures of PAE in screening(91). The variable specificity of FAEEs in meconium led to a concern about a high number of false positives which could lead to a burden on both mother and healthcare(91). It was concluded that while there was insufficient evidence to support the use of objective measures of PAE in clinical practice, biomarkers in meconium merited further investigation.

1.5 Literature review

A literature search was undertaken based on current Cochrane guidelines for literature reviews. The aim of the search was to review infant biomarkers of PAE, specifically appropriate cut off values of specific biomarkers. The search for studies was undertaken from two databases: Embase 1947 to 2021 and Ovid Medline 1947 to February 2021. Discussion was undertaken with a librarian at the University of Glasgow and other databases were not used to avoid overlap and duplication. Journals were restricted to English language, full text. The key words utilised from the search terms included: foetal, fetal, pregnancy, infant, FASD, FAS, fetal alcohol syndrome, fetal alcohol spectrum disorder, alcohol, ethanol drink, biomarkers, fatty acid ethyl esters, ethyl glucuronide, phosphatidylethanol, FAEEs, EtG, PEth. Boolean logic was utilised for search terms to focus the search.

The study generated 618 references; once duplicates were removed this left 504 studies of which 399 were excluded based on their title. A further eight relevant studies were identified subsequent to the literature search. Included studies were primarily population recruitment of maternal-infant dyads with collection of meconium and/or blood spot cards for analysis of biomarkers (FAEE, EtG and/or PEth) as a measure of PAE.

Articles which had titles of relevance (113) had their abstracts reviewed, and from them 59 were selected for full paper review. 20 relevant articles were categorised by their primary biomarker of interest and reviewed by two researchers (including myself and one of my supervisors) to extract relevant information. Papers were tabulated and evidence summarised including methods and cut-off values utilised for each biomarker. Additionally, we considered whether the cited cut-off value had been derived from self-reported PAE within the study populations or adopted based on previous studies. All data were reviewed after being compiled into a table.

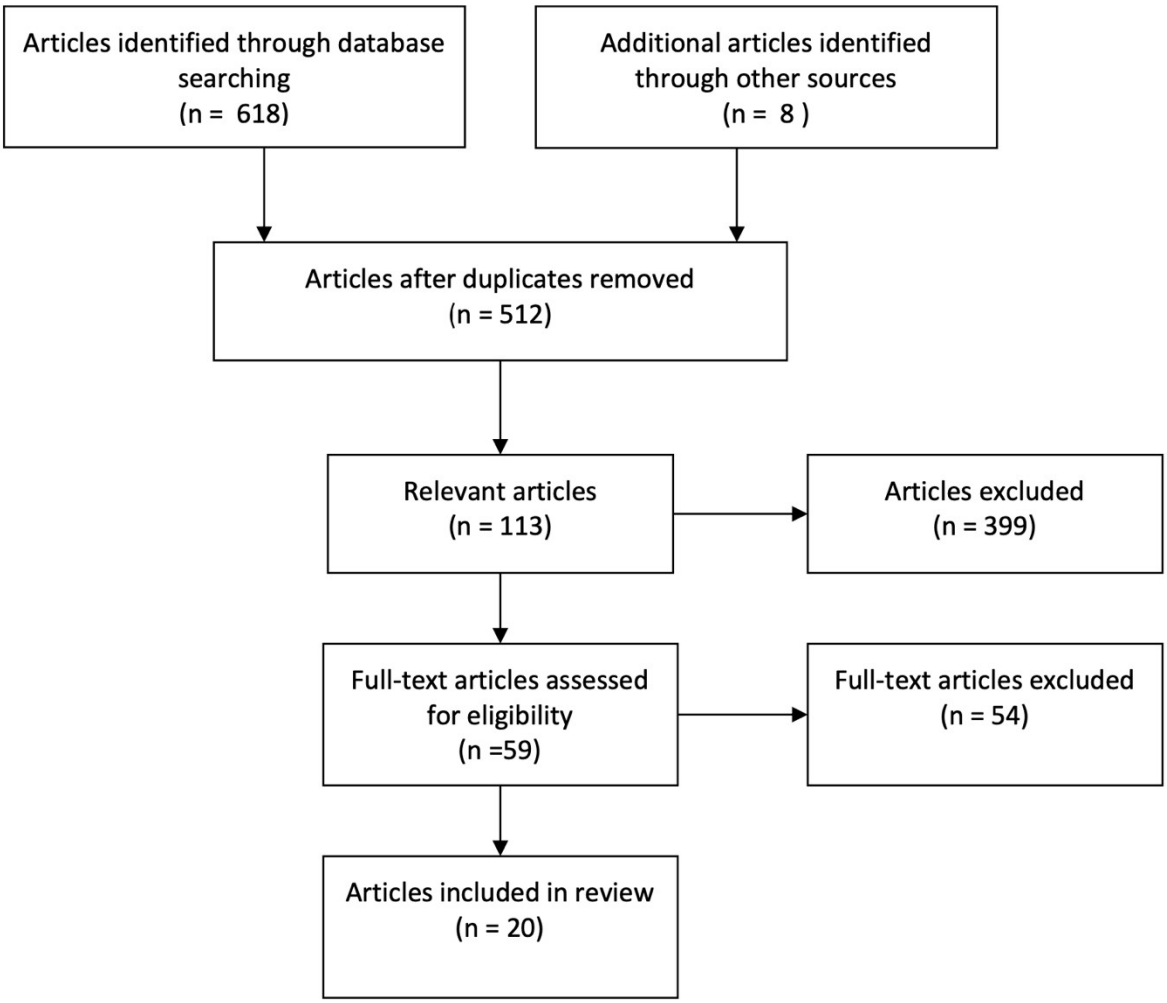

Figure 1.3 PRISMA diagram of literature review for infant biomarkers of prenatal alcohol exposure

Within the 20 studies included studies were: six studies with primarily FAEEs measured in biomarkers, seven studies of EtG in meconium (one also included FAEEs) and eight studies of PEth measurement in blood spot cards. Papers describing the same population were excluded.

1.5.1 Fatty Acid Ethyl Esters

FAEEs are direct metabolites of ethanol, formulated through non-oxidative pathways in the presence of FAEE synthase and ethanol acyl-O-transferase enzymes. They can be produced from both exogenous and endogenous ethanol. FAEEs are more hydrophobic than regular fatty acids and therefore can cross the cell membrane with relative ease(108). They are transported in blood by lipoproteins(109). As FAEEs cannot cross the placenta, FAEEs present in any fetal samples represent fetal production(110). FAEEs affect cellular proliferation and impair the synthesis of proteins(109). It is hypothesised that FAEEs may themselves be mediators in the pathogenesis of alcohol-related end organ damage and therefore may contribute to the teratogenicity of ethanol(90).
Higher levels of FAEEs in meconium have been correlated with lower educational attainment and lower IQ scores(89). Within a population of 191 mainly African American children of low socioeconomic background followed up through childhood and adolescence, higher concentrations of 4 FAEEs (ethyl myristate, ethyl oleate, ethyl linoleate and ethyl linolenate) in meconium were related to lower Wechsler intelligence scores and full-scale IQ scores(89). The author acknowledged limited correlation with maternal self-report of alcohol consumption in pregnancy and also noted that higher concentrations of FAEEs in meconium were related to higher birth weights.

Endogenous alcohol production results in a low level of FAEEs in meconium, therefore requiring the establishment of a cut-off values for the assessment of maternal alcohol consumption(111). Endogenous alcohol production occurs as a by-product of the normal microflora of an adult gut and ethanol is also a by-product of yeast and bacterial infections, with increased production of FAEEs in chronic infections(111).There are additionally some dietary effects as olive oils contain FAEEs in varying concentrations(111). Production of FAEEs in meconium may occur due to bacterial contamination as gut flora are established in the infant(112).

The first description of FAEEs in meconium was in an abstract by Mac *et al.*, published in 1994. They reported on 25 meconium samples, of which 15 were from PAE newborns, and demonstrated higher FAEEs (ethyl laurate, palmitate,

stearate) in the alcohol exposed samples. Their conclusion was that FAEEs in meconium might be a promising biomarker for PAE.

Subsequently there have been a number of population studies seeking to establish the prevalence of PAE by measuring FAEEs in meconium. These studies have investigated a range of between three and nine FAEEs, either comparing individual concentrations or cumulative concentrations of differing combinations of FAEEs. They have utilised various values to define a 'positive' sample, ranging from 50 to 10,000 ng/g. There has been variability in study populations although all have been hospital based, either single sites or multi-centred. Some studies have included control populations allowing comparison of meconium from alcohol exposed infants and those that had not been exposed. Ostrea *et al*. focused on individual biomarkers and found ethyl linoleate correlated with maternal self-report(113). Pichini *et al*. recruited from seven different hospitals across Italy and Spain and were able to demonstrate significant geographical variance in FAEEs in meconium(114). They related these findings at least in part to public health work; despite repeated analyses no FAEEs were found in meconium from babies born in areas in which a strong public health message had been delivered regarding the dangers of PAE(114).

One important consideration is the definition of a positive sample; research papers which have compared concentrations of FAEEs in meconium with maternal self-report are summarised in table 1. These five studies have used differing populations and differing methods for ascertainment of self-reported alcohol consumption as well as having differing proportions of PAE and non-exposed newborns.

Bearer *et al*. carried out testing on 248 meconium samples screening for eight FAEEs, and categorised mothers by alcohol exposure following postnatal questioning(115). They identified that ethyl linoleate was the FAEE of the eight which had the most presence in meconium of those infants whose mothers reported one or more drinks in the last trimester, but they did not establish any cut-off values for the biomarkers(115). The same research group then carried out a small study of 27 mothers and infant dyads screening meconium for three

individual FAEEs (ethyl palmitate, ethyl oleate and ethyl linoleate) compared to time line follow back ascertainment of alcohol consumption in the second and third trimester and found that ethyl oleate had a sensitivity of 84.2% and specificity of 83.3% for an alcohol intake of at least 42 g per day(116).

Chan *et al*. in the same year compared meconium concentrations of FAEEs to maternal self-report of PAE to establish appropriate cut-off values and sensitivity(111). They compared meconium samples from two non-drinking pregnant populations in Toronto and Jerusalem (208 mothers in total) with six meconium samples from known PAE infants(111). FAEEs were present in all meconium samples and a cut of off 600 ng/g (2 nmol/g) for a cumulative concentration of 4 FAEEs (ethyl palmitate, ethyl stearate, oleate and linoleate) provided the best sensitivity (100%) and specificity (98.4%) for the detection of PAE. These 4 FAEEs were utilised by English *et al* with a cut off 600ng/g in a Ugandan population of 505 women and infants, however found higher detection of PAE by TWEAK questionnaire than FAEE in meconium (16% *vs* 2%)(117). Bearer's work on ethyl linoleate was further confirmed by Ostrea *et al*. in 2006; in a larger case control study of 124 maternal infant dyads ethyl linoleate was linearly associated with PAE as ascertained by screened by MAST, CAGE and TACE screening tools(113). The sensitivity of ethyl laurate >0.05 μg/g (LOD) was 26.9% and the specificity 96.8%(113).

Yang *et al*. carried out a prospective cohort study including 182 meconium samples from infants of alcohol abstaining mothers and 54 samples from "light to moderate" drinking mothers(118). At 34 weeks' gestation mothers completed an electronic questionnaire with regards to their alcohol intake, to try to reduce response bias. Women were classified as light risk (< three drinks per week) and moderate risk (3-7 drinks per week) as per the United States National Institute on Alcohol Abuse and Alcoholism (USNIAAA) with 1 drink equating to 14 g of ethanol (just less than two UK units). Utilising a total of nine FAEEs (ethyl laurate, myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate, arachidonate), the authors found no difference between meconium samples from infants of abstainers and drinkers. They noted a linear increase in FAEEs above a threshold of three drinks per week, but this was based on only two cases. Yang's

conclusion was that low to moderate alcohol intake during pregnancy cannot reliably be assessed by measurement of FAEEs, but that there may be a linear relationship between FAEE concentration in meconium and PAE above a threshold of three drinks (approximately 42 g) per week(118).

Kwak also measured the total of nine FAEEs in a Korean cohort study(119). Mothers provided an alcohol history at 34 weeks' gestation via an electronic form and were categorised based on their self-report as low, moderate, and high-risk drinkers as per the USNIAAA. 38.1% of mothers reported alcohol consumption in at least one trimester of pregnancy, the majority of whom were light drinkers. The authors explored different cut off values for a total of FAEEs in predicting PAE as self-reported; 600 ng/g had a sensitivity of 22.2% and specificity of 73.6%, 3000 ng/g a sensitivity of 5.6% and a specificity of 94.5% and 6000 ng/g a sensitivity of 3.7% and a specificity of 98.4%. The authors found no correlation overall between self-report of alcohol consumption in mid-late pregnancy and presence of any FAEEs in meconium(119). With a cut off of 6000 ng/g however, total FAEEs in meconium correlated with both the amount and the frequency of self-reported alcohol consumption. Their conclusion was that assumption of PAE in the absence of self-report could only safely be made with a cumulative FAEE concentration of >6000ng/g.

Himes *et al*. related meconium FAEEs to maternal self-report of alcohol consumption in pregnancy for 107 mother and infant dyads(120). They utilised the timeline follow back method to establish maternal PAE, considering alcohol type, amount, and duration. They applied questionnaires four times between six weeks' gestation and one month post-delivery; mothers were also questioned about pre-pregnancy alcohol consumption. Alcohol consumption beyond 19 weeks of pregnancy was designated as positive exposure (i.e., during the period of meconium formation). 58 women drank after 19 weeks' gestation, of whom 45 women drank into their third trimester. Nine FAEEs were assayed; the authors measured individual FAEEs then carried out summation of four, six and nine FAEEs. A cut off of 200 ng/g for four FAEEs (myristate, palmitate, oleate and stearate) yielded the highest prediction of PAE with a sensitivity of 64.9% and specificity of

51.4% (prevalence 34.6%). Himes *et al.* acknowledged that self-report was likely to be unreliable, and suggested EtG as the gold standard(120).

Bakhireva in 2014 in a study including both PAE infants and non-PAE infants established PAE by detailed maternal self-report through timeline follow back method over a two-week period from the month prior to the last menstrual period(121). Non-alcohol exposed control subjects were recruited for comparison. The study group compared various cut offs and combinations of FAEEs that had previously been reported in the literature including 600 ng/g (four FAEEs), 10,000 ng/g (seven FAEEs) and 100,000 ng/g (nine FAEEs). They concluded that a cumulative concentration of four FAEEs (ethyl palmitate, stearate, oleate, linoleate) ≥ 600 ng/g was the best outcome of all the combinations with a sensitivity of 100% for detection of PAE, although with much lower specificity (12.5%) than reported by Chan.

The evidence indicates that significant amounts of FAEEs in meconium are reflective of PAE, but only reliably so with higher consumption of alcohol (40 g per week) While there is some lack of consensus in the literature regarding the best cut off for the concentration of FAEEs, a cumulative concentration of ≥ 600 ng/g for four FAEEs is probably the most reliable(111, 121). Pichini *et al.* described the method of liquid chromatography-tandem mass spectrometry (LC-MS/MS) when comparing prevalence rates between different European populations and found less than 10% degradation of the samples on defrosting and analysis, ensuring the feasibility of storing frozen samples for batch analysis(122).

Potential problems with measurement of FAEEs:
The majority of studies undertaken to date do not discuss potential consent bias within the recruited population. Zelner *et al.* undertook a population study in 2012 reviewing mothers' willingness to take part in a study with clinical follow up of infants based on the meconium results and compared this to a previous anonymous study in a geographically similar population(123). The number of meconium samples positive for FAEEs dropped from 30% to 3% between the two studies, potentially reflective of 'tactical refusal' by alcohol consuming mothers. Mothers may be reluctant to disclose a history of alcohol consumption to

healthcare workers due to guilt and/or fear of repercussion as evidenced by Lange *et al.*, who found significant differences between PAE measured by self-report and by meconium biomarkers. Therefore, biomarkers including FAEEs in meconium are more likely to accurately reflect PAE in a population if collected anonymously(83).

FAEEs measured in meconium can be falsely positive, more commonly in delayed sampling(112). Zelner *at el*. demonstrated by serial testing of 136 meconium samples from 30 neonates (all initially negative) that the average time for FAEEs to become positive in meconium samples was 59.2 hours(112). FAEEs are thought to be produced by bacteria in the meconium in samples when analysis has been delayed. While meconium is sterile *in utero*, once the infant has passed through the birth canal and feeding is established, natural gut flora will begin to colonise the gut(112). Spiking meconium samples with glucose *in vitro* results in previously negative samples of meconium becoming positive for FAEEs. Therefore, to avoid false positive results, samples should be taken as early as possible after birth and frozen (discussed in Methods). Many studies do not report the timing of collection and analysis of the meconium samples, which result in confounding in those studies with delayed sampling.

Author/year	Study design	Study population	Number of participants	Cut off used	Cut off identified	Meconium biomarkers	Self-report alcohol
Bearer *et al* 1999 (115)	cohort study		248	n/a	no	8 FAEE ethyl laurate, myristate, palmitate, stearate, oleate, linoleate, linolenate and arachidonate	Post-natal interview
Bearer *et al* 2003 (116)	population study	Cape Town, South Africa	27	>32 ng/g Ethyl oleate	yes	ethyl oleate, ethyl palmitate and ethyl linolenate	TLFB
Chan *et al* 2003 (111)	population-based cohort study	Jewish and Canadian	207 non-drinkers and 6 heavy drinkers	>600 ng/g (4 FAEEs)	yes	ethyl stearate, oleate, linoleate, laurate, palmitate, myristate	
Ostrea *et al* 2006 (113)	Cohort study		124		no	Ethyl laurate, myristate, palmitate and stearate	MAST, TACE and CAGE
Kwak *et al* 2014 (119)	cohort study	Korea	294 (182 abstainers)	2, 10 and 20 nmol/g (600, 3000, 6000 ng/g)	yes	9 FAEEs ethyl laurate, myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate and arachnidonate.	custom electronic questionnaire at 34 weeks' gestation - abstainers, light, moderate, heavy
English *et al* 2016 (117)	population study	Uganda	505	>600 ng/g	no	4 FAEEs ethyl stearate, oleate and linoleate, myristate	Questionnaire & TWEAK
Himes *et al* 2015 (120)	cohort study	USA/South Africa - vulnerable cohort	107; 33 abstainers; 58 drank after 19 weeks	≥200 ng/g	yes	4, 7, 9 FAEEs ethyl laurate, myristate, palmitate, oleate, stearate, linoleate, linolenate and arachidonate	TLFB at 4 periods of pregnancy
Yang *et al* 2015 (118)	cohort study	Republic of Korea	236; 182 abstainers, 54 light-moderate drinkers	LOQ	yes	9 FAEEs ethyl laurate, myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate and arachnidonate.	electronic questionnaire at 34 weeks' gestation

Table 1.1 Summary of articles measuring fatty acid ethyl esters (FAEEs) in meconium with proposed cut-off values (111,113,115-120)

1.5.2 Ethyl Glucuronide

EtG in a non-oxidative metabolite of alcohol which has been measured in urine, blood, placenta, and meconium. EtG has been reported to be a more reliable alcohol biomarker than FAEEs, as it is less prone to variation due to nutritional factors and/or endogenous production(120). With fewer false positives, specificity of EtG as an alcohol biomarker is greater than for FAEEs. EtG is measured by LC-MS/MS and is stable at room temperature and will remain stable for up to three freeze-thaw cycles, although samples should be well mixed prior to analysis(106, 124).

EtG in meconium has been linked to developmental outcomes with IQ lower on average by six points in a cohort of primary school age children with ≥154 ng/g EtG in their meconium. In the same study there was a correlation between meconium EtG concentration and ADHD related behaviours(125). Clinical findings were not related to maternal self-report of alcohol consumption. Elevated EtG in meconium has also been correlated with methylation of genes associated with cognition and attention deficit, suggesting that PAE in late pregnancy may effect epigenetic changes(126).

Identifying a cut off value for EtG in meconium

There are a number of publications which overlap in the analysis of both FAEEs and EtG in meconium. Several of these papers come from the same team based in Italy and Spain and include some overlap in subjects which made data extraction difficult. Authors have sought to validate EtG in meconium either by comparison with maternal self-report or by comparison with FAEEs in meconium or a combination of the two. Relevant papers are summarised in table 1.2; I will briefly discuss both the method(s) of validation and suggested cut off values.

The first report of EtG being detected in meconium as a potential marker of in utero exposure to alcohol was by Pichini *et al.* in two populations in Spain and Italy. They analysed 173 meconium samples from infants admitted to neonatal units and reported the presence of EtG as well as FAEEs(127). There was a significant difference between the two populations in the likelihood of meconium being positive for FAEEs, as defined by a total concentration of seven FAEEs of >600

ng/g). Babies born in Barcelona were more likely to be positive for FAEEs in meconium compared to those born in Reggio Emilia (41.9 *vs* 8.3% of samples); median concentrations of EtG in meconium in these two populations were 152.3 ng/g and 17.6 ng/g, respectively. There was no direct correlation between EtG and FAEE concentrations.

The same research group carried out a population study using both maternal self-report and FAEEs in meconium to try to define cut-off values for EtG in meconium(128). 185 meconium samples were obtained with mothers divided into two cohorts based on a structured questionnaire regarding alcohol consumption. Unexposed infants were defined as those with no PAE reported and a meconium FAEE concentration of <600 ng/g. EtG was detected in 83.3% of all meconium samples(128). A cut of value of 440 ng/g for EtG in meconium was reported to be 100% sensitive and specific for PAE as determined by both FAEEs in meconium and maternal self-report. In 47 meconium samples collected from infants in a single Spanish centre there was a correlation between FAEE and EtG concentrations in meconium above an EtG cut-off of 50ng/g.

A study in a randomly selected German population found 16.3% of 596 samples to have detectable EtG where 10 ng/g was the limit of detection (LOD) for EtG. Samples were kept frozen (-20C) and retested at 3 months and found to be relatively stable in terms of EtG concentration(106). By comparison, analysis of the same meconium samples found 7.1% of samples were positive for FAEEs as defined by a total concentration of >500 ng/g for four FAEEs(106). The authors found a good correlation between the two biomarkers when including all results (FAEE >100 ng/g and EtG >10 ng/g) (Pearson's co-efficient 0.367; P=0.003). Optimal agreement between the two biomarkers was described with FAEEs > 500 ng/g and EtG > 274 ng/g(106). Two standard questionnaires (FRAMES and CAGE) were used to determine maternal self-report of alcohol consumption in pregnancy but only six subjects were positive via CAGE. There was very poor correlation between EtG in meconium and maternal self-report, interpreted as inaccurate self-report.

In a further prospective cohort of 557 women in Germany, 21.2% reported low to moderate alcohol intake in pregnancy (FRAMES)(129). Alcohol intake was assessed in detail both during and after pregnancy (soon after delivery and at 6-8 months

postpartum). EtG concentration in meconium was compared with self-report and with multiple sociodemographic factors, using either presence or absence of EtG or a cut of 120 ng/g to delineate a positive. There was no difference in sociodemographic factors between the mothers who did or did not consume alcohol, apart from alcohol consuming mothers being less likely to be married. It is not clear how the cut of 120 ng/g of EtG was selected. A further study published in 2016 by the same research group reviewed 180 meconium samples from a randomly recruited population(79). EtG concentration varied from 17 ng/g to 10,235 ng/g. A cut off value of 120 ng/g was utilised which correlated with self-report of prenatal alcohol consumption (14% of samples; $p<0.01$)(129). When women reported any alcohol consumption, EtG concentration in infant meconium was on average 1.8 time higher than for infants of mothers that did not report any alcohol consumption. Women were more likely to self-report alcohol consumption in pregnancy directly after birth than retrospectively, at 6-8 years post-partum(79). Agreement between FAEE and EtG was identified when Carbarcos *et al* analysed both biomarkers in the same sample, utilising the cut off value of 600ng/g for FAEEs but not identifying a cut of value for EtG(130).

Up to this point cut-off values identified for EtG were conservative, focussing primarily on specificity. Himes *et al*. analysed 107 meconium samples as part of the Safe Passage study from the Northern Plains, USA and Cape Town, South Africa(120). Detailed self-report by timeline follow back method was carried out on five occasions throughout pregnancy to establish alcohol intake and four cohorts were identified (no alcohol consumption, nil in third trimester, average 3-10 drinks per drinking day (DPDD) after 24 weeks' gestation and average >10 DPDD (equivalent to approximately 140 g alcohol) after 24 weeks' gestation. There was a weak association between FAEEs (sum of either four, seven or nine) and EtG as well as a dose concentration relationship with EtG for alcohol consumption after 19 weeks' gestation. A cut-off concentration of EtG of ≥ 30ng/g yielded the highest sensitivity (81.8%) and specificity (75%) compared to other biomarkers including ethyl sulfate and cumulative FAEE combinations(120). In validating maternal hair EtG, Joya *et al*. from the Spanish research group subsequently acknowledged that the cut-off value for EtG in meconium should be reduced to 30 ng/g (106, 120, 131).

Since Himes *et al*. there have been a number of population studies describing the prevalence of PAE based on the use of EtG as a biomarker in combination with either self-report or another alcohol biomarker(120). These have included a Navajo population (prevalence of any EtG in meconium 5.1%) and a Glasgow population (prevalence of EtG $\geq$ 30 ng/g in meconium 15%)(78, 132). Measurement of EtG by enzyme-linked immunosorbent assay (ELISA) is a more rapid and inexpensive measurement than LC-MS/MS; in this study by Pichini *et al*., a cut off value of 200 ng/g (0.9 nmol/g) EtG as measured by ELISA was 100% sensitive and 78% specific for the detection of PAE in a cohort of babies born to known alcohol consuming mothers(133).

Author/year	Study design	Study population	Number of participants	Population	Compared to FAEEs	Correlation with FAEEs	Comparison to self-report	Correlation with self-report	Questionnaire	Cut off
Pichini *et al* 2009 (127)	Random population study	Spain/Italy	173	Random	Y	N	N	N	n/a	n/a
Bakdash *et al* 2010 (106)	Random population study	Germany	596	Random	Y	Y	N	n/a	FRAMES/ CAGE	≥10 ng/g (LOD) and ≥30 ng/g
Morini *et a*l 2010 (128)	Prospective cohort study	Spain/Italy	185	Random - PAE/ Non-exposed	Y (7 FAEEs)	N	Y	n/a	Structured questionnaire on three occasions in pregnancy	>2 nmol/g (440 ng/g)
Goecke *et al* 2014 (129)	Prospective population study	Germany	1100 participants, 557 participants	Random	Y	N	Y	Y	FRAMES	120 ng/g
Cabarcos *et al* 2014 (130)		Spain	47		Y	Y	N	N	n/a	50 ng/g
Himes *et al* 2015 (120)	Prospective cohort study	USA/South Africa	107	Categorised PAE/ non-exposed	Y	Y (weak)	Y	Y	Modified TLFB	LOQ, 5 ng/g, ≥30 ng/g
Eichler *et al* 2016 (79)	Prospective population study	Germany	180	Random	N	n/a	Y	Y	FRAMES/ FRANCES Franconian cognition and emotion studies	>10 ng/g (LOD) and >120 ng/g

Table 1.2 Summary of articles measuring ethyl glucuronide (EtG) in meconium with proposed cut of values (79, 106, 120, 127-130)

1.5.3 Phosphatidylethanol (PEth)

Phosphatidylethanol (PEth) is a glycerophospholipid present within the red blood cell membrane, which is formed from ethanol by the enzyme phospholipase(134). Fatty acid chain lengths can be either 16, 18 or 20. PEth 16:18.1 (1-palmitoyl-2oleolyl-sn-glycero-3-phosphoethanol) is a reliable biomarker in the adult population with a dose-response relationship(134). In healthy adult subjects PEth has a half-life of four to 12 days, however this half-life varies; in alcoholic subjects the half-life of PEth is four days. While PEth measured in adult blood is a very sensitive measure of high alcohol exposure, caution has been expressed about the detection of low to moderate alcohol exposure, with cut off values close to the LOQ. Several studies have reported that assay of PEth concentration in the newborn may provide a measure of PAE in the last trimester of pregnancy with a reported specificity of 100%(121).

Details of seven papers and one abstract exploring the use of PEth assayed from dried blood spot cards obtained from newborn infants as a measure of PAE are presented in Table 3. As a validated method of ascertaining PAE, PEth measured from a dried blood spot card would be particularly useful in population studies since dried blood spot cards are already obtained from almost all of the newborn population and routinely stored for retrospective testing. Synthesis of PEth has been reported not to occur in blood spot cards post collection(135).

Bakhireva *et al*. undertook a feasibility study in 2013 of 201 randomly obtained blood spot cards for measuring PEth with a view to population screening for PAE(102). 87.6% of blood spot cards had sufficient blood and yielded a PEth result. The LOD for PEth was 8 ng/ml. This paper also reviewed the stability of PEth in dried blood spot cards over time, reporting 96.3% stability at three months from collection(102). The cost of analysis of dried blood spot cards was approximately half that of analysis of meconium for FAEEs ($61.10 *vs* $112.69).

There is variation between published studies in cut off values used to define positive samples. Both 8 and 20 ng/ml have been utilised by Bakhireva's group whereas 20 ng/ml is suggested by the United States drug testing laboratories

(USDTL) as a positive result in adult samples(102, 121).

The same group tried subsequently to validate PEth in dried blood spot cards by using 60 dried blood spot cards from 28 PAE newborns and comparing them with 32 non-alcohol exposed newborns. PAE was also assessed by meconium testing for FAEEs and maternal self-report (AUDIT-C & time line follow back-14 days)(121). 32.1% of blood spot cards obtained from PAE newborns had a PEth concentration ≥ 8ng/ml (LOD) compared to none in the control group. The specificity of PEth in dried blood spot cards was 100% compared to a specificity of 12.5% for a FAEE concentration of >600 ng/g (ethyl palmitic, stearic, oleic, linoleic) in meconium within the same population. The sensitivity of PEth was 32.1% compared to 100% for FAEEs(121).

Baldwin *et al*. presented an abstract describing 346 blood spot cards collected anonymously and at random in the Midwest. These dried blood spot cards were filled from umbilical cord blood at the time of delivery. 19.7% of samples were deemed positive (PEth >8 ng/ml)(136). The same team went on to publish a study of 250 residual dried blood spot cards taken at the same time as newborn screening (24-48 hours of age) from a similar population and obtained from the department of public health; PEth concentration was ≥8 ng/ml in 4%(137). PEth concentration was not compared to maternal self-report in this anonymised study which established the feasibility of carrying out retrospective testing on dried blood spot cards obtained for the purpose of newborn screening(137). Stability of dried blood spot cards when stored at room temperature over a 12 month period was 65.9%, with evidence that stability begins to diminish after six months at room temperature(137).

Bakhireva *et al*. carried out a larger, anonymised study of 1000 residual dried blood spot cards obtained within the state of Texas (40% Hispanic population)(138). Utilising a cut-off of ≥20 ng/ml as per the USDTL, the positivity rate was 8.4%. Large regional differences were detected within this population with a higher prevalence of raised PEth in urban areas and within the Hispanic population, and a trend to increased prevalence of a PEth concentration ≥20 ng/ml in more affluent

areas. This study did not attempt to correlate PEth values with self-reported PAE as the samples were obtained anonymously from the newborn screening lab.

In a recent (2019) cohort of 162 infants, dried blood spot cards were used to compare PEth in umbilical cord blood with EtG in umbilical cord tissue in a department where practice was to screen cord tissue for EtG at physician discretion. 26.5% of dried blood spot cards had a PEth concentration of ≥8 ng/ml compared to universally negative EtG cord tissue. Results were not compared to maternal self-report. There was no association with maternal demographics or infant Apgar scores and PEth concentration in newborn blood spot cards(139).

The largest study published to date measuring PEth in dried blood spot cards was carried out across two sites (Uruguay and Brazil) and utilised dried blood spot cards obtained from both mothers and their newborns(140). Infant blood spot cards were obtained at 24–48 hours of life. Using a cut-off of ≥8 ng/ml PEth to define a positive sample, there was variation between maternal and infant blood spot cards; Uruguay 86.8% positive in newborns and 45.8% positive in maternal blood spot cards and Brazil 76.9% positive in newborns and 43.9% positive in maternal blood spot cards. The explanation for this difference is not clear although mothers would only be expected to have a positive sample if they had consumed alcohol within 48 hours of delivery(140). Only 13% of mothers reported third trimester alcohol consumption.

Umer *et al*. published in 2000 a large study of 1830 residual dried blood spot cards obtained in West Virginia which were anonymously linked with maternal demographics(141). PAE as defined by PEth >8 ng/ml was associated with smoking, preterm births, lower birth weight (<2000g *vs* >3000 g) and with mothers not wishing to exclusively breastfeed(141).

In summary, the assay of PEth in infant dried blood spot cards is relatively new and has only been validated in a small study of 28 PAE infants and 32 controls(121). The timing of blood sampling has been either at birth or between 24 and 48 hours of life, coincident with routine newborn screening(102, 142). Of note, this is different timing from the obtaining of dried blood spot cards within the UK which is

carried out between 96 and 120 hours of age. To date no study has reported PEth values in dried blood spot cards obtained within 96 to 120 hours of life. Neither has any study to date reported on developmental outcomes of infants with detectable PEth in blood samples at, or soon after birth.

Author/year	Study Design	Study location	Number of participants	Population type	Timing of sample	Cut off	Prevalence of positive results	Sensitivity (%)	Specificity (%)
Bakhireva *et al* 2013 (102)	Prospective cohort study	USA	201	Random, anonymised	24-48 hours of life	>20 ng/ml	6.50%	n/a	n/a
Bakhireva *et al* 2014 (121)	Prospective cohort study	USA	60	Exposed (28) *vs* control (32)	24 - 48 hours of life	>8 ng/ml (LOD)	32.1% exposed group, 0% control group.	32.10%	100%
Baldwin *et al* 2014 (136)	Prospective population study	USA	346	Random, anonymised. Midwest	birth (umbilical cord blood)	≥8 ng/ml	19.70%	n/a	n/a
Baldwin *et al* 2015 (137)	Retrospective cohort study	USA	250	Random, anonymised. Midwest	24 - 48 hours of life	≥8 ng/ml	4%	n/a	n/a
Bakhireva *et al* 2017 (137)	Retrospective cohort study	USA	1000	Stratified random sampling. Multi-ethnic (40% Hispanic), anonymised	24 - 48 hours of life	>20 ng/ml	8.40%	n/a	n/a
Maxwell *et al* 2019 (139)	Prospective cohort study	USA	162	West Virginia; known risk of substance/alcohol misuse or physician discretion	birth (umbilical cord blood)	≥ 8 ng/ml	26.5%.	n/a	n/a
Baldwin *et al* 2020 (140)	Prospective cohort study	Brazil and Uruguay	1140	Population study, two sites urban	24 - 48 hours of life	≥ 8 ng/ml	86.8% newborns in Uruguay (maternal 45.8%) and 76.9% Brazilian newborns (maternal 43.9%)	n/a	n/a
Umer *et al* 2020 (141)	Retrospective cohort study	USA	1830	Population study - West Virginia. Poor and rural	24 - 48 hours of life	≥ 8 ng/ml		n/a	n/a

Figure 1.3 Summary of articles measuring PEth from dried blood spot cards with proposed cut-off values, sensitivity and specificity (102,121, 136-141)

1.5.4 Conclusions

There are significant concerns around the accuracy of maternal self-report of alcohol consumption in pregnancy, especially when sought retrospectively. While it might be anticipated that sociodemographic factors are associated with consumption of alcohol in pregnancy, this has not been consistently demonstrated. Identifying sociodemographic factors predictive of at-risk populations would allow focused public health interventions.

Meconium has potential for measurement of alcohol biomarkers, and both FAEEs and EtG in meconium have been correlated with neurodevelopmental outcomes. FAEEs in meconium can however be unstable, related to dietary factors and affected by bacterial contamination(120, 143). Similarly, to screening for smoking in pregnancy with self-report and CO measurement, it may be that alcohol biomarkers can complement maternal self-report of alcohol consumption. This is most likely for unidentified high consumers of alcohol who have not disclosed alcohol consumption. Using appropriate, validated cut-off values for alcohol biomarkers is key, and there is evidence that use of more than one biomarker may be helpful.

The potential use of newborn blood spot cards to measure PEth needs further investigation. As these cards are routinely collected and stored in the UK, there is potential to measure retrospectively PEth concentration in the newborn period in children presenting with features of FASD, particularly if a maternal history of alcohol consumption is unavailable.

2.0 Methods

2.1 Aim

The primary aim of this observational population-based study was to examine the pattern and prevalence of alcohol consumption in pregnancy as determined by infant biomarkers among women delivering at the Princess Royal Maternity (PRM) in Glasgow. Additionally, the research study sought to examine the potential for newborn blood spot cards to be tested retrospectively as a marker of PAE.

2.2 Study population and protocol development

PRM is a tertiary level maternity facility serving the relatively deprived and culturally diverse north and east of the city of Glasgow and its suburbs. The annual delivery rate is approximately 6000 births. Within the PRM, the neonatal unit is a tertiary referral centre, admitting around 500 babies per annum.

The eligible population comprised mothers delivering a live singleton infant at any gestation within each specified 24-hour collection window. In order to achieve a random sample of the population delivering at the PRM while dealing with the practicalities of recruitment, the study was designed to run over two 20-week periods within a year, during which as many mothers as possible would be recruited during each fourth 24-hour period (00:01 to 24:00 hours). Collection of samples every day for a full year was impractical because of the need for informed, written consent and the involvement of only one investigator. Every fourth day avoided potential weekend variation in drinking alcohol.

The study was delivered at point of care in sites including prenatal, labour ward, postnatal and neonatal unit in order to minimise study burden to delivering mothers and therefore optimise recruitment rates. Women were excluded if the infant was thought not to be likely to survive, or if they lacked capacity or if their state of mind meant that either that approaching them to discuss the study was not appropriate or informed consent was unable to be achieved. The midwives looking after the mothers guided the researcher with regards to the appropriateness and timing of approaching mothers, appreciating that delivery can be a sensitive time.

The design of the study was informed by a previous feasibility study undertaken in the same department by other members of the PRM research team and alluded to in the introduction(78). Additionally, we set out to explore the usefulness of assaying alcohol biomarkers in newborn dried blood spot cards. Routine newborn blood spot screening is undertaken between 96 and 120 hours of life (generally on day five of life) by the community midwives. Once newborn screening has been carried out the cards are routinely stored indefinitely, unless there is specific parental request otherwise(144). Should alcohol biomarkers in dried blood spot cards correlate with meconium biomarkers and/or maternal alcohol history, this raises the potential usefulness of retrospective measurement of alcohol biomarkers in dried blood spot cards either as a diagnostic tool for children suspected to have FASD or for anonymised national population screening to assess PAE.

2.3 Choice of biomarkers

The advantages and disadvantages of different alcohol biomarkers measurable in the newborn have been fully described in the introduction, together with their diagnostic accuracy. There are multiple FAEEs including around 20 variants. Different researchers have reported differing combinations of FAEEs in studies of PAE; our decision to use a total of four FAEEs including ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate was for two reasons: pragmatic, to allow direct comparison with the feasibility study; and because Himes *et al*. found the sum of these four FAEEs to have better agreement with self-reported PAE than the sum of either all nine FAEEs, or the sum of seven FAEEs excluding ethyl laurate and myristate(78, 120).

An appropriate cut-off value for a total of four FAEEs in infant meconium as a marker of regular prenatal alcohol consumption is a balance between sensitivity and specificity. A cut off value of 600 ng/g has consistently been reported to have a sensitivity of 100%, but with a specificity varying from 13 - 98.4% using detailed maternal self-report as the standard(78, 111, 120, 121).

EtG > 30 ng/g was reported by Himes *et al*. as superior to summated FAEEs in terms of agreement with self-reported alcohol consumption beyond 19 weeks' gestation, so this was included in our analyses(120). Since the two biomarkers may be more

useful in combination, we chose to analyse both from the same meconium sample as in the feasibility study(145-147).

As a pilot arm of this study, we sought to analyse newborn blood spot cards for PEth, with the aim of investigating the potential use of routinely collected and retained newborn blood spot cards as a retrospective measure of PAE. Measurement of PEth has not been previously carried out in blood spot cards collected in the UK infant population. We sought REC permission to obtain blood spot cards additional to those obtained for routine newborn screening so as not to interfere with potential clinical use of routinely retained blood spot cards for retrospective infant testing for other conditions (*e.g.* cytomegalovirus exposure)(144).

2.4 Funding

Funding was obtained in summer 2014 by competitive application to the Yorkhill Children's Charity (now Glasgow Children's Hospital Charity) for a one-year Clinical Research Fellow post, commencing August 2015. Ideally, we would have sought two years' funding, but at the time of application, YCC was only awarding one-year fellowships, with no guarantee of a second year's funding. A grant of £57,808 was awarded to cover a Clinical Research Fellow salary for one year, the costs of analysis of 750 meconium samples and blood spot cards and in addition University of Glasgow MD fees (one-year full time, two years part-time).

During the twelve-month period prior to the start of the fellowship, I finalised the study protocol in conjunction with my supervisor and submitted it for approval by both Research Ethics Committee (REC) for the West of Scotland 3 and the Research and Development (R&D) department of NHS Greater Glasgow and Clyde. This enabled recruitment for the study to start within three weeks of the beginning of my fellowship and allowed recruitment to occur over the majority of one full calendar year. More details of my direct involvement in REC and R&D approval are given below.

2.5 Research Ethics Committee Approval

Following awarding of funding, REC approval was sought through the Integrated Research Application System (IRAS). I personally completed the IRAS form under supervision by my research supervisor Dr Mactier and prepared all of the accompanying documentation, including information leaflets and consent forms.

As previously noted, a smaller, feasibility study of meconium biomarkers had previously been completed by a different members of my supervisor's research team and included 253 mother and infant dyads within the same research centre: this feasibility study confirmed maternal willingness to take part in a study examining infant alcohol biomarkers and facilitated the IRAS application(78). Following completion of the IRAS application, together with my research supervisor I attended a West of Scotland REC 3 meeting on 28/05/2015. The REC declared initially that written informed consent should be obtained from all participants prior to delivery, but the practicalities of this decision troubled us. During subsequent discussion with the REC panel, we highlighted that this would a) make executing the study very difficult therefore limiting recruitment and probably making the study not reflective of the general population and b) (more importantly) seriously undermine the scientific validity of the study. Specifically, we were concerned that approaching women prior to delivery might result in mothers changing their pattern of alcohol consumption during pregnancy, as well as potentially discouraging those with significant alcohol consumption from taking part. We agreed to display posters in antenatal clinics advising prospective mothers of the study, emphasising the anonymity of the study, and pointing out that mothers-to-be had a 25% chance of being eligible for the study (collection periods every fourth day). REC approval was also obtained to allow collection of the first meconium sample prior to written consent, to ensure that a minimum number of samples would be missed. The acute nature of maternity services means that predicting labour and delivery is very difficult, and we wanted all eligible mothers to have the opportunity to participate in the study. Written information was provided antenatally to mothers and their partners both in poster format and information leaflets, which had been reviewed and approved by the REC. Lastly the researcher was not involved in clinical work, therefore any information given to the researcher regarding alcohol consumption during pregnancy would not be shared

with the clinical team. R&D approval was obtained via IRAS from NHS Greater Glasgow and Clyde and the study registered with the R&D department on 05/08/2015.

2.6 Study Aims and research questions as described in the research protocol:

Aims:

- To describe the pattern and prevalence of alcohol consumption by pregnant women in Glasgow during the second and third trimesters of pregnancy by assay of FAEEs and EtG in meconium.
- To compare FAEEs and EtG measured in meconium with PEth measured from a dried blood spot collected along with routine blood spot screening as a marker of alcohol consumption in pregnancy.
- To relate postnatally acquired data, including a confidential maternal interview and results of FAEE, EtG and PEth assays, to routinely collected antenatal information regarding maternal drinking in pregnancy.

Research questions:

- What are the levels of FAEEs and EtG in meconium samples from singleton term and preterm infants born in a single maternity hospital in Glasgow?
- What are the relationships between high levels of FAEEs and EtG in infant meconium and maternal age, parity, smoking, ethnicity, and socioeconomic status?
- What are the relationships between high levels of FAEEs and EtG in infant meconium and infant gestation, birth weight and head circumference?
- What are the relationships between high levels of FAEEs and EtG in infant meconium and maternal alcohol intake during pregnancy as self-reported at booking and immediately post-delivery?
- What are the levels of PEth in dried blood spot cards from singleton term and preterm infants born in a single maternity hospital in Glasgow?
- What are the relationships between high levels of PEth in dried blood spot cards and maternal age, parity, smoking, ethnicity, and socio-economic status?
- What are the relationships between high levels of PEth in dried blood spot cards and infant gestation, birth weight and head circumference?

- What are the relationships between high levels of PEth in dried blood spot cards and maternal alcohol intake during pregnancy as self-reported at booking and immediately post-delivery?
- What is the relationship between levels of FAEEs and EtG in meconium and levels of PEth in dried blood spot cards in singleton term and preterm infants?

2.7 Recruitment

2.7.1 Preparation for recruitment

During the period between obtaining REC and R&D approvals I met with hospital and community midwives to provide and discuss information in regard to the study protocol and day to day running of the study, including recruitment and sample collection. Folders providing detailed study information including a copy of the study protocol and consent forms were made available in each ward at PRM, including maternity assessment and the community midwifery base. Posters containing information about the study were displayed in clinical areas including labour ward, maternity assessment, and the postnatal wards. These posters provided eligible mothers as well as clinicians with outline study information, where to find further information and research team contact information. (Appendix 1)

Immediately prior to each study collection day the night team in the maternity assessment ward, labour ward, postnatal wards, and neonatal unit were contacted to ensure staff knew that a study day was scheduled the following day; this information was repeated at every change of shift during the 24-hour recruitment period and highlighted in the safety brief at each change of shift to ensure all members of staff on shift were aware. Pre-arranged study dates (every fourth calendar day) were made widely available.

In total there were 71 study days - the first collection period was slightly shorter at 19 weeks (second collection period 21 weeks in duration) due to closure of the clinical research facility laboratory during the Christmas period.

2.7.2 Study recruitment

Antenatally:

Potentially eligible mothers were approached after 35 weeks' gestation, either in the maternity assessment unit, antenatal wards or antenatal clinics and offered an information leaflet detailing the study, noting that there was a one in four chance of them being eligible. (Appendix 1) This information leaflet had been approved by the REC. Additionally, I made personal contact with all mothers who were planned for induction of labour or elective caesarean section to provide verbal and written information about the study.

Postnatally:

Eligible mothers admitted in labour were provided with written information about the study by the admitting labour ward midwife as well as a yellow plastic bag to collect the baby's first meconium-filled nappy in. Subsequently, if contact was deemed appropriate by the attending midwife, I spoke to each mother in the labour ward. At this stage, mothers were provided with further study information and given the opportunity to ask questions about the study. The majority of eligible mothers were seen by me prior to delivery; a few others were first seen by myself in the postnatal wards. I did not approach the few mothers without capacity to consent or where contact was not deemed appropriate. I sought to approach all eligible mothers within 24 hours of delivery (generally within a few hours) and to ask for consent to enrol them in the study. If a mother declined to participate and a soiled nappy had already been collected, this was discarded as per local clinical waste policy.

Pending formal consent, any yellow plastic bags containing meconium soiled nappies were placed in a dedicated area in the sluice in the postnatal ward with a patient addressograph label attached. The ward sluices are free from natural light, ensuring that the samples were kept safely in the dark and in a clinical area. The reason for only taking written, informed consent after delivery was that it would be inappropriate to consent mothers in active labour. The consent process ensured that participating mothers had ample opportunity to ask questions about the study before consenting. For those mothers for whom English was a second language,

foreign language interpreters or (in two cases) British sign language interpreters were used to obtain consent.

After obtaining written consent, I administered a health questionnaire to each participating mother. This questionnaire included the gestation at which the mother found out she was pregnant (weeks), how many previous pregnancies and how many previous live births she had had, any medication taken in pregnancy and her smoking status. I also asked if any alcohol had been consumed during pregnancy. If mothers reported alcohol consumption in pregnancy, they were asked more details including the timing of alcohol consumption and the type and amount of alcohol consumed; this was recorded using a calendar style method. Participants were encouraged to utilise telephones, diaries and, where relevant, social media to prompt recollection of timing of alcohol consumption. Particular focus was made on special occasions such as birthdays and anniversaries to act as prompts with the aim of identifying in which trimester alcohol had been consumed. I also documented whether alcohol consumption was prior to or after knowledge of pregnancy.

Maternal and infant medical notes were reviewed in order to obtain demographic information including parity, post code of residence, ethnicity, and documented alcohol intake at booking appointment. These demographic details were recorded on a clinical research file (CRF) (Appendix 2) and subsequently transcribed by me into an Excel spreadsheet, categorised to allow for statistical analysis.

2.8. Collection, storage, and transportation of samples

2.8.1 Meconium

Meconium samples were either given directly to the researcher or placed in the designated area in the sluice (see above) to reduce the likelihood of accidental disposal. Meconium samples were subsequently placed in a plain universal container directly from the nappy; no apparatus was utilised in order to avoid contamination. Care was taken to avoid any meconium which had come into contact with baby nappy wipes as some contain alcohol. In theory meconium contaminated with bacteria could result in metabolism of alcohol in baby wipes to produce FAEEs. Meconium samples were labelled with a study number and

maternal date of birth; a handwritten list matching study number to maternal CHI was stored securely in a locked office.

Meconium samples were taken to the laboratory within the Clinical Research Facility at Glasgow Royal Infirmary (adjacent to PRM) up to four times per collection day, depending on the number of samples. The meconium samples were then frozen at -40°C in a designated freezer. If samples were obtained out of hours (i.e., between 17:00 and 08:00 on weekdays and all day at weekends) they were frozen at -20ºC at the biochemistry department based at Glasgow Royal Infirmary and transferred to the Clinical Research Facility freezer during the next working day. The longest time interval between collecting any meconium sample and it being frozen would have been between my final evening check at 23:00 and subsequent early morning round at 08:00 (9 hours). During the midweek collection days most samples reached the -40ºC freezer within 4 hours.

The exact duration of time that each meconium sample was stored for prior to freezing was not documented – in hindsight this was a minor weakness of the protocol. However, had sample times been documented, these would have reflected the time the nappy was changed and, in many cases, not the time the infant passed the meconium. Thus, documenting sample collection time would have been of limited value. Meconium samples were shipped to the University of Padova, Italy on dry ice with temperature monitoring to ensure that the temperature remained less than -40ºC during transit. The samples subsequently underwent LC-MS/MS for the measurement of FAEEs and EtG (see laboratory analyses below).

2.8.2 Blood spot cards

When a mother consented to participate in the study, a blood spot card labelled with the study number and maternal date of birth was attached to the maternal handheld notes which were subsequently taken home by the mothers and used by the community midwives. Specifically, this blood spot card was attached to the page in the handheld notes where consent is documented for routine newborn blood spot screening, ensuring that it was visible to the midwife at the time of taking consent for newborn screening. Blood spot cards were only issued for

participants who had provided written consent; mothers had the right subsequently to withdraw or to refuse consent at the time of newborn blood spot sampling. On day five, if mothers had consented to the study and were still in agreement, the community midwife tried to obtain an additional blood spot on the second card following routine newborn blood spot screen. This second blood spot card was dependent upon the baby continuing to bleed after the routine blood spot screen. Further heel stick was not performed. Routine demographic data collected at this time were transcribed to the study blood spot card including method of feeding and if the infant had undergone treatment for jaundice or had a blood transfusion. The blood spot card was dried in room air and collected at PRM, either deposited in a designated box at PRM or posted back. The samples were kept out of direct sunlight and vacuum packed to reduce exposure to humidity prior to being frozen at -40°. Unfilled blood spot cards were returned with information regarding whether the mother had declined consent or whether the midwife had been unable to obtain sufficient blood.

It is common practice for staff to utilise alcohol gel and then apply gloves prior to taking a blood spot card sample. Use of an alcohol wipe to cleanse the heel prior to obtaining a heel prick sample is not mandated. If an alcohol wipe is utilised to cleanse the area it should be allowed to dry and therefore the alcohol evaporate prior to the sample being obtained. The study blood sample was obtained after the routine blood spot sample and we were measuring a metabolite of alcohol (PEth) rather than alcohol *per se*. For all these reasons, contamination of the study blood spot card with alcohol was considered extremely unlikely. Since the study aim was to see if routinely obtained blood spot cards could be utilised retrospectively to determine PAE, while there may have been more caution around the use of alcohol hand gel, changing sampling practice was not appropriate.

Blood spot cards were shipped on dry ice in a thermally regulated package at -40° to the University of Padua (*Universita degli Studi di Padova)* in Italy together with meconium samples. They were subsequently analysed there for the presence of PEth. Blood spot cards were linked anonymously via the study number to meconium samples to enable comparison of results from different samples from the same baby.

2.9. Demographic Data

One of the study aims was to identify if there were demographic differences in the population presenting with high alcohol biomarkers compared to low or absent alcohol biomarkers. The previously described feasibility study with a smaller study population suggested that PAE may be more common in more affluent mothers(78). We were aware of a common assumption that poorer mothers are more likely to misuse alcohol, but this is not evidence based.

It was intended to compare mothers recruited into the study with the entire population delivering at PRM during the study period in order to ensure that the recruited population was truly representative. An anonymised download of data from the Pregnancy and NewBorn Screening (PNBS) database via the Safehaven was planned, to determine overall population demographic details for comparison with the recruited study population. Unfortunately, this data download proved to be uninterpretable on a pilot download, therefore a further download was not carried out. Scottish Birth Record was screened to identify eligible patients who had delivered in each 24-hour period and could be approached by the researcher. Basic demographic information including the community health index (CHI) number (from which age can be obtained), postcode and mode of delivery were used to identify eligible mothers; these data allowed identification of which ward mothers were being cared for in, and prioritisation of those mothers who had delivered vaginally (predicted earlier hospital discharge). These basic, anonymised data were entered into a secure spreadsheet on the NHS server and compared between mothers who participated in the study and those who declined to participate, to ensure that the mothers who declined to participate were not different from the population as a whole. A more comprehensive comparison would have been carried out as per REC approval had the planned data download from PNBS been successful.

When a mother consented to inclusion in the study, I reviewed both maternal and baby clinical notes in order to obtain demographic information including maternal age, parity, ethnicity, postcode of residence and smoking status at booking, together with baby's birth weight, gestation, APGAR scores and occipital frontal circumference (OFC). Postcode was converted to an SIMD16 score to give a measure of socioeconomic deprivation(148). Parity was documented based on self-report

and was described in a single digit as the number of previous live birth(s) and/or stillborn children delivered after 24 weeks' gestation. Maternal ethnicity was recorded as had been reported by the mother at booking and documented in the maternal hand-held notes. Thus, ethnicity was self-identified.

2.9.1 Measure of socioeconomic deprivation

The DEPCAT socioeconomic score based on postcode of residence had been used in the feasibility study, but I chose to use a more recent measure of social deprivation for the current study, Scottish Index of Multiple Deprivation (SIMD)(148). SIMD was originally published by the Scottish government in 2004 and the most up to date version published in 2016 (SIMD16)(149). SIMD is a measure of deprivation across Scotland based on postcode. In assigning a score, SIMD considers more variables than DEPCAT, including employment, income, crime, housing, health, education and access to transport links and healthcare within a given postcode. These seven categories are further subdivided with 38 variables which are combined to provide a SIMD score between 1 (most deprived) and 6976 (least deprived). Individuals who do not have a postcode, *e.g.*, homeless, prisoners and travelling communities are given a generic postcode to generate a deprivation scale estimate which is therefore of limited value. An additional problem is that new build houses which have not been issued a postcode will not register a SIMD score(149).

Demographic data were collected onto CRFs and then transcribed to the anonymised master Excel spreadsheet. The original CRFs were stored in a locked cabinet in a locked office at PRM.

2.10 Laboratory analyses

2.10.1 FAEEs and EtG in meconium

To ensure stability, samples were transferred in a frozen state to the University of Padua in Italy for analysis(147). LC-MS/MS was carried out for the measurement of FAEEs (ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate) and EtG. 200 mg of meconium was sonicated for 15 minutes with 20 ng EtG-d5 and 200 ng of FAEE d-5. The supernatant was added to an aminopropyl solid-phase extraction cartridge, preconditioned with 2 ml methanol, water and acetonitrile (ACN). FAEEs were eluted with 2 ml hexane and EtG elution carried out with 2 ml water. The

two mixtures were dried using nitrogen stream and recovered with 50 µl of ACN (FAEEs) and 50 µl methanol (EtG). FAEEs were detected following separation using a C8 reversed-phase column. A C18 reversed-phase column was used in isocratic mode for EtG detection. Acquisition was in multiple reactions monitoring for all the analytes in positive mode for FAEEs and negative mode for EtG. Lower limit of quantification (LOQ) values were 10-15 ng/g for FAEEs and 10 ng/g for EtG.

2.10.2 PEth in dried blood spot cards

PEth and the internal standard (pentadeuterated PEth) were dissolved in dichloromethane at a concentration of 1 mg/ml. Working solutions were prepared at a concentration of 20 µg/ml with methanol. All standard and working solutions when not in use were kept at -20°C. From each dried blood spot card, a blood spot (30 µL) was removed by a manual punch (5 mm diameter) and placed in methanol for extraction (2 ml). After an hour, the solvent was transferred and evaporated to dryness in a nitrogen stream. The dried extract was dissolved with isopropanol (100 µl) and after stirring transferred to the vial for the LC-MS/MS analysis. The LC-ESI-MS/MS system consisted of an Acquity UPLC chromatograph coupled to a Xevo TQ-S Micro Mass Spectrometer (Waters). 5 µl of the extracts were injected on a Waters - Acquity BEH C8 column (2.1 x 50 mm, 1.7 µm) with a flow of 400 µl/min at a temperature of 55 °C. Gradient elution was performed with 10 mM ammonium acetate buffer (0.5% formic acid) and 2-propanol (10% tetrahydrofuran).

The mass spectrometer was operated in electrospray ionization in negative ion mode. The desolvation temperature was set at 350 °C, the gas nebulizer flow at 600 l/min, the ionization potential was 3.5 kV. Calibration curves were prepared on DBS by spotting 30 µl of blank blood, obtained from an alcohol abstinent subject, fortified at a concentration of 20, 40,100, 400, 800 and 1600 ng/ml of each PEth homologue. Quality controls are routinely added including blanks and samples at known concentrations; if the highest concentration was higher than that of controls they were diluted and then reanalysed. The laboratory undergoes bi-annual proficiency testing for the biomarkers.

2.11 Statistical analyses

Data were collected from patient questionnaires and clinical notes as appropriate and entered into a CRF (Appendix 2); data were subsequently transcribed to an Excel spreadsheet. SIMD16 scores were calculated from postcodes as described previously. Patient data were then merged with laboratory results, matched by study number. Once all data entries had been double-checked the spreadsheet was locked. Using Excel 2016, I extracted descriptive data and calculated mean, median, standard deviation, range, percentages as relevant in order to construct data tables. The completed tables were examined to identify potential differences between variables.

For those demographics and/or laboratory results which appeared to differ, statistical analysis was undertaken in collaboration with Dr. David Young, statistician. To avoid types 1 errors, statistical analysis was not undertaken where no obvious difference was observed. Analyses were carried out utilising Mini-tab (version 18). Categorical variables, *e.g.*, SIMD16 quintiles, were compared using a CHI square test. For those categories with two relatable observations *e.g.*, smoking status (yes or no) and ethnicity (White British vs non-white British) a paired t-test was undertaken. Pearson's co-efficient was used to investigate relationships between (non-linear) biomarkers.

I personally calculated the sensitivities, specificities, positive and negative predictive values of each of the infant biomarkers (FAEE, EtG and PEth). I used as the gold standard PAE as reported to me by the mother at the time of the confidential postnatal questionnaire.

3.0 Results

3.1 Recruitment

The study ran from 12/08/2015 to 22/12/2015 and from 20/01/2016 to 16/06/2016 inclusive. This gave a total of 71 collection days.

1045 infants were born on these 71 study days (Figure 3.1) Of the seven ineligible singleton births, two babies were born at home, one baby was transferred out for specialist neonatal care, one baby died within the first seven days of life, one baby was placed for adoption, one mother did not have capacity to consent, and one mother was too unwell to approach for consent.

I approached 908 (89.5%) mothers of the 1014 eligible singleton infants, of whom 840 (92.5%) agreed to participate.

Sample collection and return is illustrated in Figure 3.2. In total 740 meconium samples were collected, and 668 dried blood spot cards were returned. A few meconium samples could not be analysed and 11.6% of the dried blood spot cards either had no blood or an insufficient sample. A full set of alcohol questionnaire, analysable meconium and a well filled dried blood spot card was collected from 445 mother/infant dyads.

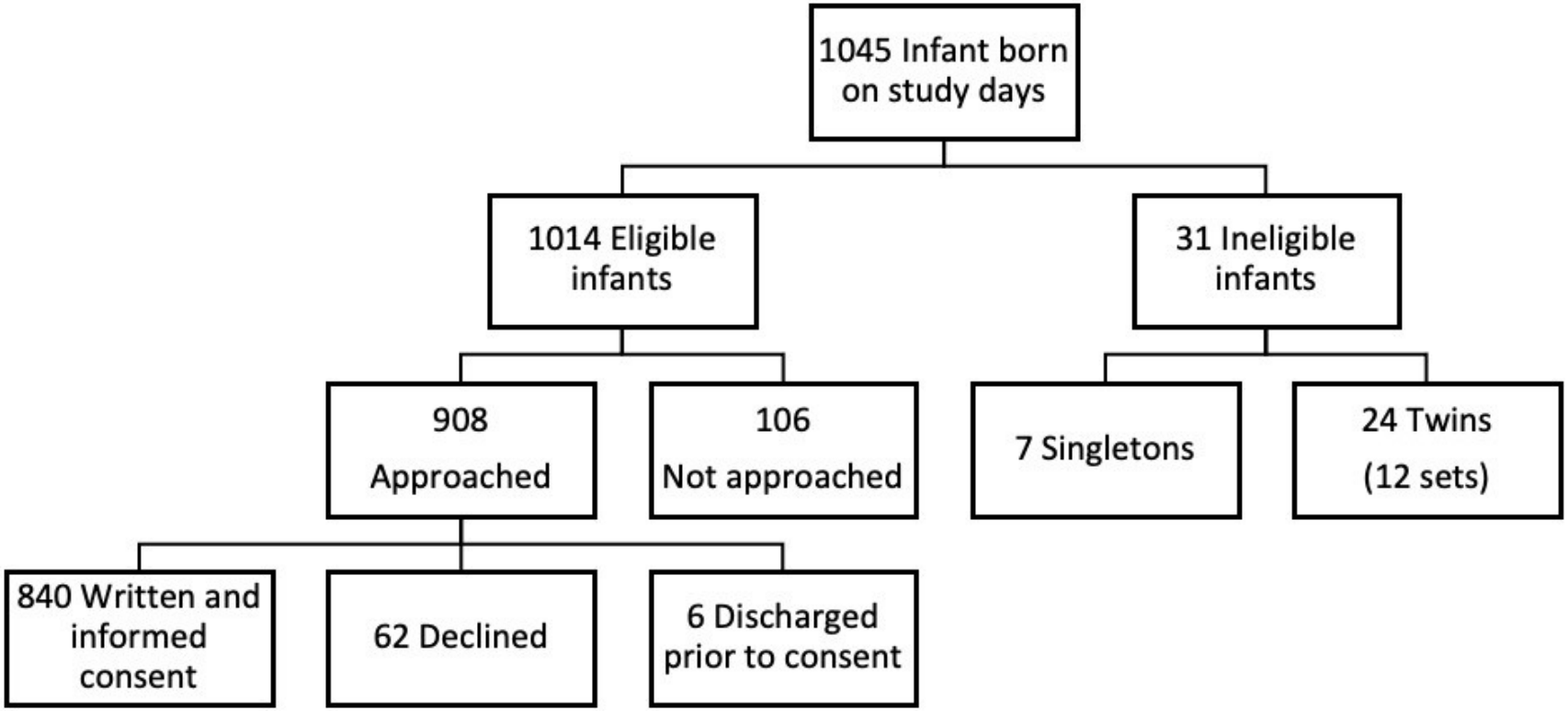

Figure 3.1 Flow diagram of study recruitment

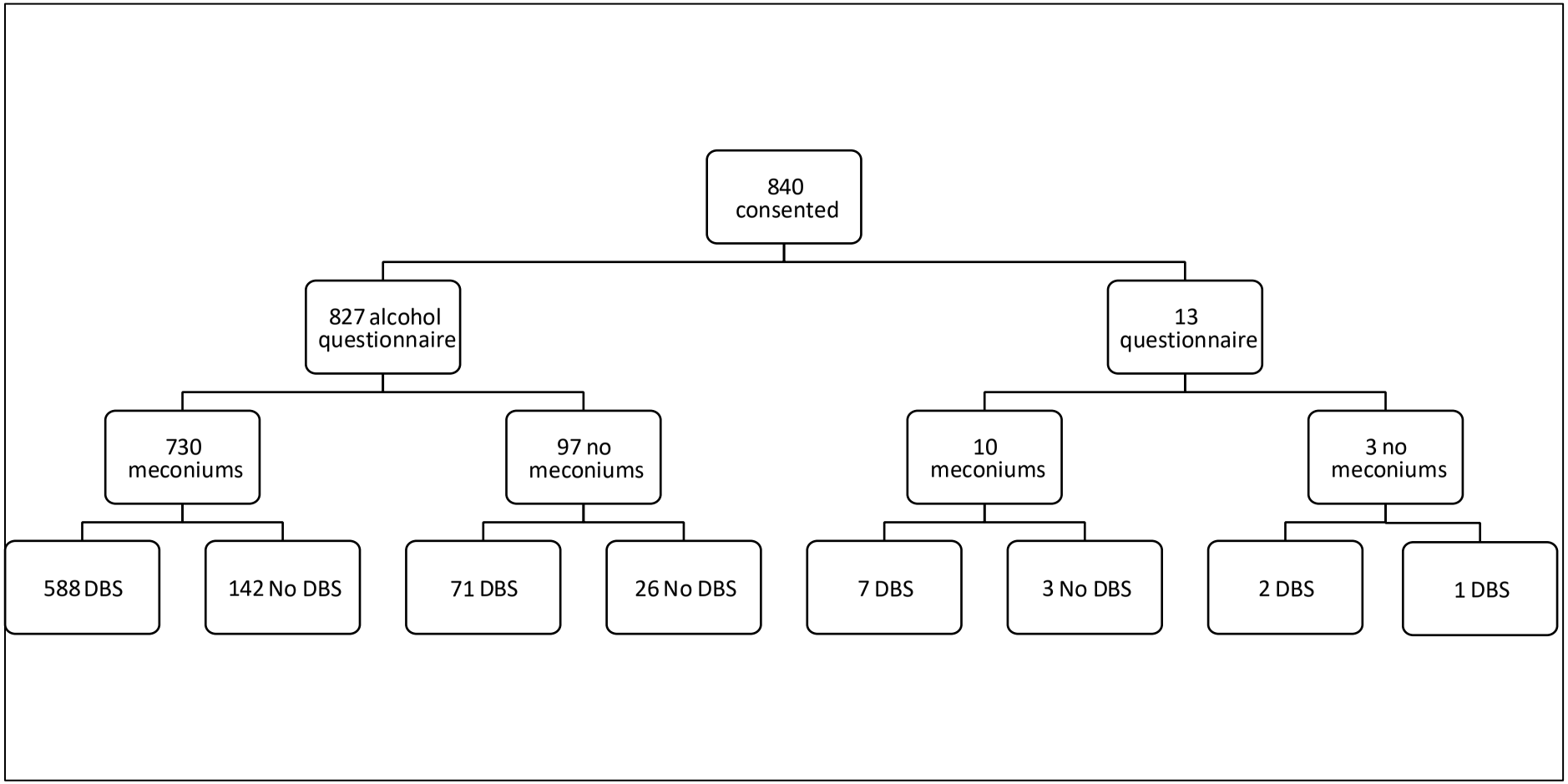

Figure 3.2 Flow diagram of collected samples (meconium and dried blood spot cards)

3.2 Maternal demographics

The demographics of recruited mothers are presented in Table 3.1. Demographic data are presented separately for those mothers who agreed to participate and who provided at least one infant sample (meconium and/or dried blood spot card) and for those mothers from whose infants no samples were obtained (potential tacit refusal). A blank dried blood spot card which was unfilled or incomplete because the baby did not bleed sufficiently well counted as sample returned. To ensure a representative population had been recruited limited anonymised data were collected from the Scottish Birth Record for those mothers who declined to take part. As described in the Methods chapter, these data were of necessity fewer than originally intended.

	ENTIRE RECRUITED POPULATION (N=840)	DECLINED TO PARTICIPATE (N=62)	CONSENTED 1 OR MORE SAMPLES (N=813)	CONSENTED NO SAMPLES (N=27)	CONSENTED MECONIUM SAMPLE COLLECTED (N=740)	CONSENTED NO MECONIUM SAMPLE COLLECTED (N=100)
Maternal age (years) mean (SD)	N=840	61	813	27	740	100
	29.8 (5.7)	29	29.8 (5.6)	30.5 (6.3)	29.8 (5.6)	29.5 (5.9)
range	16 – 50.3	16 – 39	16 – 50.3	18.9 – 42.7	16.0 – 50.3	18.9 – 45.4
Maternal BMI (kg/m^2) Median (IQR)	N=822		795	27	724	98
	25.8 (22.9 - 30.3)		26.0 (22.8 - 30.3)	25.0 (23.2 - 29.2)	26.0 (22.8 - 20.2)	25.6 (23.3 - 30.1)
range	15.2 – 59		15.2 – 59	18 – 52.5	15.2 – 59	17.9 – 52.5
SMID decile median (IQR)	N=831	60	804	27	731	100
	3 (1 – 6)	3 (1-10)	3 (1 – 6)	3 (1 – 5)	3 (1 – 6)	3 (1 – 7)
range	1 – 10	1 – 10	1 – 10	1 – 10	1 – 10	1 – 10
Ethnicity (% White British)	N=803		779	24	709	94
	80.3%		80.6%	70.8%	80.3%	80.9%
Parous mums (%)	N=822		796	26	727	95
	56.3%		56.4%	53.8%	55.8%	61.3%
Mode of delivery (% vaginal)	N=839	60	812	27	739	100
	61.5%	60%	61.5%	63%	61.3%	63%

Table 3.1: Maternal demographics according to sample collection

Maternal age ranged from 16 to 50.3 years. For those mothers who were approached for consent for the study and agreed to participate (n = 840), mean age on the day of delivery was 29.8 (+/- SD 5.7) years. There was no difference in age between those eligible mothers who were approached for consent and either agreed or declined to participate, or between those mothers who consented to the study and from whose infants a sample of meconium and/or dried blood spot card was obtained, and those mothers who consented to the study but who did not supply either a meconium sample or dried blood spot card (Table 3.1).

Body mass index (BMI) had been documented for 822/840 (97.9%) mothers recruited to the study and ranged from 15.2 to 59 kg/m^2. Data were slightly skewed; median

BMI was 25.8 kg/m^2 (IQR 22.9 to 30.3) and mean BMI was 27.2 kg/m^2 (SD 6.3). BMI was so poorly documented in the Scottish Birth Record that it could not have been investigated for those mothers who declined to participate.

SIMD 16 scores calculated from maternal postcode ranged from 1 to 6976 and were ranked into deciles. The lowest decile represents the areas of most socio-economic deprivation. SIMD 16 score was available for 831 of 840 women recruited to the study (98.9%). Not all postcodes were able to be translated to SIMD 16 scores due either to not having been written in the correct format (n=3) or data missing (n=1) or not having a corresponding SIMD 16 score (n=5). The latter scenario applied particularly to new build properties for which the postcode was not present at time of formulation of the SIMD scoring system. The population of recruited mothers was highly skewed towards the more deprived postcodes with 247 (29.7%) residing in areas with SIMD 16 scores in the most deprived decile, consistent with the maternity hospital location and catchment area. (Figure 3.3) The median SIMD decile was 3 (IQR 1 to 6).

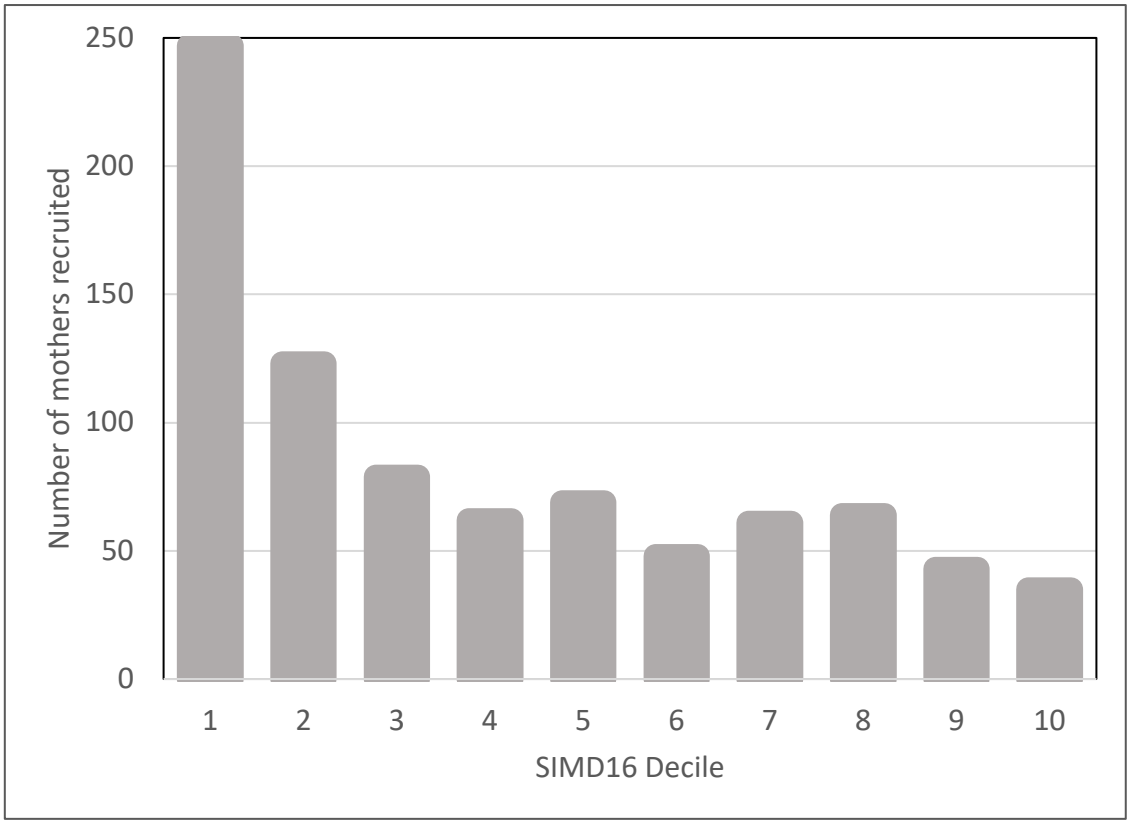

Figure 3.3: Distribution of SIMD 16 deciles within recruited study population (n=831)

Socioeconomic status as described by SIMD 16 did not vary between mothers who consented or declined, or between mothers who supplied a sample compared to those that did not.

Ethnicity data (self-reported) were available for 803 of the 840 women recruited to the study (Tables 3.1 and 3.2). Mothers who provided at least one sample tended towards an ethnicity of white British compared to mothers who provided no samples 80.6 *vs* 70.8%; this difference was not significant (p=0.23). 688 (81.2%) mothers had their first language documented at booking, of whom 591 (86%) were recorded as having English as a first language.

Ethnicity	Number of Mothers
African/African Caribbean	35
South Asia	36
South East Asia (China, Malaysia, Thailand)	23
Other non-European (Middle east, South America, North Africa)	19
Southern and Other European (Poland, Russia, Sardinia, Greece)	37
White British	645
Northern European	8
Mixed	0
Other Ethnicity	0
Total	803

Table 3.2: Self-declared maternal ethnicity

Previous pregnancies were documented from the handheld maternity notes. 270 (32.4%) of the 809 mothers recruited to the study for whom data were available had previously had at least one fetal loss before 24 weeks' gestation, of whom 81 (10% of recruited mothers) were documented as having had more than one fetal loss. 251/809 (31%) mothers stated in the confidential postnatal health interview that they were pregnant for the first time. 463/822 mothers had previously delivered a baby after 24 weeks' gestation or a live baby prior to 24 weeks' gestation (56.3%). For the purposes of this thesis, I defined only these 463 mothers as "parous". While a previous early fetal loss also confers the status of "parous", early fetal loss data were less complete and potentially under-reported. For the purposes of comparing biomarkers, women were divided into two group – 463 "parous" mums as described above and 359 "first time mums" who had not previously delivered such a baby.

Mode of delivery was documented for 839 mothers: 407 had a spontaneous vertex delivery (SVD), 109 had an assisted vaginal delivery and 323 were delivered by caesarean section. Of the caesarean deliveries, 143 were categorised as emergency and 180 as elective caesarean section. For those mothers who were approached for consent and declined, mode of delivery was ascertained from the Scottish Birth Record as part of screening and recruitment prioritisation. Theoretically variation in mode of delivery may have affected recruitment, due to earlier discharge from the maternity unit and thus less opportunity to obtain study consent from mothers who delivered vaginally. There was no difference in the mode of delivery of mothers who participated in the study compared to those who declined to participate in the study (Table 3.3). Mode of delivery is simplified in Table 3.1 into vaginal or caesarean birth for presentation in relation to sample collection.

Mode of Delivery	Recruited	Percentage (%)	Declined	Percentage (%)
Total (number)	839		60	
Spontaneous Vertex Delivery (SVD)	407	48.5	30	50
Ventouse	55	6.6	3	5
Forceps	54	6.4	3	5
Emergency caesarean section	143	17.0	7	11.7
Elective caesarean section	180	21.5	14	23.3
Uncategorised caesarean section	0	0	3	5

Table 3.3: Mode of delivery

3.3 Infant demographics

Of the 840 infants recruited to the study, 421 (50.1%) were male (Table 3.4). Gestation was documented for 837 of 840 babies recruited to the study and ranged from 28 to 42 weeks with a mean gestation of 38.9 (SD 1.7) weeks and a median of 39 weeks. 53 (6.3%) babies were of less than 37 weeks' gestation at delivery.

	ENTIRE RECRUITED POPULATION (N=840)	CONSENTED; 1 OR MORE SAMPLES (N=813)	CONSENTED; NO SAMPLES (N=27)	CONSENTED; MECONIUM SAMPLE COLLECTED (N=740)	CONSENTED; NO MECONIUM SAMPLE COLLECTED (N=100)
Infant sex (% male)	N=840	813	27	740	100
	50.1%	50.3%	44.4%	51.1%	43%
Gestation (weeks) mean (SD)	837	810	27	737	100
	38.9 (1.7)	38.9 (1.7)	38.6 (1.7)	38.9 (1.6)	38.9 (1.8)
range	28 – 42	28 – 42	33 – 41	30 – 42	28 – 41
Birthweight (g) mean (SD)	838	811	27	738	100
	3367 (526)	3374 (529)	3162 (401)	3373 (525)	3330 (538)
range	960 – 4905	960 – 4905	2086 – 3702	1320 – 4950	960 – 4450
OFC (cm) Mean (SD)	825	801	24	728	97
	34.6 (1.5)	34.6 (1.5)	34.2 (1.2)	34.6 (1.4)	34.5 (1.9)
Range	24.8 – 41.5	24.8 – 41.5	31.2 – 35.6	29.5 – 41.2	24.8 – 41.5
APGAR score mean (SD)	829	802	27	731	98
	9 (1.3)	9 (1.3)	9 (1.3)	9 (1.3)	9 (1.2)
range	0 – 10	0 – 10	4 – 9	0 – 10	1 – 10

Table 3.4: Infant demographics in relation to sample collection.

Birth weight was documented in the Scottish Birth Record for all but two babies recruited to the study and ranged from 960 to 4905 g with a mean of 3367g (SD 526g). 41 babies had a birth weight of less than 2500g (4.9%). Babies from whom no samples were obtained tended to be a little lighter at birth compared to those from whom at least one sample was obtained (mean 3162 *vs* 3374 g). Consistent with slightly lower birth weight, head size, measured as occipito-frontal circumference (OFC) tended to be less in those infants whose mothers consented to the study but from whom no samples were obtained (34.2 *vs* 34.6 cm).

Birth was more common between 09:00 and 12:00, reflective in part of the timing of elective caesarean sections (Figure 3.4).

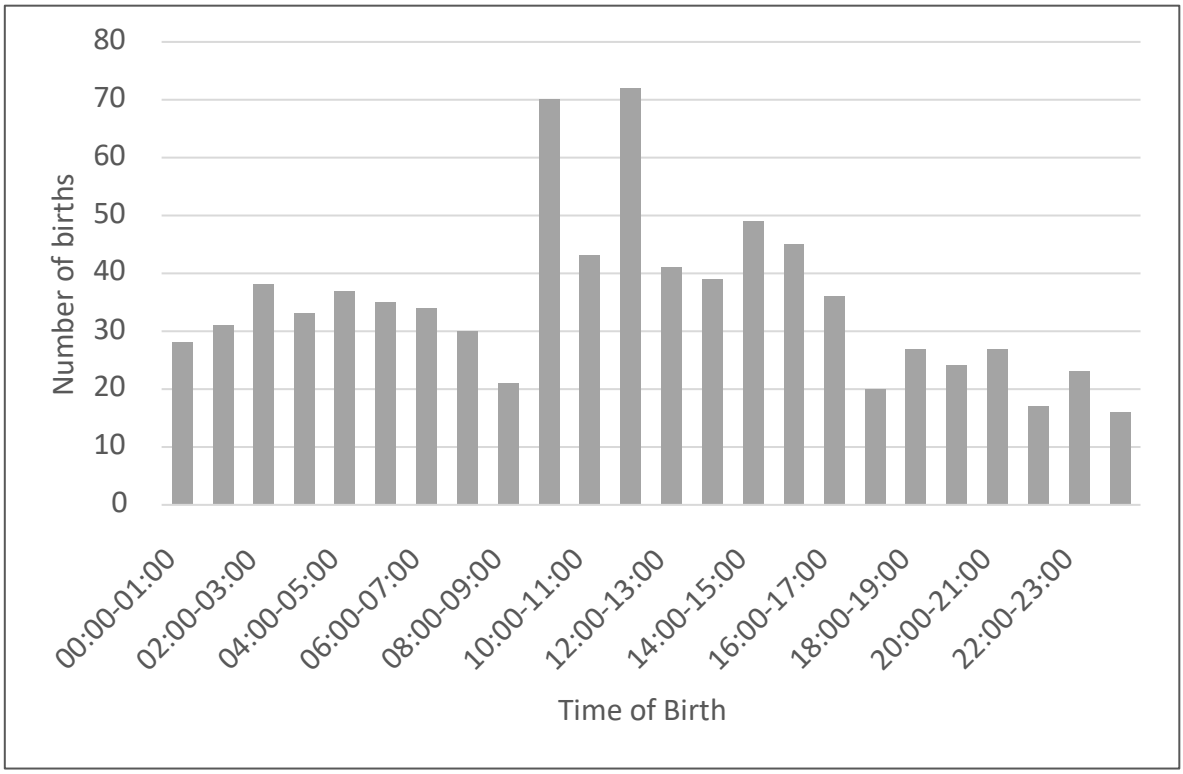

Figure 3.4: Time of birth of recruited infants.

3.4 Maternal smoking and alcohol consumption

As part of the confidential postnatal health questionnaire, smoking status (continued to smoke in pregnancy, stopped smoking in pregnancy or never smoked) was asked directly by the researcher of 828/840 mothers recruited to the study. 223 mothers (26.9%) declared smoking prior to pregnancy, of whom 145 (65%) continued to smoke in pregnancy. A further two mothers started smoking in this pregnancy. Thus 17.8% of all recruited mothers for whom data were available smoked in pregnancy (Table 3.5).

	ENTIRE RECRUITED POPULATION (N=840)	CONSENTED; 1 OR MORE SAMPLES (N=813)	CONSENTED; NO SAMPLES (N=27)	CONSENTED; MECONIUM SAMPLE COLLECTED (N=740)	CONSENTED; NO MECONIUM SAMPLE COLLECTED (N=100)
Smoked prior to pregnancy	N=828	802	26	731	97
	26.9%	26.8%	30.8%	26.8%	27.8%
Smoked during pregnancy	N=828	802	26	731	97
	17.8%	17.6%	23.1%	17.8%	17.5%
Stopped smoking in pregnancy	N=828	802	26	731	97
	9.4%	9.5%	7.7%	9.2%	11.3%
Never smoked	N=828	802	26	731	97
	72.8%	72.9%	69.2%	73.1%	71.1%
CO level mean (SD) (ppm)	N=621	601	20	549	72
	2.5 (3.7)	2.5 (3.7)	2.9 (4.2)	2.5 (3.6)	2.8 (4.2)
range	0 – 25	0 – 25	0 – 16	0 – 22	0 – 25
% > 5ppm	12.7	12.6	15	12.8	12.5
Ever drank prior to pregnancy	N=828	798	26	727	97
	80.6%	80.3%	84.6%	80.7%	78.4%
Drank prior to knowledge of pregnancy	N=828	779	22	713	92
	30.6%	31.6%	23.1%	32.4%	22.8%
Self-reported alcohol > 20 weeks' gestation	N=827	801	26	730	97
	13.8%	13.6%	19.2%	13.2%	18.6%
No alcohol in pregnancy	N=828	801	26	730	97
	53.7%	53.8%	53.8%	53.6%	55.7%

Table 3.5: Self-declared maternal smoking and alcohol consumption in relation to sample collection.

Smoking status was incompletely documented in the maternal handheld record and very poorly documented in the Scottish Birth Record. Despite being a universal screening test for smoking, carbon monoxide level as ascertained by a breath test had been documented at the booking visit for only 621 (73.9%) of the 840 recruited women. 93 (15%) mothers had a CO level of greater than or equal to 4 ppm (trigger for referral to the smoking cessation services). Of the women who declared at the postnatal interview never to have smoked, 93/524 (17.7%) of those tested returned a positive CO test ($\geq$4 ppm).

Overall, at least one sample was obtained from the infant for 813/840 recruited mothers (96.7%) although not all blood spot cards had sufficient blood for analysis. 141/147 (95.9%) mothers who smoked in pregnancy provided at least one of a meconium sample and/or a dried blood spot card compared to 661/668 (99%) mothers who either never smoked or who gave up smoking in pregnancy, this difference was significant (p=0.006). In terms of meconium samples alone, 130/147 (88.4%) of mothers who smoked in pregnancy provided a sample from their infant, compared to 601/681 (88.3%) of mothers who did not smoke in pregnancy.

Compared to those mothers from whose infants at least one sample was obtained, a higher proportion of mothers who did not provide either a meconium sample or dried blood spot card reported smoking prior to pregnancy (30.8 *vs* 26.8%) or smoking during pregnancy (23.1 *vs* 17.6%). (Table 3.5) These differences were not however significant (p=0.65).

827 women were asked during the confidential postnatal health questionnaire if they had consumed any alcohol during pregnancy (Table 3.5). 443 (53.6%) mothers stated that they had never consumed alcohol in pregnancy. Of the 384 mothers who reported consuming alcohol at any point in pregnancy, 252 (65.6%) stated that they only drank prior to knowing that they were pregnant. 114 (13.8%) of all recruited mothers who completed the questionnaire) reported drinking alcohol beyond 20 weeks of gestation of whom three reported one or more episode of binge drinking (*i.e.* five or more units of alcohol on one occasion). Fewer mothers reported drinking alcohol in mid-pregnancy. (Figures 3.5 and 3.6)

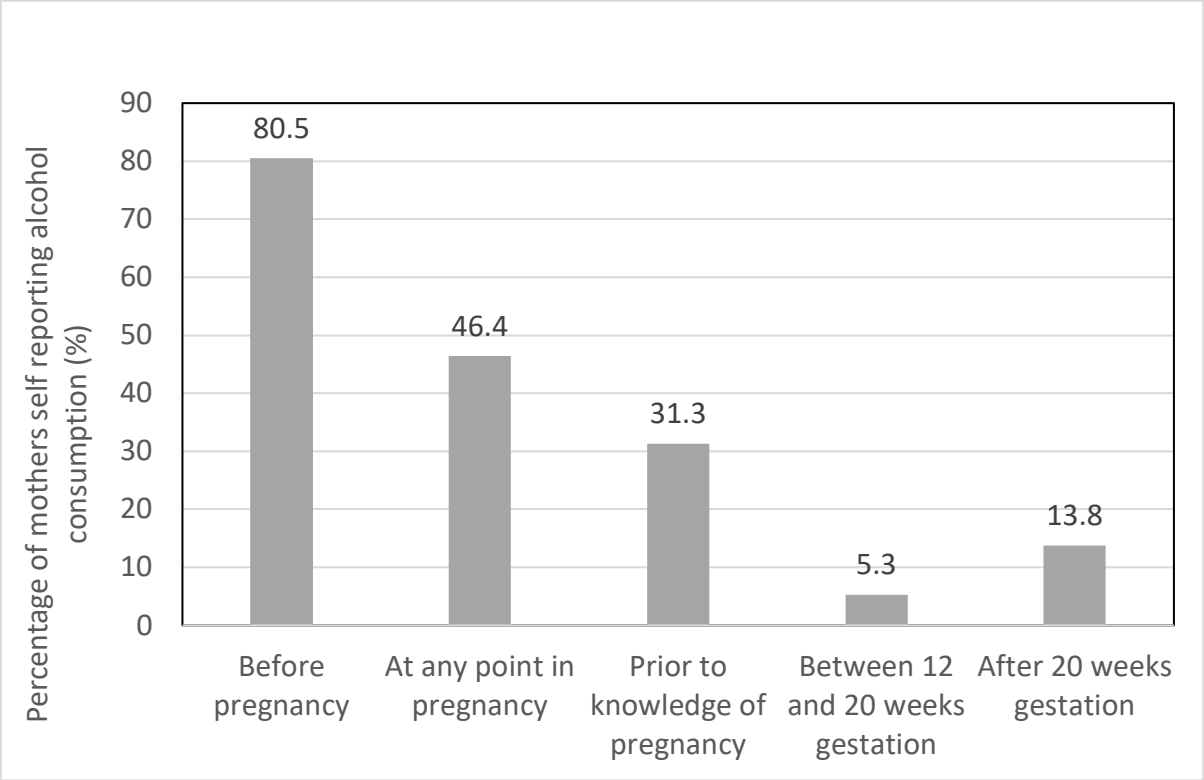

Figure 3.5 Percentage of mothers reporting any alcohol intake in relation to stage of pregnancy.

Women who self-reported any alcohol consumption in pregnancy (n=384) were more likely to identify as white British and to have smoked in pregnancy compared to those women who self-reported no alcohol consumption in pregnancy (n=443), (p= <0.0001). Women who self-reported alcohol consumption in pregnancy beyond 20 weeks' gestation were more likely to be aged > 35 years (p = 0.0004) and were also more likely to identify as white British compared to those mothers who did not report alcohol consumption in pregnancy after 20 weeks' gestation (p <0.05) (Table 3.6).

Babies born to mothers who declared alcohol consumption after 20 weeks' gestation had a mean weight of 3470 g compared to 3352 g (p=0.032); this was not explained either by gestation or by maternal BMI. Heavier birthweight was not accompanied by greater OFC.

	ENTIRE RECRUITED POPULATION* (N=840)	Ever drank alcohol in pregnancy (N=384)	Never drank alcohol in pregnancy (N=443)	Drank alcohol after 20 weeks' gestation (N=114)	Did not drink alcohol after 20 weeks' gestation (N=713)
Maternal age (years) mean (SD)	N=840	384	443	114	713
	29.8 (5.7)	29.5 (5.8)	30 (5.5)	31.3 (5.7)	29.5 (5.6)
range	16 – 50.3	16 - 45.4	17 - 50.3	16 – 45.4	16.1 – 50.3
% aged > 35 years	19.5	20.6	18.3	31.6+	17.4+
Maternal BMI (kg/m^2) median (IQR)	N=822	380	429	112	697
	25.8 (22.9 – 30.3)	27.0 (16 - 30.3)	26.2 (23 - 30.2)	25.4 (23 – 29.8)	25.8 (22.8 – 30.3)
range	(15.2 – 59)	(22.8 - 53)	(15.2 - 59)	(19.1 – 53)	(15.2 – 59)
SMID decile median (IQR)	N=831	379	440	110	709
	3 (1 – 6)	4 (1 - 7)	3 (1 - 6)	5 (2 - 8)	4 (1 – 6)
range	1 – 10	1 - 10	1 - 10	1 – 10	1 – 10
Ethnicity (% White British)	N=803	370	423	107	686
	80.3%	90.5%+	71.6%+	91.6%+	79.7%+
Smoked during pregnancy	N=828	383	442	113	712
	17.8%	20.1	15.8	13.3	18.5
Parous mums (%)	N=822	375	435	111	699
	56.3%	54.1%	57.7%	54.1%	56.8%
Mode of delivery (% vaginal)	N=839	383	443	114	712
	61.5%	62.9%	60.3%	60.5%	61.7%
Infant sex (% male)	N=840	384	443	114	713
	50.1%	50.8%	49.9%	48.2%	50.6%
Gestation (weeks) mean (SD)	N=837	382	442	113	711
	38.9 (1.7)	38.9 (1.5)	38.9 (1.8)	39 (1.3)	38.9 (1.7)
range	28 – 42	32 - 41	28 - 42	34 – 41	28 – 42
Birthweight (g) mean (SD)	N=838	N=383	442	113	712
	3367 (526)	3369 (511)	3368 (538)	3470 (474)+	3352 (532)+
range	960 – 4905	1774-4672	3075 - 3705	2235 – 4675	960 – 4905
OFC (cm) Mean (SD)	N=825	375	438	111	702
	34.6 (1.5)	34.7 (1.5)	34.6 (1.5)	34.7 (1.5)	34.6 (1.5)
Range	24.8 – 41.5	30.4 – 41.5	24.8-39	30.5 – 41.2	24.8 – 41.5
APGAR score mean (SD)	N=829	381	436	112	705
	9 (1.3)	9 (1.2)	9 (1.3)	9 (1.3)	8.6 (1.5)
range	0 – 10	1 - 10	0-10	1 – 9	0 – 10

Table 3.6 Maternal self-report of alcohol consumption beyond twenty weeks' gestation in comparison to maternal and infant demographics

* self-report of alcohol consumption available for 827 mothers
+ P=<0.05

3.5 Infant biomarkers

3.5.1 Analysis of Meconium for Fatty Acid Ethyl Esters (FAEE)

740 meconium samples were sent for analysis of both fatty acid ethyl ester (FAEE) and ethyl glucuronide (EtG) concentrations, of which 712 meconium samples were successfully analysed for ethyl-myristate, ethyl palmitate, ethyl oleate and ethyl stearate as well as EtG. The concentrations of each of the FAEE metabolites were summed, to give total FAEEs (ng/g). (Table 3.7)

	Fatty acid ethyl ester concentrations (ng/g)				
	Ethylmyristate	Ethylpalmitate	Ethyloleate	Ethylstearate	Total of 4 FAEEs
Min	3.6	5.9	7.3	2.4	22.2
Max	2384.0	2166.2	2683.4	1266.6	7549.8
Median	120.5	121.9	139.7	85.2	533.9
Interquartile (25th)	57.5	57.7	66.2	34	271.6
Interquartile (75th)	204.5	209.9	241.6	161.2	773.6
Mean (SD)	162.4 (211)	166.2 (193)	190.4 (237)	124 (135)	642.8 (669)

Table 3.7 Individual and total fatty acid ethyl esters (FAEEs) in meconium samples.

Every meconium sample yielded a positive reading for FAEEs with the total concentration of FAEEs ranging from 22.2 to 7549.8 ng/g. Using a cut off value of ≥600 ng/g total FAEEs, 282 (39.6%) meconium samples were positive, and 430 (60.4%) samples were negative for FAEEs (Table 3.8). Of the positive meconium samples, 21 (2.9% of the samples analysed, 2.5% of all recruited mothers) yielded a total FAEE concentration of ≥2000 ng/g.

	Meconium sample resulted (N=712)	FAEEs ≥600 ng/g (N=282)	FAEEs <600 ng/g (N=430)	STATISTICS
Maternal age (years) mean (SD)	N=712	282	430	
	29.8 (5.6)	30.1 (5.3)	29.7 (5.8)	
range	16 – 50.3	16.0 – 43.6	16.1 – 50.3	
% >35 years	19.5	19.9	19.3	P=0.855
Maternal BMI (kg/m^2) median (IQR)	N=696	274	422	
	25.7 (22.8 – 30.1)	25.6 (23 – 30.6)	26.9 (22.5 – 30)	
range	15.2 – 59	15.2 – 50.4	16.0 – 59	p=0.360
SIMD decile median (IQR)	N=704	280	424	
	3 (1 – 6)	3 (1 – 7)	3 (1 – 6)	
range	1 – 10	1 – 10	1 – 10	P=0.387
Ethnicity (% White British)	N=682	276	406	
	80.2%	79%	81%	P=0.513
Smoked during pregnancy	N=703	279	424	
	17.9%	14.7%	20%	P=0.071
Parous mums (%)	N=699	280	419	
	55.8%	57.9%	54.4%	P=0.368
Mode of delivery (% vaginal)	N=711	282	429	
	61%	61.7%	60.6%	
Infant sex (% male)	N=712	282	430	
	51.3%	51.4%	51.2%	
Gestation (weeks) mean (SD)	N=709	282	427	
	38.9 (1.6)	39 (1.5)	38.8 (1.7)	
range	30 – 42	30 – 42	31 – 40	
Birthweight (g) mean (SD)	N=710	282	428	
	3373 (526)	3425 (523)$^{+}$	3331 (525)$^{+}$	P=0.032^{+}
range	1320 – 4905	1600 – 4886	1320 – 4905	
OFC (cm) Mean (SD)	N=700	276	424	
	34.6 (1.4)	34.7 (1.4)	34.6 (1.5)	
Range	29.5 – 41.2	30.4 – 38.0	29.5 – 41.2	
APGAR score mean (SD)	N=703	278	425	
	8.6 (1.2)	9 (1.2)	8.6 (1.3)	
range	0 – 10	1 – 10	0 – 10	

Table 3.8 Maternal and infant demographics in relation to meconium sample positive or negative for FAEEs.

There was no relationship between maternal age, BMI, SIMD score, parity or ethnicity and the likelihood of the infant's meconium being either negative or

positive for FAEEs. Within the 20 mothers whose infant's meconium sample yielded a total FAEE concentration of ≥2000 ng/g and for whom SIMD was available, there was a slight preponderance of postcodes in SIMD16 deciles 2 and 4, but numbers were too small to make meaningful statistical comparison.

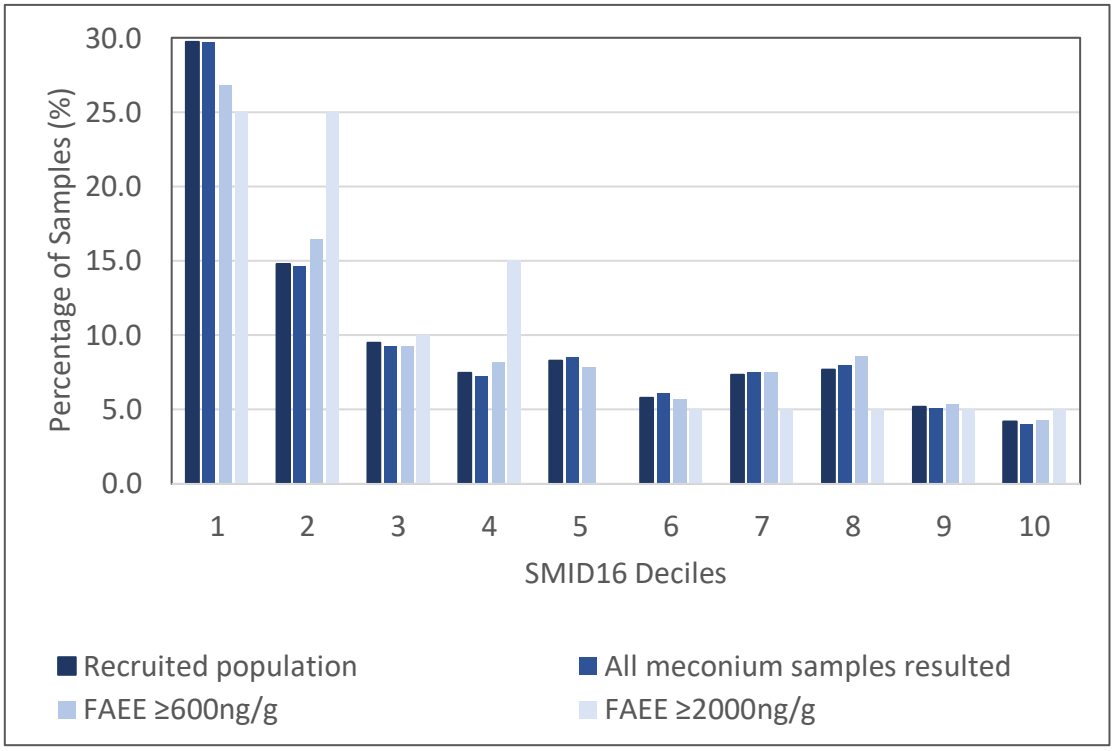

Figure :3.6 Socioeconomic profile in relation to meconium sample being positive for FAEEs.

Mothers tended to have been less likely to smoke during pregnancy within the group whose infant's meconium was positive for FAEEs, compared to the group whose infant's meconium contained <600 ng/g FAEEs (14 *vs* 20%, p=0.071). Among infants of the 126 mothers who declared smoking during pregnancy total FAEE concentration in meconium was ≥600 ng/g in 32.5%. By comparison, 23/63 (36.5%) meconium samples from infants whose mothers reported stopping smoking in pregnancy were positive for FAEEs, compared to 215/514 (41.8%) for infants of women who had never smoked. This difference was not significant.

The likelihood of infant meconium being positive for FAEEs was not related to sex or gestation, but mean birth weight was greater for those infants whose meconium contained ≥600 ng/g total FAEEs (3425g (SD 523) compared to 3331g (SD 525) for

infants with a negative meconium sample (P=0.032). Mean OFC did not however differ between these two groups.

Self-reported alcohol consumption as determined by confidential interview after delivery could be linked with 702 meconium samples. When infant meconium was positive for FAEEs, as defined by a concentration of ≥600 ng/g, 41/280 (14.5 %) mothers had reported alcohol consumption later than 20 weeks of pregnancy. When the concentration of FAEEs in infant meconium was <600 ng/g, 54/422 (13%) mothers had reported alcohol consumption after 20 weeks' gestation, this difference did not reach significance. Thus, maternal self-report of any alcohol consumption in later pregnancy did not predict an infant meconium total FAEE concentration of ≥600 ng/g. Infants of mothers who reported alcohol consumption beyond 20 weeks' gestation tended to have a higher mean total FAEE concentration in meconium compared with those infants whose mothers did not report alcohol after 20 weeks' gestation (699 ng/g *vs* 638.5 ng/g). Of the eight mothers that reported drinking at least three units of alcohol on any one occasion beyond 20 weeks of gestation, three had an infant whose meconium was positive for FAEEs using a cut off of ≥600 ng/g (concentrations 707, 847 and 996 ng/g respectively)

3.5.2 Analysis of meconium for ethyl glucuronide (EtG)

740 meconium samples were sent for measurement of ethyl glucuronide (EtG) concentration, of which 712 samples were successfully analysed and were eligible for comparison with maternal and infant demographics. EtG concentration in meconium ranged from 0 to 8894.9 ng/g. EtG was detected in 293 samples, of which EtG concentration was below the LOQ (*i.e.*, <10 ng/g) in 96. In the remaining 419 samples no EtG was detectable. A cut off value of ≥30 ng/g EtG was designated a positive result.(116) Of the 197 samples in which EtG was detectable above the limit of quantification, 103 had an EtG concentration of ≥30 ng/g ("positive"). This represented 14.5% of the total number of analysable samples. For all the 712 samples, the median concentration of EtG was 0 ng/g, and the IQR 0 to 12.4 ng/g. When EtG was detectable above the limit of quantification (n=197), the mean concentration was 162.1 ng/g (SD 871) and the median concentration was 32.2 ng/g, IQR 17.9 to 55.8 ng/g.

	Meconium sample resulted (N=712)	EtG ≥30 ng/g (N=103)	EtG <30 ng/g (N=609)	STATISTICS
Maternal age (years) mean (SD)	N=712	103	609	
	29.8 (5.6)	29.4 (6.5)	29.9 (5.6)	
range	16 – 50.3	17.7 – 41.3	16.0 – 50.3	
% > 35 years	19.5	17.5	19.9	P=0.557
Maternal BMI (kg/m^2) median (IQR)	N=696	100	596	
	25.7 (22.8 – 30.1)	26.9 (22.8 – 30)	27.1 (22.7 – 30.2)	P=0.893
range	15.2 – 59	17.7 – 44.6	15.2 – 59	
SIMD decile median (IQR)	N=704	101	603	
	3 (1 – 6)	2 (1 – 6)	3 (1 – 6.5)	P=0.371
range	1 – 10	1 – 10	1 – 10	
Ethnicity (% White British)	N=682	101	581	
	80.2%	71.3%	81.8%	P=0.028
Smoked during pregnancy	N=703	103	600	
	17.9%	13.6%	18.5%	P=0.174
Parous mums (%)	N=699	101	598	
	55.8%	52.5%	56.4%	P=0.516
Mode of delivery (% vaginal)	N=711	102	609	
	61%	64.7%	60.4%	P=0.409
Infant sex (% male)	N=712	103	609	
	51.3%	45.6%	52.2%	
Gestation (weeks) mean (SD)	N=709	103	606	
	38.9 (1.6)	38.8 (1.8)	38.9 (1.6)	
range	30 – 42	34 – 42	30 – 42	
Birthweight (g) mean (SD)	N=710	103	607	
	3373 (526)	3418 (524)	3365 (526)	P=0.349
range	1320 – 4905	2220 – 4750	1320 – 4905	
OFC (cm) Mean (SD)	N=700	101	599	
	34.6 (1.4)	34.8 (1.5)	34.6	
Range	29.5 – 41.2	31.8 – 41.2	29.5 -38.5	
APGAR score mean (SD)	N=703	100	603	
	8.6 (1.2)	8.5 (1.1)	8.6 (1.3)	
range	0 – 10	4 – 9	0 – 10	

Table 3.9 Maternal and infant demographics in relation to meconium sample positive or negative for EtG

There was no difference in either maternal age, BMI, parity, or SIMD score when meconium samples were grouped into EtG positive (n=103) or EtG negative (N=609) (Table 3.8). Infants whose meconium sample was positive for EtG were however less likely to have a mother who identified as white British (71.3 *vs* 81.8%, p=0.028).

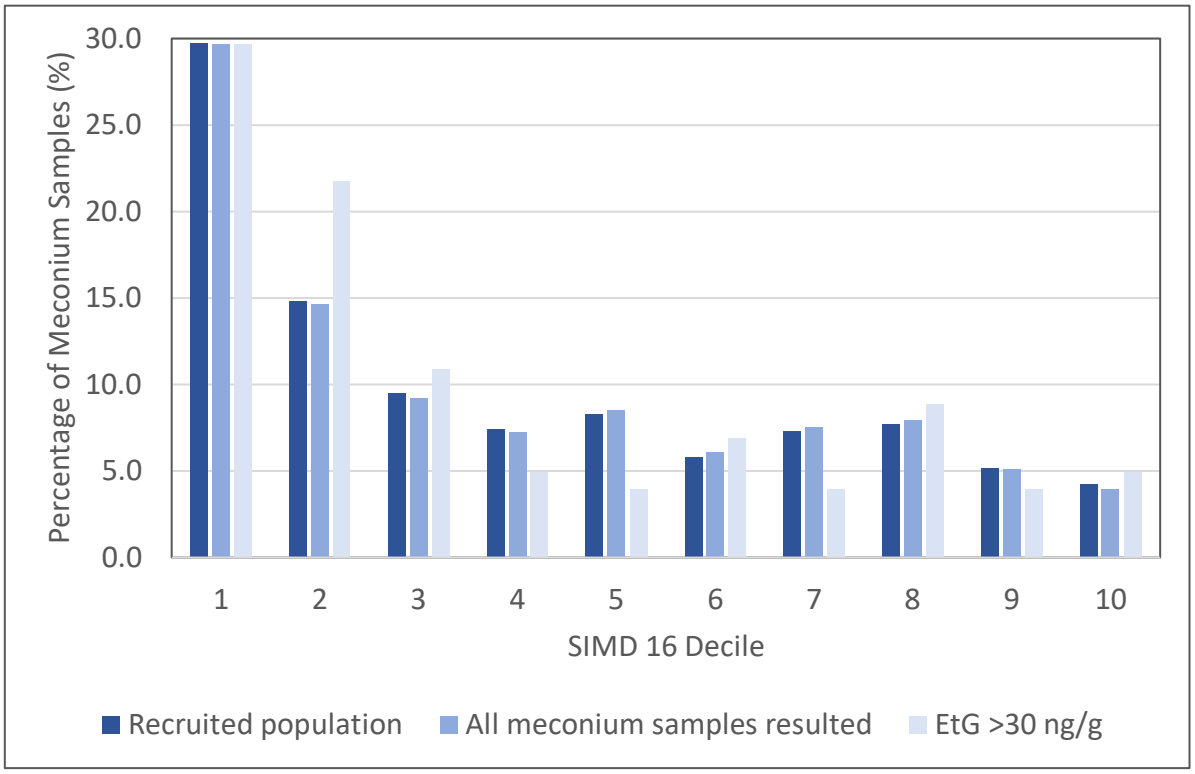

Figure 3.7 Socioeconomic profile in relation to meconium sample being positive for EtG.

When SIMD 16 data were combined into deciles, roughly equal percentages of samples with EtG concentration ≥30 ng/g were obtained from babies whose mothers resided in deprived or relatively affluent postcodes (figure 3.7). A similar preponderance of positive EtG samples from SIMD decile 2 as was noted for FAEEs was observed, but numbers were too small to make useful statistical comparison.

There was a lesser likelihood of the mother having smoked during pregnancy when the infant meconium was positive for EtG (13.6 *vs* 18.5%) but this difference did not reach statistical significance (Table 3.9). Of the mothers who stopped smoking in pregnancy (N=63), 7.9% infant meconium samples were positive for EtG, compared to 16.3% of samples from infants of mothers who never smoked. Thus, having stopped smoking during pregnancy was associated with a trend towards a lesser likelihood of PAE.

Slightly fewer girls yielded meconium samples positive for EtG (45.6 *vs* 52.2%). Gestation was not different between infants whose meconium samples were positive or negative for EtG; similarly to FAEEs, birth weight tended to be higher

when meconium was positive for EtG but this difference did not achieve statistical significance (mean 3418 (SD 524) *vs* 3365 (SD 526) P = 0.349). This finding was mirrored by a marginal increase in mean OFC for newborns whose meconium was positive for EtG (mean 34.8 (SD 1.5) *vs* 34.6 (1.4) cm).

Self-reported alcohol consumption as determined by confidential interview after delivery could be linked with 702 meconium samples. When infant meconium was positive for EtG, as defined by a concentration of ≥30 ng/g (N =103), 11 (10.7 %) mothers had reported alcohol consumption later than 20 weeks of pregnancy. When the concentration of EtG in infant meconium was <30 ng/g (N=599), 84 (14%) mothers had reported alcohol consumption after 20 weeks' gestation. Thus, maternal self-report of any alcohol consumption in later pregnancy did not predict an infant meconium EtG concentration of ≥30 ng/g. In fact, infants of mothers who reported alcohol consumption beyond 20 weeks' gestation tended to have a lower mean EtG meconium concentration compared with those infants whose mothers did not report alcohol consumption after 20 weeks' gestation (20.1 ng/g *vs* 50.2 ng/g). Of the eight mothers that reported drinking a least three units of alcohol on any one occasion beyond 20 weeks of gestation, one had an infant whose meconium was positive for EtG using a cut off of ≥30 ng/g

Correlation between FAEEs and EtG

EtG concentration in meconium was compared with total FAEE concentration in the same sample. All meconium samples yielded some FAEEs but only 293 samples had detectable EtG, of which 96 were below the LOQ (Figure 3.8). There was no correlation between EtG and total FAEEs. However, when only samples positive for both FAEEs (≥600 ng/g) and EtG (≥30 ng/g) (n=51) were considered (Figure 3.9) there was a weakly positive correlation (Pearson's coefficient= 0.283, p value=0.044).

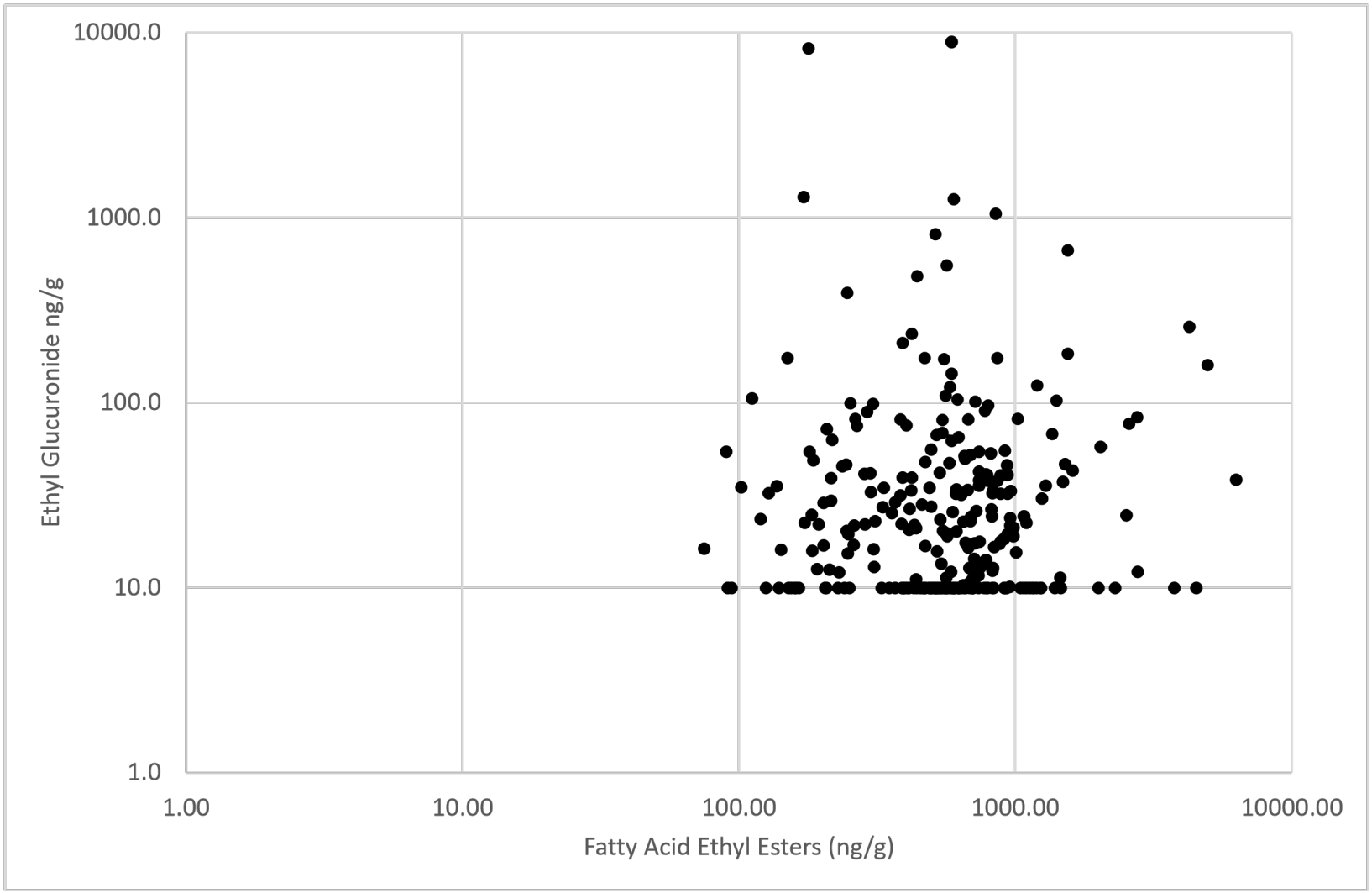

Figure 3.8 EtG concentration in relation to total FAEEs for all resulted meconium samples (ETG LOQ <10 ng/g)

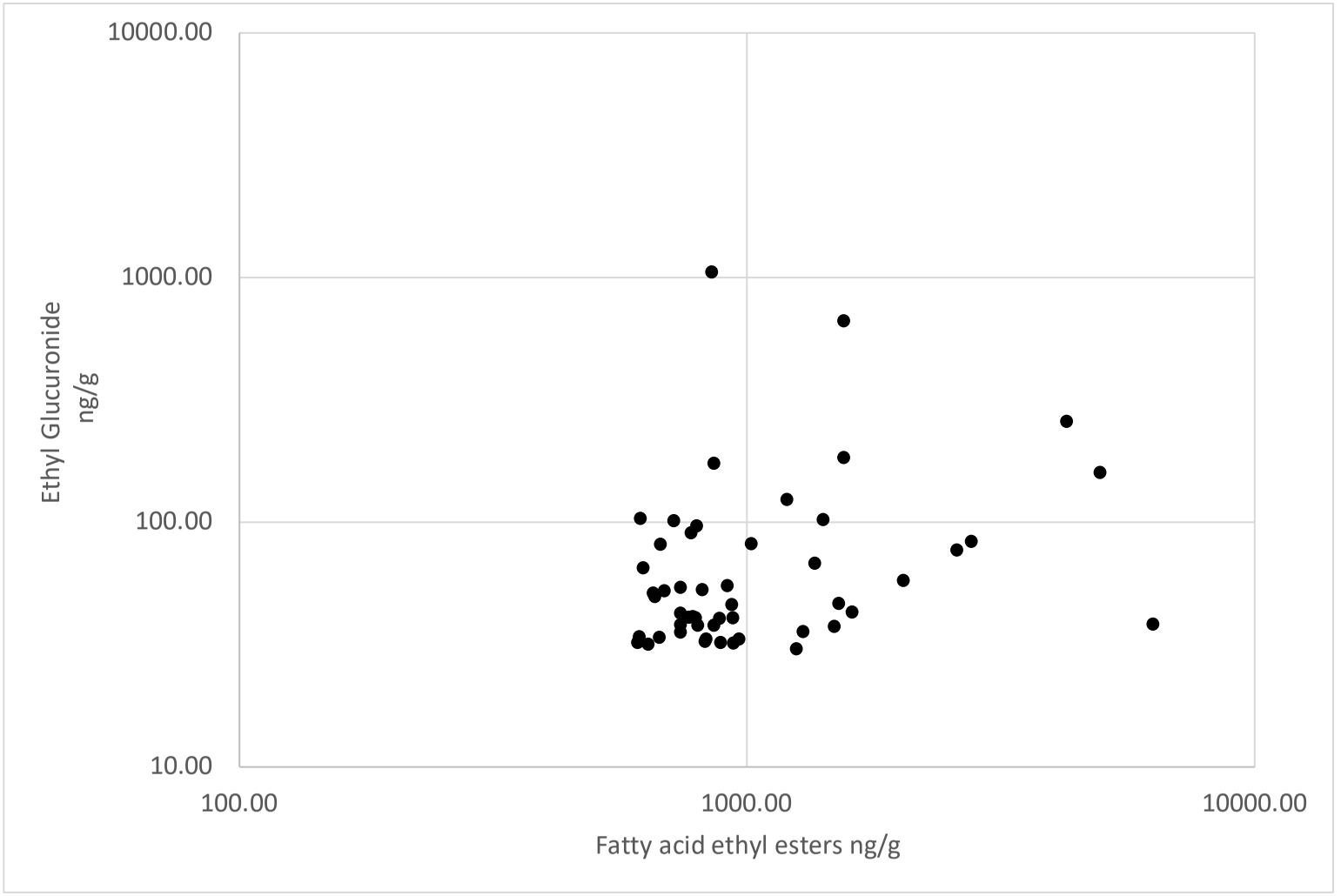

Figure 3.9 EtG concentration in relation to total FAEEs for meconium samples when both EtG and FAEEs were positive (30 ng/g and 600 ng/g respectively)

3.5.3 Analysis of infant blood obtained on day five for phosphatidylethanol (PEth)

510 blood spot samples were sent for assay of PEth concentration, of which 502 samples were suitable for analysis. PEth was detectable in 262 (52%) samples, with concentrations ranging from 2.4 to 3991.6 ng/ml. The mean PEth concentration was 100.5 ng/ml (SD 416.3, median 5 ng/g). Comparison with maternal and infant demographics was made utilising two cut off values for PEth, ≥8 ng/ml and ≥20 ng/ml (121, 137, 150).

3.5.3.1 PEth cut off ≥8 ng/ml

216 (43%) meconium samples had a total PEth concentration of ≥8 ng/ml. Results were highly skewed with a mean concentration of 120.7 ng/ml and a median concentration of 29.5 (IQR 16.3, 57.5) ng/ml).

	Blood spot card resulted (N=502)	PEth ≥8 ng/ml (N=216)	PEth <8 ng/ml (N=286)	STATISTICS
Maternal age (years)	N=502	216	286	
mean (SD)	29.6 (5.7)	29.3 (6)	29.9 (5.5)	
range	16 .1– 45.4	16.8 – 45.5	16.1 – 43.6	
% >35 years	19.3	22.7	16.1	p=0.102
Maternal BMI	N=489	211	278	
(kg/m^2) median (IQR)	27.3 (22.9 – 30.1)	27.8 (23 – 31)	27 (22.8 – 30)	p=0.301
range	16.2 – 59	16.5 - 59	16.2 – 53	
SIMD decile median	N=497	214	283	
(IQR)	3 (1 – 6)	3 (1 – 6)	3 (1 – 7)	p=0.157
range	1 – 10	1 – 10	1 – 10	
Ethnicity	N=482	209	273	
(% White British)	82.4%	86.6%	79.1	p=0.028
Smoked during	N=497	215	282	
pregnancy	18.3%	22.3%	15.2%	p=0.047
Parous mums	N=491	212	279	
(%)	59.5%	60.4%	58.8%	p=0.721
Mode of delivery (%	N=502	216	286	
vaginal)	60%	61.6%	58.7%	
Infant sex	N=502	216	286	
(% male)	48.2%	46.8%	49.3%	
Gestation (weeks)	N=500	216	284	
mean (SD)	38.9 (1.6)	39 (1.4)	38.8 (1.8)	
range	28 – 42	34 – 42	28 – 42	
Birthweight (g)	N=501	216	285	
mean (SD)	3389 (540)	3428 (500)	3360 (567)	p=0.052
range	960 – 4905	1912 – 4886	960-4905	
OFC (cm)	N=492	213	279	
Mean (SD)	34.7 (1.6)	34.8 (1.5)	34.6 (1.7)	
Range	24.8 – 41.5	30.5 – 38.5	24.8 – 41.5	
APGAR score	N=492	215	227	
mean (SD)	8.6 (1.3)	8.6 (1.3)	8.6 (1.3)	
range	0 – 10	1 – 10	0 – 9	

Table 3.10 Maternal and infant demographics in relation to PEth concentration in dried blood spot cards (8 ng/ml cut off).

There was no relationship between maternal age, parity, BMI, SIMD16 decile and the likelihood of the infant's blood spot card having a PEth concentration ≥8 ng/ml or <8 ng/ml. There was a trend toward mothers of infants with a blood spot positive for PEth to be more likely to be over 35 years of age, 22.7 *vs* 16.1%,

p=0.102. When the infant blood spot card contained ≥8 ng/ml PEth, the mother was more likely to have self-identified as white British (86.6 *vs* 79.1%, p=0.028) and to have smoked during pregnancy (p=0.047).

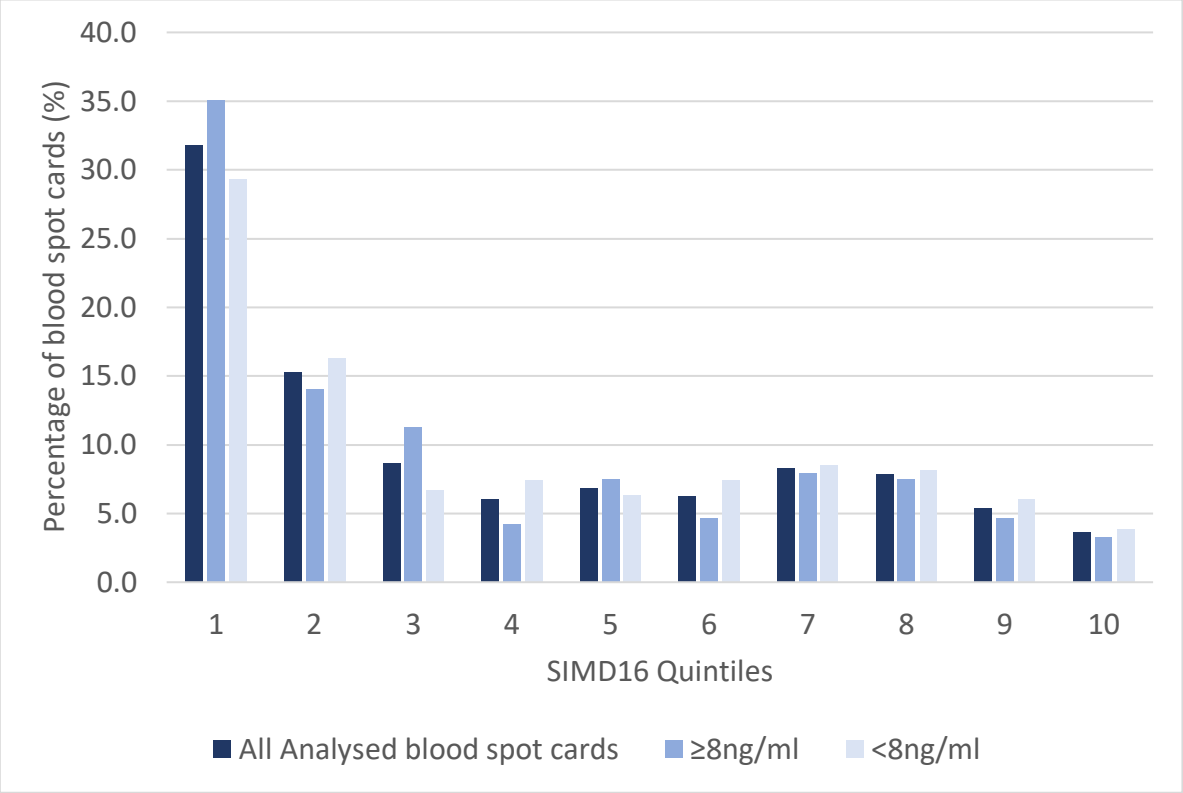

Figure 3.10 Socioeconomic profile in relation to blood spot sample being positive for PEth (8 ng/ml cut off).

When SIMD 16 scores were combined into deciles, roughly equal numbers of those samples with PEth concentration ≥8 ng/ml compared to PEth samples with <8 ng/ml were obtained from babies whose mothers resided in deprived or relatively affluent postcodes.

Those infants with a positive blood sample as defined by PEth concentration ≥8 ng/ml tended to be heavier at birth (p=0.052); there was however no difference in OFC.

Self-reported maternal alcohol consumption was linked with 502 blood spot cards. Of those infants whose blood concentration of PEth was ≥8 ng/ml, 32 (14.8%) mothers reported alcohol consumption later than 20 weeks of pregnancy. When the infant blood spot contained <8 ng/ml PEth, 35 (12.2%) mothers reported

alcohol consumption after 20 weeks' gestation; this difference was not significant (p=0.396).

3.5.3.2 PEth cut off ≥20 ng/ml

148 (29.5%) of blood spot samples had a PEth concentration of ≥20 ng/ml.

	Blood spot card resulted (N=502)	PEth ≥20 ng/ml (N=148)	PEth <20 ng/ml (N=354)	STATISTICS
Maternal age (years) mean (SD)	N=502	148	354	
	29.6 (5.7)	28.7 (5.9)	30.0 (5.6)	p=0.023
range	16 .1– 45.4	16.8 – 42.8	16.1 – 45.4	
% over 35 years	19.3%	18.9%	19.5%	
Maternal BMI (kg/m^2) median (IQR)	N=489	144	345	
	27.3 (22.9 – 30.1)	28.4 (23.2 – 32)	26.9 (22.7 – 29.9)	p=0.038
range	16.2 – 59	16.5 – 59	16.2 – 53	
SIMD decile median (IQR)	N=497	147	350	
	3 (1 – 6)	2 (1 – 5)	3 (1 – 7)	p=0.026
range	1 – 10	1 – 10	1 – 10	
Ethnicity (% White British)	N=482	141	341	
	82.4%	85.1%	81.2%	p=0.291
Smoked during pregnancy	N=497	147	350	
	18.3%	24.5%	15.7%	p=0.021
Parous mums (%)	N=491	145	346	
	59.5%	60%	59.2%	p=0.877
Mode of delivery (% vaginal)	N=502	145	354	
	60%	64.2%	58.2%	
Infant sex (% male)	N=502	148	354	
	48.2%	47.3%	48.6%	
Gestation (weeks) mean (SD)	N=500	148	352	
	38.9 (1.6)	39.1 (1.3)	38.8 (1.8)	
range	28 – 42	34 – 42	28 – 42	
Birthweight (g) mean (SD)	N=501	148	353	
	3389 (540)	3457 (491)	3360 (557)	p=0.068
range	960– 4905	1912 – 4886	960 – 4905	
OFC (cm) Mean (SD)	N=492	146	346	
	34.7 (1.6)	34.9 (1.4)	34.6 (1.7)	
Range	24.8 – 41.5	30.5 – 38.5	24.8 – 41.5	
APGAR score mean (SD)	N=492	147	345	
	8.6 (1.3)	8.6 (1.5)	8.6 (1.2)	
range	0 – 10	1 – 10	0 – 9	

Table 3.11 Maternal and infant demographics in relation to PEth concentration in dried blood spot cards (20 ng/ml) cut off

Mothers of infants whose blood spot card was positive for PEth (≥ 20 ng/ml) were younger (p=0.023) and their BMI was greater (p=0.038). They were also more likely to have a lower SIMD score (p=<0.05). Slightly more mothers identified as white British among those infants whose blood spot card contained ≥ 20 ng/ml PEth but this difference was not significant.

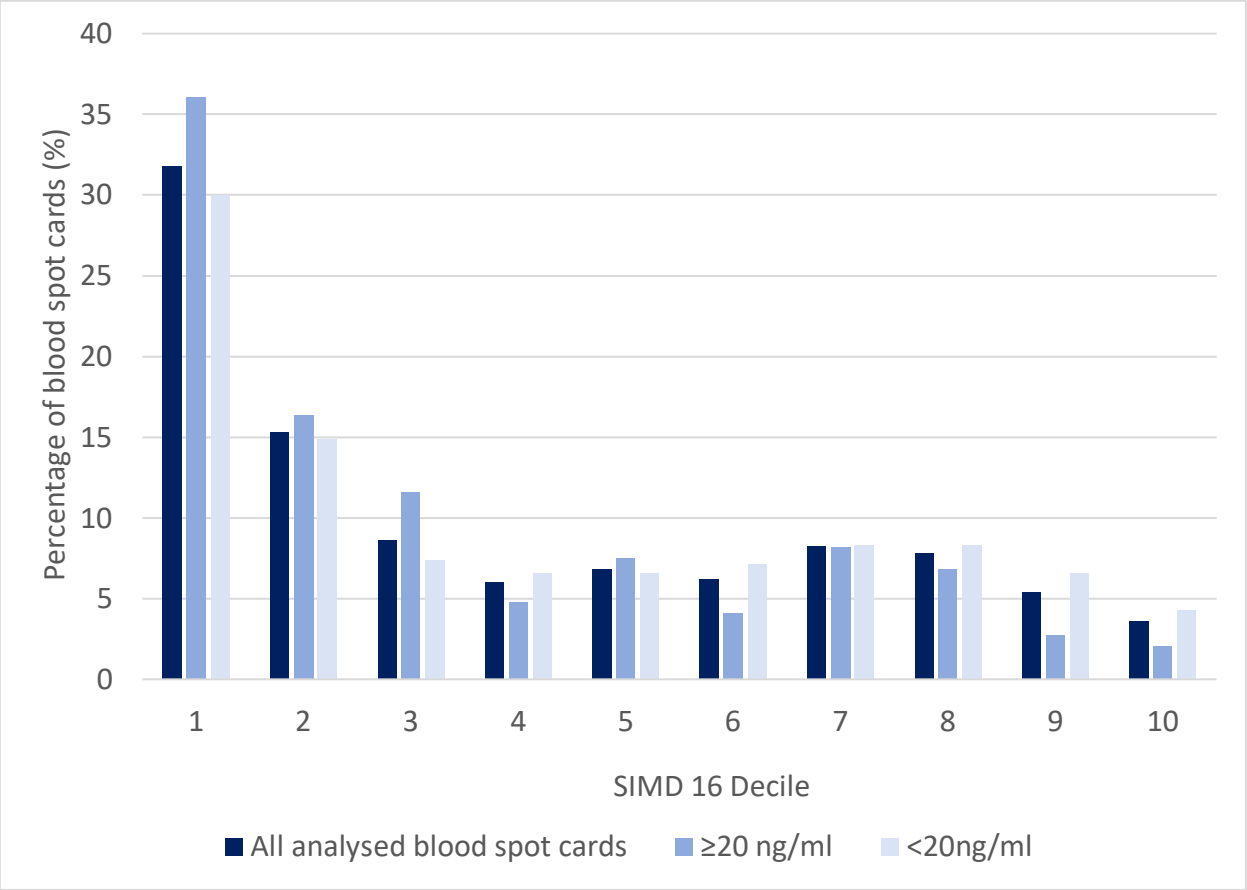

Figure 3.11 Socioeconomic profile in relation to blood spot sample being positive for PEth (20 ng/ml cut off).

When the infant's blood spot sample PEth concentration was ≥20 ng/ml, the mother was more likely to have smoked (p=<0.0215).

Infants with a PEth concentration of ≥20 ng/ml in their blood spot card tended to have a higher birth weight (3457 *vs* 3360 g, p=0.068). OFCs was marginally higher in infants with a positive PEth sample, however this was not of clinical significance.

When the infant's PEth concentration was ≥20 ng/ml 19 (12.8%) mothers reported alcohol consumption later than 20 weeks of pregnancy; this was very similar to the

48 (13.6%) of mothers who declared alcohol consumption after 20 weeks' gestation when the infant's PEth concentration was <20 ng/ml.

There was no correlation between PEth measured in dried blood spot cards and either FAEEs (Pearson co-efficient-0.019, p= 0.695) or EtG measured in meconium (Pearson co-efficient -0.009, P= 0.843) (Figures 3.12 and 3.13).

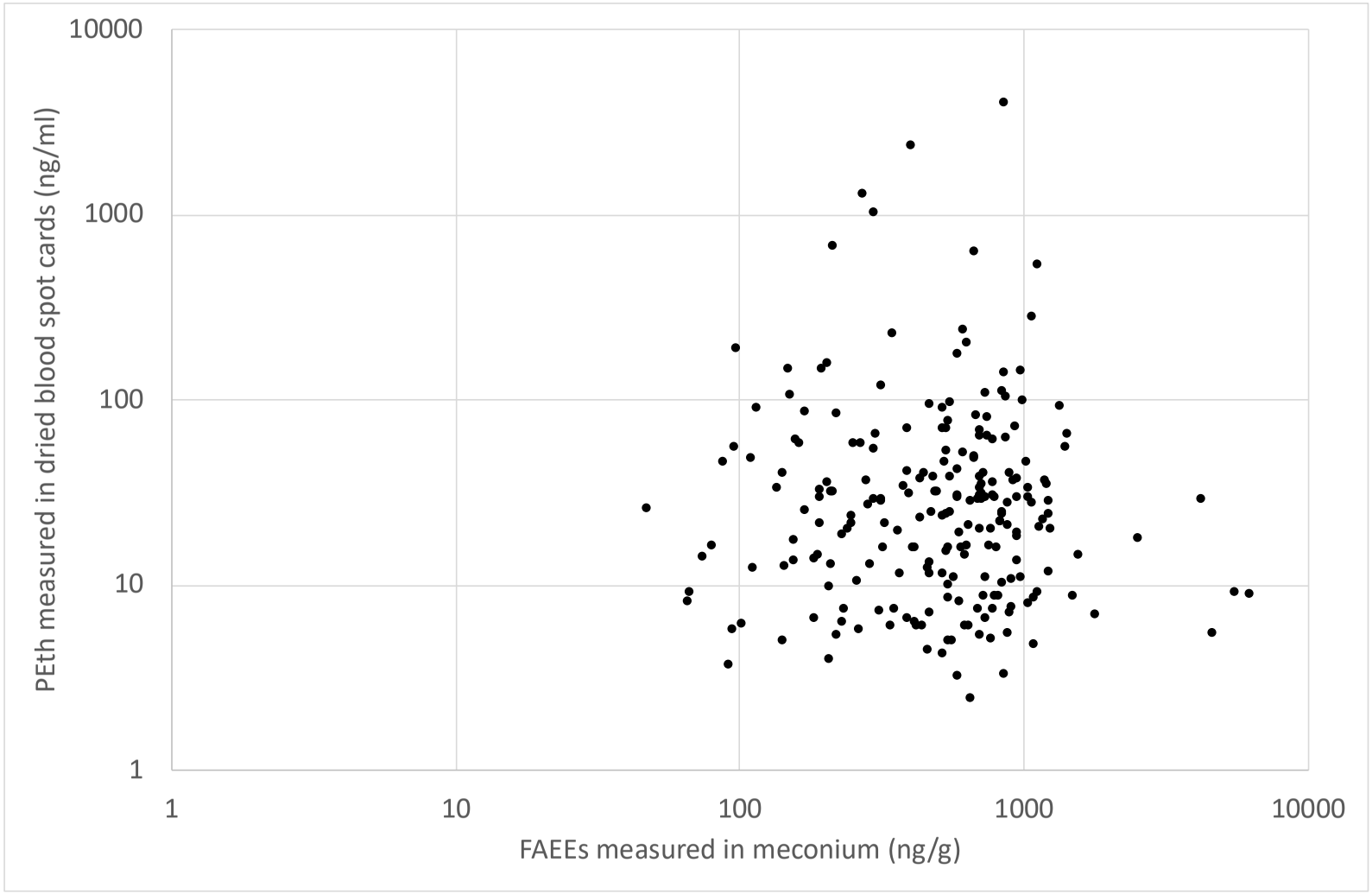

Figure 3.12 PEth concentration as measured in dried blood spot cards compared to FAEE concentration in meconium

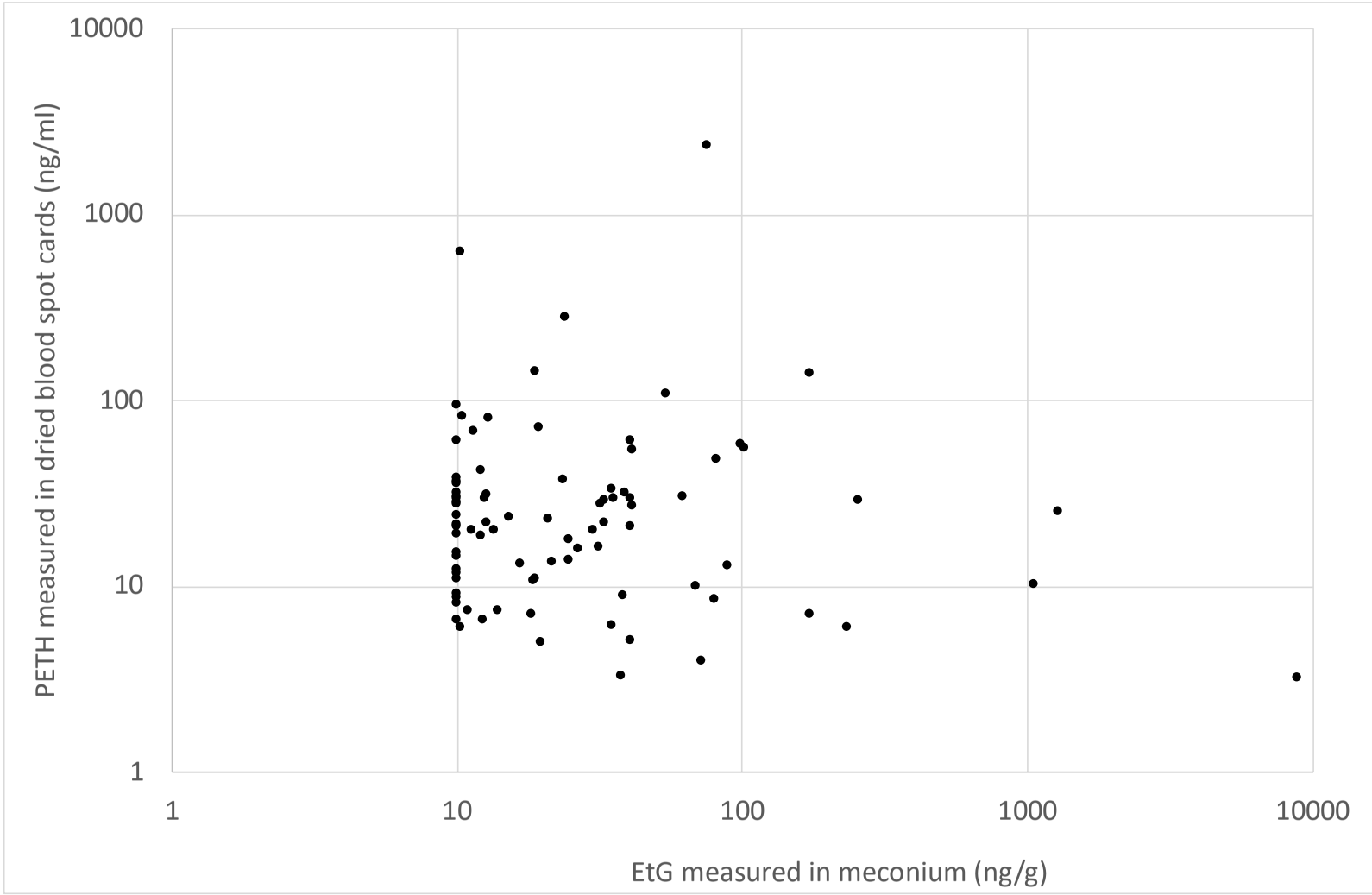

Figure 3.13 PEth concentration in dried blood spot cards compared to EtG in meconium (EtG LOQ=10ng/g)

3.6 Sensitivity and specificity of alcohol biomarkers

The sensitivity and specificity of the infant biomarkers were calculated using maternal self-report as the standard (Table 3.11).

	Self-reported alcohol consumption in pregnancy at any time (n=328)				Self-reported alcohol consumption in pregnancy after 20 weeks' gestation (n=95)			
	sensitivity	specificity	PPV	NPV	sensitivity	specificity	PPV	NPV
FAEEs ≥600ng/g	41.2%	61.2%	48.2%	54.3%	43.1%	60.6%	14.6%	87.2%
EtG ≥30ng/g	13.1%	84%	41.7%	52.4%	11.6%	84.8%	10.7%	86%
FAEEs ≥600ng/g and EtG ≥30ng/g	6.7%	92.2%	43.1%	53%	5.3%	92.4%	9.8%	86.2%
PEth ≥8ng/ml	44%	57.1%	45.8%	55.3%	47.8%	57.3%	14.8%	87.6%
PEth ≥20ng/ml	29.3%	71.1%	46.6%	55.4%	28.4%	70.1%	12.8%	86.3%

Table 3.12 Sensitivity, specificity, positive predictive value, and negative predictive value of infant biomarkers for PAE as ascertained by confidential postpartum maternal interview.

Sensitivities of the various alcohol biomarkers ranged from 11.6% for EtG (≥ 30 ng/g) to 47.8% for a PEth concentration of ≥8 ng/ml, and specificities ranged from 57.1% (PEth ≥8 ng/ml) to 84.8% (EtG). Using a combination of total FAEEs ≥600 ng/g and EtG ≥30 ng/g had a very high specificity for alcohol consumption either ever in pregnancy or only after 20 weeks' gestation, but a very low sensitivity of 6.7 and 5.3% respectively.

Consistent with the lower reported incidence of drinking alcohol in later pregnancy, the positive predictive values of all biomarkers were less, and the negative predictive values higher for this reported pattern of drinking. The positive predictive value for self-reported alcohol consumption after 20 weeks' gestation of both biomarkers being present in significant concentrations in meconium was 9.8%. Conversely, a combination of FAEEs <600 ng/g and EtG <30 ng/g had a high positive predictive value of 87% for no alcohol consumption after 20 weeks of gestation.

4.0 Discussion

The primary aim of the study was to assess the pattern and prevalence of alcohol consumption in pregnancy by use of infant biomarkers in both meconium and dried blood spot cards. FAEEs were universally present in meconium, and in a concentration of ≥600 ng/g in 39.6% of samples. By comparison, EtG could be detected in only 41.1% of meconium samples and in a concentration of ≥30 ng/g in 14.5%. These figures were broadly comparable to the results of a smaller feasibility study conducted a couple of years previously in the same maternity unit, suggesting consistency of methodology(78). There was correlation between FAEEs and EtG when utilising the cut-off values of 600 ng/g and 30ng/g retrospectively ($p=0.044$), however there was no correlation between PEth and FAEE or EtG. This may be for a number of reasons, such as genetic variation, different metabolite pathways or instability of the biomarkers. However, it is more probable that this is due to different windows of detection of each biomarker, with PEth likely to represent the last few weeks of pregnancy, and FAEE and EtG representing beyond 16-20 weeks(151).

As one of the largest studies to date comparing self-reported maternal alcohol history with alcohol biomarkers in meconium as well as dried blood spot cards, this study demonstrates the feasibility of collecting and analysing meconium and infant blood spot samples on a large scale. Mothers were generally keen to take part and recruitment matched other studies which did not require informed written consent(152). This was likely due to a combination of researcher enthusiasm and study participation at place of care, both in the maternity unit (meconium) and at home (dried blood spot cards). By executing the study in the labour ward, postnatal wards and neonatal unit, and obtaining infant blood in the community coincident with routine newborn blood spot screening, the burden of participation for new mothers was greatly diminished. We had excellent cooperation from both hospital and community midwives, further enhancing recruitment and study participation.

4.1 Recruitment

Despite there being only a single researcher, more than 90% of eligible mothers were approached of whom only 6.8% declined to participate. Sample collection

was high; meconium samples were obtained from infants of 86.9% of mothers who had consented to the study and a dried blood spot card was obtained from 79.5%. Almost all (97.2 %) meconium samples were analysable, as were 75% of the dried blood spot cards. An issue arose in matching study numbers to a few meconium samples in the laboratory, attributable to laboratory use of only one identifier. To avoid erroneous results, we chose not to include results from these 2.8% of meconium samples. The reason for not being able to analyse all of the blood spot cards which were collected was insufficient or no blood on the card. As part of the study protocol approved by REC, we did not ask for infants to have further heel pricks if the original heel prick for the routine newborn blood spot screening card did not continue to bleed sufficiently.

Comparable studies have reported enrolment rates of 83% in a Ugandan population, 64% in a French population and 93% in Prince Edward Island, Canada. The latter study did not require written consent(117, 152, 153). It has been reported that the most accurate time for maternal self-report of alcohol consumption is around the time of pregnancy rather than when the child is aged 6-8 years(79). Despite providing written consent, mothers may tacitly decline to participate by not providing infant samples, thereby potentially biasing the study population; other studies have not considered this. In order to establish the impact of this on the results it was hoped to compare demographic data from recruited mothers (with or without sample collection) to the entire maternity booking population by carrying out an anonymous download from PNBS, however this information proved not to be usable. The only comparisons possible were data necessarily collected during screening of mothers for study eligibility. Mothers recruited to this study were not different in terms of age, SIMD16 score or mode of delivery from the general population and therefore the study population can be considered broadly representative.

4.2 Alcohol biomarkers in relation to sociodemographic factors

We found no associations between infant alcohol biomarkers and maternal age, parity or socioeconomic deprivation as measured by postcode of residence for FAEEs, EtG or PEth (cut off 8 ng/ml). This is consistent with the findings of others in relation to FAEEs(118). There was no relationship with maternal age

when infant blood spot cards tested positive for PEth in studies in New Mexico, Texas, Uruguay, and Brazil(121, 138, 140). However, when mothers themselves were tested during pregnancy those mothers who tested positive for PEth were significantly older than those that did not test positive(154).

In our study, there was no difference in ethnicity between mothers of infants whose meconium was or was not positive for FAEEs but when EtG concentration in meconium was ≥30 ng/g, the mother was less likely to be white British. This was the opposite of what we found when infant PEth was ≥8 ng/ml, where the mother was more likely to be white British. This difference was not significant for families with infant PEth concentrations ≥20 ng/ml. Women who self-reported alcohol consumption in pregnancy were more likely to be white British (90.5 *vs* 71.6%) as seen in previous studies(155). The results may be the consequence of a type 1 error or might represent tactical refusal (although the latter is less likely since the association was inconsistent between FAEEs, EtG and PEth). It is also possible that non-white British women are less likely to declare alcohol consumption in pregnancy for fear of repercussions. Women who consented to take part in the study but who did not provide any infant samples tended to be less likely to be white British (70.8% *vs* 80.6%), perhaps a reflection of language difficulties or feelings of disempowerment. Given the inconsistency in results, and the widespread acceptance that white British women are more likely to consume alcohol in pregnancy, it would be inappropriate to draw conclusions from these data about any association between ethnicity and alcohol consumption in pregnancy.

When infant meconium was negative for FAEEs or EtG, the mother tended to have been less likely to smoke in pregnancy (p= 0.071, p=0.174 respectively), however the opposite finding was seen in PEth results (p=0.047, p=0.021 respectively). The former result was surprising as smoking is commonly associated with alcohol intake. Smoking is routinely the subject of behaviour modification in pregnancy and mothers in Scotland are screened for smoking at booking both by self-report and CO breath tests, with either self-report or a CO ≥4 ppm triggering referral to smoking cessation services(156-158). In our population, mothers who did not provide an infant meconium sample tended to

be more likely to have smoked prior to pregnancy (30.8% *vs* 26.8% of mothers who did provide a meconium sample). Consistent with this, mean CO levels were 2.9 ppm for those mothers who did not provide a meconium sample compared to 2.5 ppm for those that did provide a sample. CO was poorly documented in the handheld notes (74% completed for recruited mothers); the focus of the analysis was therefore based on maternal self-report. It is possible that smoking mothers practised a degree of tacit refusal in regard to collection of meconium samples, but this cannot be concluded with certainty. The opposite relationship with smoking was seen in PEth measured from dried blood spot cards on day 5; tactical refusal would have been more difficult as the dried blood spot cards were collected by the community midwife.

Mothers who reported any alcohol consumption in pregnancy were more likely to have smoked than those that reported no alcohol consumption (20.1 *vs* 15.8%), but for self-reported alcohol consumption after 20 weeks' gestation this association was reversed (13.3 % *vs* 18%). This may suggest successful behaviour modification within the smoking population consequent upon smoking cessation services(158).

We did not find any consistent association between socioeconomic status and infant alcohol biomarkers. When PEth was ≥20 ng/ml, mothers were more likely to be socioeconomically deprived, but this result was the opposite to self-reported alcohol consumption. Women who self-reported alcohol consumption in pregnancy tended to be more affluent (mean SIMD16 score 4 *vs* 3). This is consistent both with findings from a Texan cohort, where higher PEth concentrations were measured from dried blood spot cards from infants of high to median income families, and with self-report within other UK populations(138, 155). However, a similar study of 60 mother infant dyads measuring PEth found no such association(121). From our data it cannot be concluded that there is any association between biomarkers and socioeconomic status.

Infants of mothers who self-reported alcohol consumption in pregnancy were heavier at birth, as were those infants whose meconium contained higher levels of FAEEs. Those infants whose dried blood spot card contained concentration of

PEth >8ng/ml and >20ng/ml also trended towards being heavier at birth (p= 0.052, and p=0.068) respectively. This association was not found with EtG. Maternal diet, particularly olive oil consumption, may affect FAEEs in meconium and maternal diet will also affect infant birth weight, but we were not able to correct for these factors in this study(111). Infant polycythaemia and/or bruising at birth may also have affected PEth concentrations, but the former data were not collected and so could not be explored. There is concern that degradation of erythrocyte membranes caused by the freeze-thaw cycle in the management of blood samples in adults can lead to release of PEth(159). This may explain previously reported variation in PEth in newborn dried blood spot cards compared to PEth measured in the mother at birth(140).

4.3 Maternal self-report of alcohol consumption in pregnancy

As part of the study protocol, newly delivered mothers completed a confidential interview including details of alcohol consumption prior to and during pregnancy. Alcohol consumption is commonplace in Scotland, and four in five mothers reported drinking alcohol out with pregnancy. Almost half of newly delivered mothers self-reported some alcohol consumption in pregnancy when they were asked in confidence by a single study researcher unrelated to the clinical team. This was considerably more than the 3% of mothers who reported having consumed alcohol in pregnancy included in a similar but smaller project in the same maternity hospital(78). In the latter case, alcohol consumption was sought in a more casual way, without use of the timeline follow back method. The self-reported prevalence of alcohol consumption in pregnancy in the current study was higher than the 35% reported by mothers in Scotland in 2010 as part of the infant feeding survey(155). Within our study population, just under one in seven mothers stated that they had consumed alcohol after 20 weeks' gestation. Amounts of alcohol reported were however very low, and consistently below the 42 g per day reported in the literature as being reflected in raised infant alcohol biomarkers, which may explain why we did not see a correlation between self-report of alcohol consumption and EtG concentration $\geq$30 ng/g(116). Interestingly, the prevalence of self-report of alcohol consumption in later pregnancy (13.8%) was very similar to the prevalence of meconium EtG $\geq$30 ng/g (14.5%).

Our results indicate that within the Scottish pregnant population alcohol consumption is much more common than the reported global prevalence of alcohol consumption in pregnancy of 9.8% (95% CI 8.9-11.1%)(13). Our results are also high compared to Europe, which has the highest alcohol use in pregnancy compared to other WHO regions at 25.6% (95% CI 21.6-29.6%)(13). Goecke *et al*. described 21.2% of mothers in a German population self-reporting alcohol use in pregnancy, of whom the majority reported only low to moderate alcohol intake(79, 129, 153). The method utilised to identify alcohol consumption is important; it is possible that mothers felt more confident talking to a researcher who was not related to the medical team providing clinical care. Having just delivered a healthy baby and knowing that there was to be an anonymised measurement of alcohol consumption through infant biomarkers may have allowed mothers more confidence in describing their alcohol consumption in pregnancy. Other workers have noted higher reported alcohol consumption soon after delivery, compared to later in the infant's childhood. The importance of documenting alcohol consumption after delivery should not be underestimated as it provides an opportunity to provide interventions for both mother and infant for the current as well as for future pregnancies. Our data suggest that an alcohol history should be undertaken postnatally as well as at maternity booking(79). It is extremely unlikely that mothers will over-report alcohol consumption in pregnancy(83).

While the prevalence of alcohol consumption was probably reasonably accurately reported, the described amounts of alcohol were very low and potentially underreported. As all reported volumes of alcohol were low, we could not categorise alcohol consuming mothers by the number of units of alcohol consumed. Although we planned to recruit purposively mothers who were known to be high consumers of alcohol (indeed we sought and were granted a substantial amendment to the study protocol to do this), we were unsuccessful in recruiting any such mothers. The reasons for this are not clear and may have included reluctance of the midwives to highlight these women to the study team. No known heavy alcohol consuming mother was identified and approached in regard to study participation.

Mothers reported variation in consumption of alcohol depending on the stage of pregnancy. 31% of pregnancies had been exposed to alcohol prior to the mother's knowledge of her pregnancy; as up to 50% of pregnancies are unplanned this fact is important and suggests that a public health focus needs to be on women of childbearing age reducing their general intake of alcohol and not only if they are contemplating pregnancy(160). Mothers reported less intake of alcohol between 12-20 weeks of pregnancy compared to after 20 weeks' gestation, which may be due to the perception that alcohol exposure after 20 weeks' gestation is safer as the fetus has almost fully developed. Unfortunately, this is not true; CNS development beyond 20 weeks is critical, and so further work is required to determine how best to target health interventions(89, 90).

4.4 Sensitivity and specificity of infant meconium alcohol biomarkers

Assuming reasonable accuracy, postnatal self-report of alcohol consumption in pregnancy was utilised to calculate the sensitivity and specificity of the individual biomarkers using cut off values identified in the literature search. In general, the sensitivity of individual infant meconium biomarkers was low, ranging from 11.6% to 43.1%. A total concentration of four FAEEs of $\geq$ 600 ng/g had the highest combination of sensitivity (41.2% & 43.1%) and specificity (60.6 and 61.2%) for detection of PAE. Bakhireva and Chan each reported a sensitivity of 100% for a total of four FAEEs, albeit a slightly different set of four FAEEs (ethyl palmitate, stearate, oleate and linoleate) and Kwak demonstrated a lower sensitivity of 22.2% for a total of nine FAEEs (ethyl laurate, myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate and arachnidonate) using a lower cut-off of 200 ng/g Himes reported a sensitivity of 64.9% for four FAEEs (91, 117, 119, 120). English used the same combination of ethyl stearate, oleate, linoleate and myristate as the current study (cut off 600 ng/g) but did not report a sensitivity. Among these previously reported studies specificities of FAEEs ranged from 13-73.6%, consistent with our specificity of 60.6% for alcohol consumed after 20 weeks' gestation(117, 119, 120).

EtG, reported by Himes *et al.* to have the highest sensitivity and specificity for PAE, demonstrated low sensitivity in our population (11.6-13.1%) although

specificity was high at 84 and 84.8%(120). The combination of both FAEEs and EtG had very poor sensitivity (5.3%), but high specificity (92.4%) for self-reported alcohol consumption after 20 weeks' gestation which probably reflects the relatively small amounts of alcohol consumed. The literature suggests the detection threshold of FAEE assay in meconium is around 40 g alcohol per week(119). Within our study population, the PPV of individual or combined biomarkers for alcohol consumption after 20 weeks' gestation was low (9.8 – 14.8%); since PPV is affected by the prevalence of the condition being sought this will not necessarily be comparable to other populations. As per Howlett *et al.*, it is concluded that meconium biomarkers alone are not suitable as an PAE screening tool(92).

4.5 Potential utility of measuring PEth in dried blood spot cards

Parents proved willing to donate a small extra sample of their infant's blood at time of routine newborn blood spot testing and a majority of blood spot cards contained an adequate sample. Measurement of PEth in blood spot cards has not previously been reported from the UK although there have been reports from both North and South America(101, 121, 138, 140). American studies of PEth in the newborn have either used blood taken from the umbilical cord or coincident with newborn screening within the first 48 hours of life whereas in the UK the routine newborn blood spot card is obtained later, between 96 and 120 hours of age; results from our population and American studies would therefore be predicted to be different. It was anticipated that the concentration of PEth would be lower in blood spot cards taken on day five compared to samples obtained at birth or within 48 hours considering the time interval from alcohol exposure, but this was not what we found. A greater proportion of blood spot cards proved positive for PEth using cut offs of both 8 and 20 ng/ml than the proportion of meconium samples positive for either FAEE or EtG. While PEth measured on day 5 may be a very reliable marker of PAE in the later stages of pregnancy, and a prevalence in excess of 40% (8 ng/ml) or almost 30% (20 ng/ml) a true reflection of maternal consumption, this seems unlikely. Other potential reasons for PEth concentrations to be higher than we predicted include variation in collection, analysis and/or storage. It is possible that the haemolysis associated with the shorter half-life of infant red blood cells and/or relative

polycythaemia results in increased PEth levels relative to the adult subject(161). This could potentially be explored by undertaking serial infant blood sampling during the first week of life and examining any relationship between infant PEth and haemoglobin concentration. Baldwin's most recent publication also highlighted significantly higher PEth values (by a factor of approximately two) in newborns compared to their mothers, although she did not offer any explanation for this(140). We found infants of higher birth weight to have higher PEth levels; the reason for this is not clear.

Utilising a cut off of 20 ng/ml we found measurement of PEth to have a sensitivity of 28.4% and specificity of 70.1% for self-reported alcohol consumption after 20 weeks of gestation. Other researchers have reported PEth $\geq$20 ng/ml to have 32.1% sensitivity and 100% specificity for PAE, albeit from blood spot cards obtained at 48 hours of life(121). As with meconium biomarkers, the likelihood of a positive PEth result would have been reduced by the relatively small amounts of alcohol declared.

One of the original aims of the study was to investigate whether stored dried blood spot cards might be able to be used to confirm retrospectively alcohol use in pregnancy when there is a clinical suspicion of FASD. Using cut off values of both 8 and 20 ng/ml, we found very low PPVs for alcohol consumption after 20 weeks of pregnancy of 14.8 and 12.8% respectively. The NPV was higher, in excess of 85% for both cut off values, which might help to point the clinician towards other diagnoses, but it must be concluded that in general retrospective testing of retained newborn dried blood spot cards for PEth is unlikely to be clinically useful(64).

4. 6 Study strengths and limitations

Strengths

Undertaken within a large single centre study population, this study had high rates of participation with a study population randomly selected and broadly representative of the maternity population as a whole.

Maternal alcohol history was obtained during a discrete and confidential interview using a modified timeline follow back method that made it as reliable as possible. The researcher was not involved with the clinical care of either mother or baby, which may have engendered confidence in disclosure.

Meconium samples were frozen in order to ensure stability, and freezing was mostly achieved soon after sample collection. Steps were taken to avoid contamination from bacterial colonisation leading to false positive FAEE results(112). While some blood spot cards may have been collected after the use of alcohol wipes or alcohol hand gel, this is unlikely to have contaminated the blood sample, as once dried, PEth levels in blood spot cards do not vary considerably(162).

Limitations

The study centre serves a relatively deprived population and so results may not be applicable to the Scottish or UK population as a whole. Carrying out this study across multiple sites would have allowed a more representative picture of the national population but would have required significantly more resource. We had access to only limited sociodemographic data with which to assess the representativeness of the recruited study population; other demographics including education and marital status were not available.

The study freezer was a distance from the site of care, resulting in some delays in sample freezing; a freezer on the post-natal wards would have made transition to freezer quicker. We did not record the exact time between sample collection and freezing, but even if recorded, this would have been the time the nappy was changed, and not the time that the meconium was passed. There is a small possibility that some meconium samples may have been affected by delayed transit to the freezer, resulting in either breakdown of FAEEs or increased production in samples with bacterial growth(112). The latter is however very unlikely in the newborn.

For logistical reasons, meconium and dried blood spot samples were analysed in Italy rather than as originally planned at the University of Glasgow. Shipping

costs were significant, and so samples were shipped and analysed in only two batches. This led to a slight delay in analysis but should not have affected the results as samples remained frozen to ensure stability.

Since the Scottish population is predominantly Caucasian, individuals were classified into only two groups, white British, and non-white British. We were unable to study different minority ethnic groups as numbers were too small.

One of the main weaknesses of this study is that, despite highlighting these mothers for recruitment at any time (including out with designated study sample collection days) we were unable to recruit any mothers with a known history of heavy alcohol consumption. Furthermore, a dose response could not be investigated due to the small volume of alcohol reported by those mothers who self-reported any alcohol consumption in pregnancy. This is in keeping with published literature which reports that lower consumers of alcohol are more likely to be inaccurate in their self-reporting compared to those with a high alcohol intake or abstainers(79).

4.7 Potential impact on public health

It has been shown than women have misconceptions with regard to the impact of alcohol on the health of the unborn child(160). In addition, it is estimated that 50% of pregnancies are unplanned and so focussing public health information on those women planning pregnancy is unlikely to be completely successful(160). Within the UK, women who are given information with regards to cessation of alcohol intake rather than reduction in consumption are more likely to give up alcohol altogether(155). The current study highlights that alcohol consumption is pregnancy occurs commonly across the population(92). While infant biomarkers did not clearly relate to maternal self-report, it is possible that infant alcohol biomarkers could be a measure of harm to the infant, rather than simply reflecting the amount of alcohol consumed by the mother. Individual differences in alcohol metabolic pathways may favour production of toxic non-oxidative metabolites including FAEEs and EtG(108). Others have shown biomarkers in meconium to be associated with childhood outcomes(89, 90).

Alcohol consumption is strongly related to the price and availability of alcohol with a $0.01 increase in beer tax reducing the incidence of low birth weight by 1-2%(163). Scotland has introduced minimum alcohol pricing and it is important to monitor the measure the impact of this upon PAE and FASD, but it is not clear at present that the best way to do this on a population basis will be via infant alcohol biomarkers.

4.8 Review of the study aims

At the outset of this project, we sought to answer a number of questions:

1. What are the levels of FAEEs and EtG in meconium samples from singleton term and preterm infants born in a single maternity hospital in Glasgow?

We found FAEEs to be universally detectable in meconium samples with concentrations ranging from 22.2 to 7549.8 ng/g. 39.6% of infants had ≥600 ng/g FAEEs in their meconium, a level described by previous workers as indicative of PAE. 21 infants demonstrated very high concentrations of FAEEs (≥2000 ng/g). Such high concentrations have previously been described in a subgroup of babies born to opioid dependent, methadone-prescribed mothers, interpreted to indicate undisclosed significant alcohol consumption in pregnancy(11). EtG was detectable in 41.2% of meconium samples and the concentration was ≥30 ng/g in 14.5% of cases. The prevalence of each meconium biomarker was similar to those found in an earlier pilot study in the same maternity hospital. When both FAEEs and EtG concentrations were positive (as defined by ≥600 ng/g and ≥30 ng/g respectively), there was a weak positive correlation between the two biomarkers (Pearson's coefficient= 0.283, P-value=0.044). Preterm infants (< 37 weeks' gestation) comprised 6.7% of the meconium samples, consistent with the national rate of preterm birth of 6.5%.

2. What are the relationships between high levels of FAEEs and EtG in infant meconium and maternal age, parity, smoking, ethnicity, and socio-economic status?

High levels of FAEEs and EtG in infant meconium were not related to maternal age, parity, BMI, or socioeconomic status as measured by SIMD 16. In regard to FAEEs, mothers tended to have been less likely to smoke in pregnancy when their infant's meconium was negative (14.7 *vs* 20%, p=0.071); for EtG this difference was less marked (13.6% *vs* 18.5%, p=0.174). Ethnicity was not associated with FAEEs in meconium, but for EtG there was a significantly less likelihood of the mother having identified as white British when the concentration of EtG in her infant's meconium was ≥30 ng/g. The relevance of this individual finding is not clear.

3. What are the relationships between high levels of FAEEs and EtG in infant meconium and infant gestation, birth weight and head circumference?

Infants with a total FAEE concentration in meconium of ≥600 ng/g tended to be of higher birth weight compared to those with FAEEs < 600 ng/g (3425 *vs* 3331 g, P= 0.032) but there was little variance in OFC (34.7 *vs* 34.6 cm). Infants with a total EtG concentration in meconium ≥30 ng/g compared to <30 ng/g did not have a significant difference in their birthweights, (birth weight 3418 *vs* 3365g, P = 0.034; OFC 34.8 versus 34.6 cm). There was no difference in gestation between groups positive or negative for either meconium or dried blood spot cards.

4. What are the relationships between high levels of FAEEs and EtG in infant meconium and maternal alcohol intake during pregnancy as self-reported at booking and immediately post-delivery?

Self-reported alcohol consumption at booking was poorly documented and therefore unable to be related to alcohol biomarkers in meconium. Mothers of infants with FAEEs ≥600 ng/g were not more likely to self-report alcohol intake beyond 20 weeks' gestation than those whose infant's meconium was negative for FAEEs (14.6% *vs* 12.8%, p=0.56) There was no relationship between EtG ≥30 ng/g and self-reported alcohol consumption in pregnancy.

5. What are the levels of PEth in dried blood spot cards from singleton term and preterm infants born in a single maternity hospital in Glasgow?

PEth levels in the population ranged from 2.4 to 3991.6 ng/ml, with all cards having some detectable PEth. Within the study population the prevalence of PEth ≥8 ng/ml and ≥20 ng/ml was 43% and 29.5% respectively.

6. What are the relationships between high levels of PEth in dried blood spot cards and maternal age, parity, smoking, ethnicity, and socio-economic status?

There was no relationship between a concentration of PEth of ≥8 ng/ml in infant blood on day five and maternal age, parity, or socioeconomic status. Mothers of infants with PEth ≥8 ng/ml in their newborn blood spot card were more likely to have smoked (p=0.047) and to have identified as being white British (p=0.028). Mothers of infants with a concentration of PEth of ≥20 ng/ml in their blood spot card were slightly younger (p=0.023), had a higher BMI (p=0.038), lived in less affluent postcodes (p=0.026) and were more likely to have smoked during pregnancy (p=0.021). There was no relationship between parity or ethnicity and PEth ≥20 ng/ml.

7. What are the relationships between high levels of PEth in dried blood spot cards and infant gestation, birth weight and head circumference?

There was no difference in gestation for infants with a significant concentration of PEth in a dried blood spot card, using cut offs of both 8 and 20 ng/ml. Infants with PETH ≥8ng/ml tended to be heavier (3428g *vs* 3360g, P = 0.052). Infants with PEth ≥20 ng/ml tended to be heavier (3457 *vs* 3360 g, P = 0.068), and OFC tended to be greater (34.9 *vs* 34.6 cm).

8. What are the relationships between high levels of PEth in dried blood spot cards and maternal alcohol intake during pregnancy as self-reported at booking and immediately post-delivery?

When PEth concentration was ≥8 ng/ml 14.5% of mothers self-reported postnatally alcohol intake in pregnancy compared to 12.2% of mothers whose

infants had a PEth concentration of <8 ng/ml. For infants with a PEth concentration of ≥20 ng/ml 12.8% of mothers reported any alcohol intake beyond twenty weeks of gestation, compared to 13.7% when PEth was ≤20 ng/ml. Most mothers self-reported very low amounts of alcohol consumption, with only three mothers reporting more than three units of alcohol on any one occasion. We did not therefore find a relationship between PEth in dried blood spot cards and self-reported maternal alcohol intake during pregnancy.

9. What is the relationship between levels of FAEEs and EtG in meconium and levels of PEth in dried blood spot cards in singleton term and preterm infants?

There was no correlation between FAEE or EtG in meconium and PEth in dried blood spot cards.

4.9 What could have been done differently?

As this was a population study, the higher the number of recruited participants, the more accurate the calculation of sensitivity and specificity, and subsequent analysis of the sociodemographic groups would have been. This could have been a larger study by recruiting over every day rather than every fourth day, and also by being carried out over two full twenty-week periods, thus not missing out on potential seasonal variation (Christmas festivities) in alcohol intake. Researcher time could potentially have been saved, therefore allowing for more recruitment, by asking mothers to carry out questionnaires on tablet devices with electronic forms; this would have reduced the need to transcribe data, but may have reduced the reliability of responses compared to face to face interview with modified timeline follow back. More analysis of data was not undertaken on the advice of a statistician, due to concerns about the introduction of type 1 errors. Thus most data are presented in descriptive format.

We tried to recruit high consumers of alcohol in pregnancy, including a significant amendment approval from REC, but were unsuccessful in this recruitment. Further study in this field needs to explore ways of recruiting this population in order to further validate biomarkers in both meconium and dried blood spot cards. Options might include carrying out targeted recruitment over a longer period or across multiple sites.

Logistically having freezers near the postnatal wards would have reduced any delay in the freezing of samples, as would having a larger, 24 hour on call research team. The latter would, however, have significantly increased costs.

Having gained further understanding of PEth in dried blood spot cards through more recent publications, it would be useful to carry out serial PEth testing over the first week of life in infants to explore the effects of variation in haemocrit and bilirubin. These assays are not routinely performed from dried blood spot cards and would therefore require different samples to be obtained. The ethics of obtaining clinically unnecessary blood samples in the newborn would make this very difficult.

4.10 Conclusions

This large study has demonstrated that alcohol consumption in pregnancy in the west of Scotland is common, with almost half of newly delivered mothers reporting postnatally that they drank some alcohol in pregnancy. While a majority of mothers stop drinking alcohol once they realise they are pregnant, one in seven pregnant women delivering in the west of Scotland reports drinking some alcohol after 20 weeks of gestation. There was no clear association with maternal age, parity, ethnicity or socioeconomic status and consumption of alcohol in pregnancy, indicating that measures to reduce alcohol consumption in pregnancy must be widely targeted.

FAEEs are commonly present in meconium, and EtG detectable in 41.2%; using cut off values of 600 and 30 ng/g respectively, alcohol biomarkers in meconium had poor PPV for any declared maternal alcohol consumption in pregnancy. A combination of FAEEs < 600 ng/g and EtG < 30 ng/g in meconium has a high NPV for self-reported alcohol consumption in pregnancy.

Routine use of meconium biomarkers for detection of alcohol consumption in pregnancy is not currently justified. Similarly, retrospective testing of stored dried blood spot samples taken on day five of life cannot be recommended as a measure of assessing PAE.

Neither infant alcohol biomarkers nor maternal self-report of alcohol consumption in pregnancy can be considered a gold standard for the detection of PAE.

Further work is required to explore relationships between alcohol biomarkers in meconium and longer-term neurodevelopmental outcomes, as well as the role of measuring PEth in the newborn.

Appendix 1. Participant information sheet

University of Glasgow

NHS Greater Glasgow and Clyde

Working out the pattern of drinking alcohol in pregnancy by examination of baby's first stool (meconium) and a blood spot card.

Lead Researcher – Dr Helen Mactier, Neonatal Unit, Princess Royal Maternity

Parental information sheet version 2 16/06/2015

You are being invited to allow your baby to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Ask us if anything is unclear or if you would like more information. Take time to decide whether or not you wish your baby to take part.

What is the purpose of the study?

Drinking too much alcohol in pregnancy may damage the unborn child. The Scottish Government and other health agencies are working hard to inform mothers-to-be about the dangers of drinking too much alcohol in pregnancy, but it is generally acknowledged that many women continue to drink at least some alcohol when they are pregnant. It is very important that we understand how many women do continue to drink once they know they are pregnant, so that we can better target our information and try to find out if small amounts of alcohol drunk in pregnancy are harmful. At present, because most women do not admit to drinking in pregnancy, accurate data are lacking. During pregnancy, alcohol drunk by the mother transfers to the baby. The baby's body processes this alcohol, and substances known as fatty acid ethyl esters (FAEEs) are laid down in the baby's internal organs. After birth we can measure these FAEEs in the baby's first stool (meconium). We can also measure other breakdown products of alcohol from blood spot cards. A small study of baby meconium samples conducted here at Princess Royal Maternity suggested that up to a quarter of mums continue to drink alcohol when they are pregnant; we now need to check these results by studying a much larger sample of babies. We are also looking to see if measuring meconium or blood spots is a better way of monitoring alcohol consumption in pregnancy. The purpose of this study is to understand how many women drink alcohol when they are pregnant, and whether this behaviour is more common in older or younger mums, or in richer or poorer areas of Glasgow. It's therefore important that we are able to include as many babies as possible.

This study is completely anonymous. We are **NOT** seeking to identify individual mums who have drunk alcohol in pregnancy or their babies. Instead, we are interested in finding out the number of women and babies affected. Your GP will not routinely be informed of your participation in this study, but if you wish us to let them know, we will do this.

Why has my baby been invited?

We are asking all mums who deliver a single baby on every 4th day (*i.e.* a random sample of the population), if their baby can take part in this study.

Does my baby need to take part?

No. It is entirely up to you. If you do decide that your baby can take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide not to take part or to withdraw, this will not affect the standard of care that your baby will receive. We will be using routinely collected hospital data to check that we have collected meconium and/or blood spot samples from a group of babies typical of the population as a whole.

What will happen to my baby and myself if he/she takes part?

We will collect a sample of meconium from your baby's nappy as soon after birth as possible. In addition, if possible, a second blood spot card will be taken at the same time as routine newborn bloodspot screening on

day 5. This will not cause your baby any additional discomfort. You will also be asked a brief, confidential questionnaire about your health and alcohol consumption in pregnancy, which will take 5-10 minutes to complete. If you and your baby are discharged early and before the baby has passed meconium, the sample may be collected at home by your community midwife. Taking part in this study will not delay your discharge home. Meconium and blood spot samples will all be kept anonymous. Samples may be retained for future research, but this would be subject to new Research Ethics approval.

What are the possible risks of taking part?
There are no risks to either yourself or your baby from taking part in this study.

What are the possible benefits of taking part in this study?
There is not expected to be any direct benefit to either yourself or your baby from taking part in this study. Taking part in the study may however help future babies if the results allow us to improve the way we help pregnant women to reduce their consumption of alcohol.

Suggestions and complaints.
If you wish to complain about any aspect of the way you have been treated during the course of this study, the normal National Health Service complaints mechanism may be available to you. You can put any complaint in writing to Mrs. Anne Snape, Patient Liaison Manager, North Glasgow University Hospitals Division, 84 Castle Street, Glasgow G4 0SF (telephone 0141 211 5112).

Will my baby's taking part in this study be kept confidential?
Yes. All information collected about you or baby during the course of this research will be kept strictly confidential. We will only use routinely collect information including the baby's gestation and birth weight, as well as your age and how many babies you have had before.

What will happen to the results of the research study?
Information gathered from this study will be analysed and the results submitted for publication in a medical journal. Information may also be presented at scientific meetings and it is planned that the study results will submitted as part of an MD thesis to the University of Glasgow. You or your baby will not be identified in any presentation or written document.

Who is funding this research?
The study is being paid for by the Yorkhill Children's Charity. The meconium and blood samples will be analysed at the University of Florence in Italy, which is also covering some of the costs. None of the persons involved receives any money when a baby joins the study.

Who has reviewed this study?
This study has been approved by West of Scotland Research Ethics Committee 3.

Contact for further information
Dr Helen Mactier, Consultant Neonatal Paediatrician, can be contacted at any time via Glasgow Royal Infirmary switchboard (0141 211 4000 or 211 5229). If you have any questions or concerns - please simply ask the midwife who is looking after you and your baby. Independent advice may be obtained from Dr. Andrew Powls, Consultant Neonatal Paediatrician at Princess Royal Maternity (0141 211 5229).

If you have concerns about alcohol consumption in pregnancy you may discuss this in confidence with your midwife or with your GP. Help is also available on-line at https://www.drinkaware.co.uk/ or by telephoning Drinkline free on **0300 123 1110**. We will seek to help you in any way that we can.

Thank you for taking time to consider this research study.

Appendix 2. Consent form

University of Glasgow

NHS Greater Glasgow and Clyde

Consent form

Working out the pattern of drinking alcohol in pregnancy by examination of baby's first stool (meconium) and a blood spot card.

Chief Investigator;
Dr Helen Mactier,
Consultant Neonatologist,
Princess Royal Maternity Hospital
Glasgow
G31 2ER

Please Initial Box

1. I confirm I have read and understand the information sheet for the above study and have had opportunity to ask questions. (Information sheet version 2 - 16/06/2015) ☐
2. I understand that my participation is voluntary and I am free to withdraw at any time, without giving a reason, and without my or my baby's medical care or legal rights being affected. ☐
3. I agree to take part in the above study, including collection of my baby's meconium (first stool), an additional blood spot card for analysis, and taking part in a health and alcohol intake questionnaire. ☐
4. I give permission for my and my baby's records to be viewed by the research team where it is relevant to taking part in the research and by representatives of the study for audit purposes. ☐
5. I understand that relevant sections of my care record and data collected during the study may be looked at by the responsible individuals from the sponsor or host organisation or from regulatory authorities where it is relevant to taking part in this research. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Researcher	Date	Signature

Version 2 16/06/2015

Appendix 3. Data collection CRF

Version 6 12/11/2015

Routinely Collected Demographic Data CRF

1	Study Number (34001 34999)	
3	Maternal Date of Birth (DD/MM/YYYY)	__/__/____
4	Maternal Postcode (first digits)	
5	Occupation	Incomplete [88] □
6	Paternal occupation	Incomplete [88] □
7	BMI at booking	_____Weight_____Height _____ Incomplete [88] □
H	Contraception use at time of conception	Yes [1] □ No [2] □ Incomplete [3] □ N/A [4] □ Notes missing [5] □ Missed patient [6] □ Unbooked [7] □
9	Type of contraception	Incomplete [88] □
10	New partner	Yes [1] □ No [2] □ Incomplete [3] □ N/A [4] □ Notes missing [5] □ Missed patient [6] □ Unbooked [7] □
11	Parity	Incomplete [88] □
12	Early pregnancy losses	Incomplete [88] □
	Family Origin Questionnaire	
13	Ethnicity (letter/number)	___/___ Incomplete [88] □
14	Partners Ethnicity (letter/number)	___/___ Incomplete [88] □
15	First Language	Incomplete [88] □
16	Religion/Faith	Incomplete [88] □
17	Refugee/Asylum seeker	Yes [1] □ No [2] □ Incomplete [3] □ N/A [4] □ Notes missing [5] □ Missed patient [6] □ Unbooked [7] □
	Other health related questions	
18	Have you been taking folic acid?	Yes [1] □ No [2] □ Incomplete [3] □ N/A [4] □ Notes missing [5] □ Missed patient [6] □ Unbooked [7] □
19	When?	Preconception [1] □ Before 12 weeks [2] □ After 12 weeks[3] □ Incomplete [4] □
24	Do you go to the dentist regularly?	Yes [1] □ No [2] □ Incomplete [3] □ N/A [4] □ Notes missing [5] □ Missed patient [6] □ Unbooked [7] □
25	How many units of alcohol did you drink each day before you were pregnant?	Units Incomplete [88] □
26	How many units a day are you drinking now?	Units Incomplete [88] □
27	How many units of alcohol do you drink in a average week?	Units Incomplete [88] □

28	If drinking where were you drinking, at home, in clubs/pubs	Incomplete [88] □
29	Have you smoked in the12 months prior to pregnancy?	Yes [1] □ No [2] □ Incomplete [3] □ N/A [4] □ Notes missing [5] □ Missed patient [6] □ Unbooked [7] □
30	CO level	Date Incomplete [88] □
	Birth details	
59	Baby Date of Birth (DD/MM/YYYY)	__/__/____
60	Time of Birth (24 hours HH:MM)	__ __:__ __
61	Sex	Male [1] □ Female [2] □ Unknown [3] □
62	Gestation	____:___
63	Weight (grams)	__ __ __ __ grams
64	OFC (cm- 1 decimal place)	____:__cm
65	Presentation	□ Cephalic [1] □ Breech [2] □ Transverse [3]
66	Onset of labour	Spontaneous [1] □ Induced [2] □ None [3] □
67	Mode of delivery	□ Spontaneous Vaginal [1] □ Venthouse/Kiwi [2] □ Forceps [3] □ Emergency c-section [4] □ Elective c-section [5]
68	Apgars at 1 minute (1-10, 11 not documented)	
69	Apgars at 5 minutes	
71	Resus required	Yes [1] □ No [2] □ Unknown [3] □
72	SCBU admission	Yes [1] □ No [2] □ Unknown [3] □
73	Meconium at delivery	Yes [1] □ No [2] □ Unknown [3] □

Health in Pregnancy Questionnaire and Modified Timeline Follow Back Interview

Study Number (34001-34999)	
Maternal Date of Birth (DD/MM/YYYY)	__/__/____
What gestation were your when you found out you were pregnant? *Timeline followback question*	
When did you hear about the study?	□ >20 weeks □ 20-30 weeks □ 30 weeks to delivery
What gestation did you consent to the study?	□ <12 weeks [1] □ 12-24 weeks [2] □ 24weeks-delivery [3] □ Post delivery [4]
How many previous pregnancies?	□ 1 [1] □ 2 [2] □ 3 [3] □ 4 [4] □ 5 [5] □ 6 [6] □ 7 [7] □ 8 [8] □ 9 [9] □ >10 [10] □ 0 [11]
How many previous babies?	□ 1 [1] □ 2 [2] □ 3 [3] □ 4 [4] □ 5 [5] □ 6 [6] □ 7 [7] □ 8 [8] □ 9 [9] □ >10 [10] □ 0 [11] □ n/a [12]
Any stillborn or preterm infants?	
Did you consume folic acid prior to conceiving?	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]
Did you consume folic acid during pregnancy?	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]
Did you take any medications during pregnancy?	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]
If yes what medications	
Did you smoke prior to pregnancy?	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]
How many cigarettes per day?	____ per day
Did you smoke during your pregnancy?	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]
How many cigarettes per day?	____ per day
Prior to being pregnant did you	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]

drink alcohol?	
With what frequency did you consume alcohol prior to pregnancy?	□ Daily [1] □ 2-3 times weekly [2] □ 4-6 times weekly [3] □ Weekly [4] □ 2-3 times monthly [5] □ Monthly [6] □ 2-3 monthly/occasionally [7] □ Yearly [8] □ N/A [8] □ Unknown [9]
Did you consume alcohol at all during pregnancy?	□ Yes [1] □ No [2] □ Unknown [3] □ N/A [4]
How often did you consume alcohol during pregnancy?	□ 1-2 times [1] *time line follow back* □ Occasionally [2] *timeline follow back* □ Weekly [3] *show week in each trimester* □ Daily [4] *quantify daily drinking* □ N/A [5]
What level of education did you achieve?	□ Left school at <16 years [1] □ Attended at 16-18 years [2] □ Higher education/college [3] □ University degree/higher degree [4]

Timeline Follow back Calendar Prompt

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday		Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
	30	31	1	Maundy Thursday	Good Friday	First day of Passover 4	Easter Sunday		5	6	7	8	9	10	11
	Easter Monday 6	7	8	9	10	11	12		12	13	14	15	16	17	18
	13	14	15	16	17	18	19		19	20	21	22	23	24	25
	20	21	22	St. George's day	24	25	26	Oct-15	36	37	28	29	30	Halloween	1
Apr-15	27	28	29	30	1	2	3		2	3	4	Bonfire night	6	7	8
	Early May Bank Holiday	5	6	7	8	9	10		9	10	11	12	13	14	15
	11	12	13	14	15	16	17		16	17	18	19	20	21	22
	18	19	20	21	22	23	24	Nov-15	23	24	25	26	27	28	29
May-15	Spring Bank Holiday	26	27	28	29	30	31		30	1	2	3	4	5	6
	1	2	3	4	5	6	7		7	8	9	10	11	12	13
	8	9	10	11	12	13	14		14	15	16	17	18	19	20
	15	16	17	Ramadan begins	19	20	Father's day		21	22	23	Christmas Eve	Christmas Day	Boxing Day	27
Jun-15	22	23	24	25	26	27	28	Dec-15	28	29	30	New year's eve	New Year's day	2	3
	29	30	1	2	3	4	5		4	5	6	7	8	9	10
	6	7	8	9	10	11	12		11	12	13	14	15	16	17
	13	14	15	16	17	18	19		18	19	20	21	22	23	24
	20	21	22	23	24	25	26	Jan-16	25	26	27	28	29	30	31
Jul-15	27	28	29	30	31	1	2		1	2	3	4	5	6	7
	Summer Bank holiday	4	5	6	7	8	9		8	9	10	11	12	13	Valentine's
	10	11	12	13	14	15	16		15	16	17	18	19	20	21
	17	18	19	20	21	22	23	Feb-16	22	23	24	25	26	27	28
Aug-15	24	25	26	27	28	29	30		29	1	2	3	4	5	6
	31	1	2	3	4	5	6		7	8	9	10	11	12	13
	7	8	9	10	11	12	13		14	15	16	17	18	19	20
	14	15	16	17	18	19	20		21	22	23	24	25	26	27
	21	22	23	24	25	26	27	Mar-16	28	29	30	31	1	2	3
Sep-15	28	29	30	1	2	3	4								

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