Screening for Prenatal Alcohol Exposure using Meconium Fatty Acid Ethyl Esters as Biomarkers

By

Irene Zelner

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmacology and Toxicology
University of Toronto

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Abstract

Diagnosis of fetal alcohol spectrum disorders (FASD) is challenging and typically requires confirmation of in utero alcohol exposure. Due to the poor reliability of maternal self-reports, biomarkers have emerged to address the problem of obtaining exposure history. A relatively novel method for detecting prenatal alcohol exposure is analysis of meconium for fatty acid ethyl esters (FAEEs), which are non-oxidative ethanol metabolites. Screening newborns using meconium FAEEs may facilitate early diagnosis and intervention in alcohol-affected individuals. The overall objective of this thesis is to further investigate, validate, and assess the clinical utility of meconium FAEE analysis as a screening tool for the identification of neonates at-risk for FASD. This objective was addressed in four separate studies. The first study assessed whether meconium FAEE concentrations can be predictive of ethanol-induced organ injury in fetal sheep, and determined that the levels of these esters could be used to identify fetuses at-risk for organ dysfunction that do not display overt physical signs of ethanol teratogenicity. The second study investigated the effect of delayed meconium collection and contamination with postnatal stool on FAEE analysis, and determined it to be a risk factor for false positive test results. In the third study, maternal willingness to partake in an open meconium screening program was assessed and found to be low enough to diminish
the utility of meconium FAEE testing for population-based open screening. Lastly, a systematic review examining the capacity for FAEE synthesis and the enzymology of this non-oxidative metabolic pathway in mammalian organs and tissues revealed that FAEE synthesis is mediated by numerous enzymes and isoenzymes, many of which have other primary physiological functions, and that their contribution to overall FAEE-synthesis may be tissue-specific. Overall, the results of this research provide new information on the benefits, limitations, and utility of meconium FAEE testing as a screening tool for identifying prenatal alcohol exposure – a test that may be of great clinical value in the diagnosis and management of FASD.
Acknowledgments

I would like to thank the following individuals for their support, guidance, and contributions during the course of my graduate studies.

My supervisor, Dr. Gideon Koren, for his exceptional mentorship, encouragement, inspiration, and for the endless support and opportunities that I have had as a member of your lab.

Dr. Bhushan Kapur, who always made time to offer valuable insight, advice, and encouragement. Dr. Shinya Ito for his constructive feedback, insight, and guidance, as well as, Dr. James Brien for taking the time to share his vast expertise. Your advice and contributions are extremely appreciated.

All of my colleagues at the Motherisk laboratory for their support, enthusiasm, and great memories. Special thanks to Dr. Katarina Aleksa, Chitra Rao, Netta Fulga, and Janine Hutson, for their technical expertise and guidance. To all the collaborators, especially the research team from Monash University and the health professionals from Grey Bruce, St. Joseph’s Health Care in London, and Mt. Sinai Hospital in Toronto. Your assistance has been invaluable.

Finally, to my family, friends, and Minja. I am grateful for your patience and unwavering support; you have been a source of motivation more than you know. I am indebted to you for helping me with this challenging endeavor.
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List of Abbreviations

3-BCP 3-benzyl-6-Chloro-2-pyrone

ACAT Acetyl-CoA:cholesterol acyltransferase

ADH Alcohol dehydrogenase

ADHD Attention-deficit hyperactivity disorder

AEAT Acyl-coA: ethanol o-acyl transferase

ALDH Acetaldehyde dehydrogenase

ALT Alanine aminotransferases

ANOVA Analysis of variance

APGAR Appearance, pulse, grimace, activity, respiration

ARBD Alcohol-related birth defects

ARND Alcohol related neurobehavioural deficits

ASQ Ages and stages questionnaires

AST Aspartate aminotransferase

ATP Adenosine triphosphate

AUC Area under the curve

AUDIT Alcohol use disorders identification test

BAC Blood alcohol concentration

BNPP Bis-para-nitrophenylphosphate

BSID Bayley scales of infant development

CADUMS Canadian alcohol and drug use monitoring survey

CAGE Cut-down, annoyed, guilty, eye-opener

CAPHC Canadian association of paediatric health centres
FAS  Fetal alcohol syndrome
FASD  Fetal alcohol spectrum disorder
FMF  Families moving forward
GC-MS  Gas chromatography mass spectrometry
GGT  Gamma glutamyltransferase
GI  Gastrointestinal
GST  Glutathione S-transferase
HBHC  Healthy babies healthy children
HIV  Human immunodeficiency virus
HS-SPME  Headspace solid phase microextraction
IL  Interleukin
IOM  Institute of medicine
IQ  Intelligence quotient
IV  Intravenous
LLT  Language and literacy
LOD  Limit of detection
LOQ  Limit of quantification
LPL  Lipoprotein lipase
LV  Left ventricle
MAST  Michigan alcohol screening test
MCV  Mean corpuscular volume
MEOS  Microsomal ethanol oxidizing system
MES  Maternity experiences survey
MSH  Mount Sinai hospital
NAD+ Nicotinamide adenine dinucleotide
NICU Neonatal intensive care unit
NLSCY National longitudinal survey of children and youth
NPV Negative predictive value
NSDUH National survey on drug use and health
OD Optical density
PAE Prenatal alcohol exposure
PCIT Parent-child interaction therapy
PCR Polymerase chain reaction
PE Phosphatidylethanolamine
PEC Plasma ethanol concentrations
Peth Phosphatidylethanol
pFAS Partial fetal alcohol syndrome
PHAC Public health agency of Canada
P-HMB P-hydroxymercuribenzoic acid
PLD Phospholipase D
PPV Positive predictive value
PSIM Parent support and management
RCT Randomized controlled trial
RNA Ribonucleic acid
ROC Receiver operating characteristics
SAMHSA Substance abuse and mental health services administration
SCFA Short-chain fatty acids
SD Standard deviation
SE Standard error
SEM Standard error of the mean
SES Socioeconomic status
SJH St. Joseph’s hospital
SJHC St. Joseph's health care
SP surfactant protein
SPLS Seattle prospective longitudinal study on alcohol and pregnancy
SULT Sultotransferases
T-ACE Tolerance, annoyed, cut-down, eye-opener
TGL Triglyceride lipase
THL Tetrahydrolipstatin
TNF-α Tumor necrosis factor alpha
TOTP Tri-o-tolylphosphate
TWEAK Tolerance, worry, eye-opener, amnesia, cut-down
UDP Uridine triphosphate
UGT Uridine glucuronosyltransferase
Chapter 1
General Introduction

1 Review of the Literature

1.1 Alcohol Consumption

1.1.1 Alcohol consumption among women

Alcohol (ethanol, ethyl alcohol) is legal, easily accessible, and widely consumed. The Canadian Alcohol and Drug Use Monitoring Survey (CADUMS) conducted in 2011 indicated that 78% of Canadians (81.9% of men and 74.3% of women) aged 15 years and older consumed alcohol in the 12 months preceding the survey (Health Canada, 2011). It was reported that 15.0% of women who consumed alcohol exceeded the recommended quantity of alcohol outlined in Canada’s Low-Risk Alcohol Drinking Guidelines to reduce chronic health risks, which suggests no more than 10 drinks/week and no more than 2 drinks/day on most days for women (Butt et al., 2011). Furthermore, 9.5% of female drinkers exceeded the recommended quantity of alcohol outlined to reduce acute health risks, which suggests no more than 3 drinks in one day (Butt et al., 2011). In 2010, 40.2% of Canadian women reported light infrequent use (<5 drinks/occasion, less than once a week), 28.2% light frequent use (<5 drinks/occasion, once or more per week), 3.6% heavy infrequent use (>5 drinks/occasion, less than once a week), while 1.8% reported heavy frequent use (>5 drinks/occasion, once or more per week) (Health Canada, 2010). In the US, 51% of adults (59% of men, 43% of women) 18 years of age or older were reported to be current regular drinkers (12+ drinks in the past year) in a national health survey conducted in 2010 (Schiller et al., 2012).

1.1.2 Alcohol consumption during pregnancy

Although women typically reduce or stop their ethanol consumption once a pregnancy is detected, many pregnant women expose the fetus to alcohol before they
are aware of the pregnancy, while others simply continue to drink even after pregnancy recognition (Ethen et al., 2009). The reported prevalence of drinking during pregnancy varies depending on the population in question, survey methodology, drinking definitions, and the patterns of drinking included or excluded in a particular study.

Using data from the 2000/1 Canadian Community Health Survey (CCHS), 13.3% of women who were pregnant at the time of the survey reported alcohol use in the week before the survey (Dell and Roberts, 2006). For the most part, women who reported drinking during their pregnancy in the 2000/01 CCHS did so infrequently, however, among the self-reported drinkers in the past year, 6.9% of pregnant women reported heavy drinking (defined as regular consumption of >12 drinks/week), with 0.5% reporting heavy drinking in the week prior to the survey (Dell and Roberts, 2006). The reported overall rates of drinking (any amount) during pregnancy in subsequent CCHS surveys are similar; 12.4% in 2003 and 10.5% in 2005 (assessed among women who gave birth in the 5 years preceding the survey) (Public Health Agency of Canada, 2008). More recent data from the 2007/8 CCHS, which also asked women who gave birth in the preceding 5 years whether they drank any alcohol during their last pregnancy, indicated a lower prevalence of drinking in pregnancy of 5.8% (Thanh and Jonsson, 2010). However, some methodological differences do exist between the 2007/8 CCHS and previous CCHS surveys in that the 2007/8 data was extrapolated based on data from only two provinces (Ontario and British Columbia).

Higher rates of maternal alcohol use in pregnancy were reported in the National Longitudinal Survey of Children and Youth (NLSCY), where 15.6-15.5% of mothers reported drinking during pregnancy in 2002–03 and 2004–05 cycles, and 9.4% in 2006-07, which is relatively similar to the previous reports of 14.4% in 1998-99, of which 4.9% drank throughout pregnancy (Government of Canada, 2011). The 2005/6 and 2006/7 Maternity Experiences Survey (MES) reported that 10.5-10.8% of women indicated drinking during pregnancy, which mainly reflects low to moderate maternal alcohol consumption (Public Health Agency of Canada, 2009; Walker et al., 2011). During the
three months prior to pregnancy or detection of pregnancy, 62.4% of women consumed alcohol with variable frequency, and this percentage decreased to 10.5%, after pregnancy recognition, the majority of which reported infrequent drinking (≤ 2-3 times/month) (Public Health Agency of Canada, 2009).

Similar numbers are reported in the United States, where the Centers for Disease Control and Prevention estimated that for 2006–2010, 51.5% of non-pregnant women of childbearing age (18–44 years) and 7.6% of pregnant women used alcohol in the past 30 days, compared to 54.6% and 11.2% estimated in 2001-2005, respectively (Centers for Disease Control and Prevention, 2009, 2012a). Binge drinking (≥ 4 drinks/occasion) was estimated at 1.4% among pregnant women and 15.0% among non-pregnant women for the 2006-2010 period; and the average frequency and intensity of binge episodes in pregnant and non-pregnant women were similar - approximately 3 times/month and 6 drinks/occasion. Similar numbers were reported in the 2010 National Survey on Drug Use and Health (NSDUH), which found that among pregnant women aged 15 to 44, 10.8% reported current alcohol use, 3.7% reported binge drinking (10.1% in the first trimester), and 1.0% reported heavy drinking (Substance Abuse and Mental Health Services Administration, 2011). Higher estimates were reported in another US study that determined the prevalence of drinking in pregnancy using data from the National Birth Defects Prevention Study from 1997-2002 (Ethen et al., 2009). Here, it was estimated that as many as 30% of all women reported drinking at some point in pregnancy, of which 8.3% reported binge drinking (4+ drinks/occasion) and 2.7% reported drinking throughout pregnancy with 7.9% during the third trimester (Ethen et al., 2009).

To conclude, the published data on women's alcohol consumption during pregnancy suggests that the rates in Canadian and US are similar, ranging between 5-15%, and have generally been declining (Dell and Roberts, 2006; Walker et al., 2011). Retrospective studies that assess drinking in pregnancy and those asking about drinking prior to pregnancy recognition report significantly higher rates of alcohol
consumption, presumably since women who report alcohol use after the fact and outside of prenatal clinics are more truthful and accurate (Alvik et al., 2006; Hannigan et al., 2010). The rates of alcohol consumption in other countries are variable, with many European countries and Australia reporting higher prevalence (Kesmodel et al., 2003; Lemola and Grob, 2007; Palma et al., 2007; Rimmer and de Costa, 2006; Walker et al., 2011). For example, in a study from France, only 53% of women reported complete abstinence during pregnancy (Malet et al., 2006); in a Spanish study, 22.7% reported alcohol consumption during pregnancy (Palma et al., 2007); in Australia, 24.6% women self-reported alcohol consumption in pregnancy in an antenatal clinic (Rimmer and de Costa, 2006); and 40% of women reported at least one episode of binge drinking since conception in a study from Denmark (Kesmodel et al., 2003). The rates in other countries also vary considerably, with 57.4% of women reporting alcohol consumption in pregnancy in a prenatal clinic in Chile (Aros et al., 2006); 46.7%-59% reporting prenatal alcohol consumption in Russian studies (Chambers et al., 2006; Kristjanson et al., 2007); and 46-73% in studies from Mexico (Backstrand et al., 2001; Montesinos Balboa et al., 2004).

1.1.3 Risk factors associated with alcohol consumption during pregnancy

Numerous factors have been shown to be related to maternal alcohol consumption during pregnancy and pre-conceptual binge drinking. These include maternal age, whether the pregnancy was planned, substance use, marital status, a history of physical and emotional abuse, mental health, self-esteem, prenatal care, nutrition, and socioeconomic status (SES) (Caetano et al., 2006; Chudley et al., 2005; Ethen et al., 2009).

Prevalence reports suggest that alcohol use in pregnancy varies depending on the age of the mother. In the CCHS surveys, women over the age of 35 and those between 15 and 19 years of age were generally more likely to report alcohol consumption than mothers between the ages of 20 and 34 (Dell and Roberts, 2006).
Being unmarried, having an unintended pregnancy, and being from socioeconomically disadvantageous groups were shown to be correlated with maternal alcohol consumption during pregnancy, as was having limited access to health care services and poor prenatal care (Naimi et al., 2003). Likewise, lower education level, prenatal use of cocaine and tobacco, paternal drinking and drug use, and inadequate nutrition were also shown to be associated with alcohol consumption in pregnancy (Chudley et al., 2005; Sood et al., 2001). However, no single profile identifies all women at risk for drinking in pregnancy, making the detection of prenatal alcohol exposure extremely challenging.

1.1.4 Doses and definitions of drinking behaviours

In many countries, alcohol intake is typically quantified in terms of number of standard drinks consumed. Although the exact definition of what constitutes a “standard drink” varies somewhat from country to country (Table 1.1), it signifies a specific amount of pure ethanol contained in the alcoholic beverage. For example, in Canada one standard drink contains 17.05 mL (13.45 g) of pure ethanol and in the US it equals to 18 mL (14.00 g) of pure alcohol. This roughly corresponds to one 12 fl. oz. (341 mL) bottle of beer, cider, or cooler (all 5% alcohol); one 5 fl. oz. (142 mL) glass of wine (12% alcohol); or 1.5 fl. oz. (43 mL) shot of 80-proof spirits or liquor (40% alcohol) (Butt et al., 2011; Centers for Disease Control and Prevention, 2012b). Likewise, the definitions of drinking behaviours vary greatly between countries, studies, surveys, and guidelines. Some terms and definitions that have been used by different government agencies and in national surveys are presented in Table 1.2.
Table 1.1 Definition of a “standard drink” or “unit of alcohol” in different countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Mass of Ethanol (g)</th>
<th>Volume of Ethanol (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>13.45 – 13.6</td>
<td>17.05 – 17.24</td>
</tr>
<tr>
<td>United States</td>
<td>14</td>
<td>17.74</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>8</td>
<td>10.13</td>
</tr>
<tr>
<td>Netherlands</td>
<td>9.9</td>
<td>12.55</td>
</tr>
<tr>
<td>Australia</td>
<td>10</td>
<td>12.67</td>
</tr>
<tr>
<td>New Zealand</td>
<td>10</td>
<td>12.67</td>
</tr>
<tr>
<td>Finland</td>
<td>11–12</td>
<td>13.94 –15.21</td>
</tr>
<tr>
<td>Italy</td>
<td>10–12</td>
<td>12.67–15.21</td>
</tr>
<tr>
<td>Japan</td>
<td>19.75–23.5</td>
<td>25.03 –29.78</td>
</tr>
<tr>
<td>France</td>
<td>10–12</td>
<td>12.67–15.21</td>
</tr>
<tr>
<td>Portugal</td>
<td>14</td>
<td>17.74</td>
</tr>
</tbody>
</table>

Table 1.2 Definitions of drinking behaviours by government agencies in Canada and US.

<table>
<thead>
<tr>
<th>Agency</th>
<th>Classification</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health Canada(^1)</td>
<td>Abstainer</td>
<td>Never used alcohol in their life.</td>
</tr>
<tr>
<td>Former drinker</td>
<td>Used alcohol in their life, but not in the past year.</td>
<td></td>
</tr>
<tr>
<td>Light infrequent drinker</td>
<td>&lt; once/week on average in a year; usually &lt; 5 drinks/occasion</td>
<td></td>
</tr>
<tr>
<td>Light frequent drinker</td>
<td>Once or more/week on average in a year; usually &lt; 5 drinks/occasion.</td>
<td></td>
</tr>
<tr>
<td>Heavy infrequent drinker</td>
<td>&lt; once/week; usually ≥5 drinks/occasion.</td>
<td></td>
</tr>
<tr>
<td>Heavy frequent drinker</td>
<td>Once or more/week on average in a year; usually ≥5 drinks/occasion.</td>
<td></td>
</tr>
<tr>
<td>Statistics Canada(^2)</td>
<td>Heavy drinking</td>
<td>≥5/ occasion, ≥12 times over the past year.</td>
</tr>
<tr>
<td>Center for Disease Control(^3)</td>
<td>Moderate drinking</td>
<td>≤1 drink/day for women, ≤2 drinks/ day for men.</td>
</tr>
<tr>
<td></td>
<td>Heavy drinking</td>
<td>On average &gt;2 drinks/day for men, &gt;1 drink/day for women, ≥5 drinks/ occasion for men or ≥4 drinks on a single occasion for women, generally within about 2 hours.</td>
</tr>
<tr>
<td>National Institute on Alcohol Abuse and Alcoholism(^4)</td>
<td>Moderate or &quot;low-risk&quot; drinking</td>
<td>≤ 4 drinks on any single day AND ≤14 drinks/week for men. \newline ≤ 3 drinks on any single day AND ≤7 drinks/week for women.</td>
</tr>
<tr>
<td></td>
<td>Binge drinking</td>
<td>≥ 5 drinks (male), or ≥4 drinks (female), in about 2 hours (BAC ≥0.08%).</td>
</tr>
<tr>
<td>Substance Abuse and Mental Health Services Administration(^5)</td>
<td>Binge use</td>
<td>≥5 drinks/occasion on at least 1 day in the past 30 days.</td>
</tr>
<tr>
<td></td>
<td>Heavy use</td>
<td>≥ 5 drinks/occasion on each of 5 or more days in the past 30 days.</td>
</tr>
</tbody>
</table>

\(^1\)CADUMS 2010; \(^2\)CCHS 2011; \(^3\)CDC, 2012; \(^4\)NIAAA 2013; \(^5\)SAMHSA, 2011.
When describing alcohol consumption and drinking behaviours, the terms alcohol use, abuse, and dependence are sometimes differentiated. The term “alcohol abuse”, although often loosely used, was defined in DSM-IV as a maladaptive pattern of drinking that leads to clinically significant impairment or distress (American Psychiatric Association, 2000). This may manifest as recurrent use of alcohol when it is physically harmful, recurrent alcohol-related legal problems, impaired ability to work and failure to fulfill major obligations, and continued alcohol use despite resulting/exacerbated persistent or recurrent social or interpersonal problems (American Psychiatric Association, 2000). Long-term alcohol abuse can lead to alcohol dependence, also known as alcohol addiction and alcoholism. Alcohol dependence is a chronic disease that typically manifests as persistent desire for alcohol, inability to cut-down, the occurrence of tolerance and withdrawal symptoms, and continued use despite repeated physical or psychological problems caused or exacerbated by drinking. Individuals with alcohol dependence may be spending a lot of time getting, using, and recovering from the effects of drinking, while giving up other social, occupational, or recreational activities as a result (American Psychiatric Association, 2000). In the newly published DSM-V, however, the distinction between “abuse” and “dependence” has been removed since it was considered somewhat arbitrary, as they appear to be the same disorder on a continuum of abuse. Instead, when diagnosing addictions, substance-related disorders and alcoholism, the DSM-V allows clinicians to determine the severity of the substance use disorder depending on the number of symptoms present (e.g., inability to cut-down, craving and urges, inability to complete obligations due to substance use, presence of tolerance and withdrawal symptoms and so on).

1.2 Pharmacokinetics of Ethanol in the Maternal-Fetal Unit

1.2.1 Absorption and distribution

When alcohol-containing beverages are ingested orally, ethanol (ethyl alcohol, C₂H₅OH, Mw= 46 g/mol) is quickly absorbed through the mucosal surface by simple diffusion in the stomach (about 20% of the ingested ethanol) and in the upper portions
of the small intestine (80% of the ingested ethanol) (Kalant and Khanna, 2007; Swift, 2003). Since ethanol absorption from the small intestine is much more rapid than from the stomach, the rate of gastric emptying is an important determinant of the rate of absorption. Thus, factors affecting gastric emptying time such as the presence and type of food, as well as other factors like the concentration of ethanol, rate of alcohol consumption, blood flow at the site of absorption, and mucosal integrity; can all influence the rate of absorption (Best and Laposata, 2003; Swift, 2003).

Being a small, uncharged, water-soluble molecule, ethanol rapidly distributes and equilibrates throughout total body of water (Norberg et al., 2003), which is approximately 50-60% of total body weight in males and 45-55% in females (Kalant and Khanna, 2007). Thus, ethanol’s volume of distribution is approximately 0.45 to 0.6 L/kg, and its distribution throughout the body is primarily dependent on the blood flow to different organs and tissues (Kalant and Khanna, 2007). As expected, body composition (which is affected by factors like gender, age, and fitness) plays an important role in determining ethanol’s volume of distribution in an individual. Typically, men, younger individuals, and leaner individuals have greater volumes of distribution compared to women, older individuals, and those with a greater percentage of body fat, respectively. Furthermore, physiological changes in body composition and total body of water that occur in pregnancy can greatly increase the volume of distribution of ethanol (Dawes and Chowienczyk, 2001).

1.2.2 Metabolism and elimination

The vast majority of ingested ethanol is metabolized, with the remainder (2-10%) excreted unchanged in urine, breath and sweat (Best and Laposata, 2003; Kalant and Khanna, 2007). Ethanol is metabolized by both oxidative and non-oxidative pathways (Figure 1.1), with approximately 85% of ethanol metabolized in the liver via enzymatic oxidation (Swift, 2003). The bulk of oxidative ethanol metabolism involves ethanol's biotransformation to acetaldehyde (a highly reactive compound that can bind macromolecules and create adducts) by alcohol dehydrogenase (ADH), and the
subsequent conversion of acetaldehyde to acetic acid by aldehyde dehydrogenase (ALDH) (Kalant and Khanna, 2007). This process takes place primarily in the liver. During each of the two steps (i.e., ethanol metabolism to acetaldehyde, and acetaldehyde metabolism to acetate), a nicotinamide adenine dinucleotide (NAD+) is reduced (Kalant and Khanna, 2007). The produced acetic acid enters Krebs cycle where it is converted to water and carbon dioxide (Kalant and Khanna, 2007). The rate-limiting step in the whole process is the oxidation of ethanol to acetaldehyde by cytosolic ADH, which has a low Km of 0.05-0.1 g/L (Kalant and Khanna, 2007; Norberg et al., 2003). The metabolism of ethanol follows Michaelis-Menten kinetics and exhibits zero order elimination for a large part of the blood-concentration time course (BAC above 20 mg/dL) due to saturation of hepatic oxidative enzymes ADH (Norberg et al., 2003). During zero-order elimination, ethanol is eliminated at a rate of approximately 7 g/hr in a 70 kg person (Swift, 2003).

Multiple isoforms of ADH and ALDH with different kinetic profiles (grouped into different classes) and numerous polymorphisms lead to significant variation in human alcohol metabolism rates (Bosron and Li, 1986). The variable expression of the different isoforms and polymorphisms may account for differences in tissue capacity to metabolize ethanol, differences in ethanol metabolism observed between individuals and between racial and ethnic groups, as well as, for variation in toxicities associated with drinking among different racial groups (Norberg et al., 2003; Osier et al., 2002; Swift, 2003; Wall, 2005). Class I ADH and Class II ALDH are considered the main isoforms responsible for ethanol metabolism in the liver -- the main site of oxidative ethanol metabolism (Norberg et al., 2003). These enzymes are also expressed extrahepatically but the contribution of these other sites to overall ethanol metabolism is considered minimal. Nonetheless, lower expression of gastric ADH in women is speculated to contribute to a higher ethanol bioavailability as compared to men (Swift, 2003). Other gender differences include higher rates of hepatic metabolism of ethanol in females, which is speculated to be due to higher relative liver volumes (Kwo et al., 1998).
Two minor pathways also exist that metabolize ethanol oxidatively and these involve the microsomal ethanol-oxidizing system (cytochrome P450 2E1) and catalase. Since CYP2E1 in the liver is inducible and thus more active in chronic heavy drinkers, it accounts for increased ethanol metabolism at high alcohol concentrations observed in chronic drinkers (Swift, 2003). Catalase, which is ubiquitously expressed, is thought to play a small role in overall ethanol metabolism, but may contribute to localized production of acetaldehyde and thus acetaldehyde adduct formation in some tissues, particularly in those with low ADH expression (Swift, 2003).

Non-oxidative biotransformation is a minor pathway of ethanol metabolism and involves the enzymatic conjugation of ethanol to endogenous substrates such as fatty acids, phospholipids, sulfate, or glucuronic acid. The derivatives of non-oxidative ethanol metabolism are termed fatty acid ethyl esters (FAEEs), phosphaditylethanol (PEth), ethyl sulfate (EtS), and ethyl glucuronide (EtG), respectively (Pawan, 1972; Zimatkin and Deitrich, 1997). The esterification of ethanol with fatty acids is the best studied non-oxidative pathway to date and the focus of this thesis. Many mammalian tissues and organs have been shown to possess the ability to synthesize FAEE upon ethanol exposure. A generally accepted terminology of FAEE-synthetic enzymes delineates two enzyme activities; FAEE synthase (FAEES) and acyl-CoA: ethanol O-acyltransferase (AEAT). The term FAEES is used to designate the FAEE-synthetic activity that uses ethanol and free fatty acids as substrates, while AEAT describes the FAEE synthetic activity that uses ethanol and fatty acyl-CoA as substrates (Treloar et al., 1996). FAEES activity has been purified from several tissues and shown to be associated with enzymes with other principal physiological functions, primarily in lipid metabolism (several lipases and carboxylesterases) (Best and Laposata, 2003). Conversely, several known enzymes have been shown to possess FAEES activity, including pancreatic lipases, lipoprotein lipase, hepatic carboxylesterase, and cholesterol esterase (Best and Laposata, 2003). Since FAEEs persist longer in the body than ethanol itself and its oxidative metabolites and can accumulate in various tissues and matrices, they have been used as biomarkers of ethanol consumption, and
this will be discussed in greater detail in later sections. Ethanol conjugation to phospholipids is mediated by the action of phospholipase D (PLD); to glucuronic acid by the action of UDP-glucuronosyltransferases (UGT); and to sulfate by the actions of sulfotransferase (Szabo et al., 2011). It should be noted that oxidative and non-oxidative pathways are metabolically linked in that there is increased production of non-oxidative metabolites in instances where oxidative metabolism is deficient or inhibited. This is due to a shift from oxidative to non-oxidative pathways by substrate loading (Werner et al., 2001, 2002). This may be of interest since ADH deficiency in chronic alcoholics may be a determining factor for the increased body burden of ethanol and its disposition via non-oxidative metabolism (Werner et al., 2001).

Figure 1.1 Oxidative and non-oxidative ethanol metabolism. Note: FAEES and AEAT refer to enzymatic activity mediating FAEE synthesis from free fatty acids and fatty-acyl-CoA, respectively. Abbreviations: ADH, alcohol dehydrogenase; AEAT, acyl-CoA: ethanol O-acyltransferase; ALDH, aldehyde dehydrogenase; CE, carboxylesterase; ChE, cholesterol esterase or carboxylester lipase; CoA, coenzyme A; EtG, ethyl glucuronide; EtS, ethyl sulfate; FAEES, fatty acid ethyl ester synthase; FAEE, fatty acid ethyl esters; LPL, lipoprotein lipase; PEth, phosphatidylethanol; PLD, phospholipase D; SULT, sulfotransferase; TGL, triglyceride lipase; UGT, UDP-glucuronosyltransferase (Best and Laposate, 2003; Kalant and Khanna, 2007; Swift, 2003).
1.2.3 Fetal exposure to ethanol

Animal studies and clinical studies conducted in pregnant women demonstrated that ethanol readily crosses the placenta and rapidly distributes into the fetal compartment resulting in similar BACs in maternal and fetal circulations (Brien et al., 1983, 1985, 1987; Idanpaan-Heikkila et al., 1972). Of interest, Brien et al. (1983), who studied the disposition of ethanol in six healthy pregnant women at 16-18 weeks gestation, found that the maximal ethanol concentration was lower (2-fold) in the amniotic fluid than in maternal venous blood, but the rate of ethanol elimination from amniotic fluid was about half the elimination rate from maternal venous blood such that 3.5 hours later, ethanol was still present in the amniotic fluid while being undetectable in maternal blood. Furthermore, the ethanol AUC\(_{0-3.5h}\) in amniotic fluid was only 16% lower than the value for maternal blood (Nava-Ocampo et al., 2004). The authors suggested that following maternal ethanol consumption, amniotic fluid may act as reservoir for ethanol exposing the fetus to ethanol for a longer time period (Brien et al., 1983; Nava-Ocampo et al., 2004). Similar findings were reported in animal studies (in near-term ewe, guinea pigs and rats) that examined the disposition of ethanol and acetaldehyde (Brien et al., 1987; Clarke et al., 1989; Hayashi et al., 1991). These studies indicated that there is bidirectional placental transfer of ethanol in the maternal-fetal unit such that the ethanol concentration in maternal and fetal blood are similar, however, fetal exposure can be prolonged since the amniotic fluid serves as a reservoir for ethanol. Because of the recurrent cycle of fetal swallowing of the amniotic fluid, metabolism (which is low in the fetus), and elimination of urine back into the amniotic fluid, fetal exposure to ethanol is prolonged.

Surprisingly little research has been done on the effects of physiologic and endocrine changes in pregnancy on maternal alcohol disposition, particularly on metabolism and clearance. Increase in the volume of distribution that occurs during pregnancy is expected to result in lower ethanol plasma concentrations after the same given dose. With respect to metabolism, in pregnant rats, increased alcohol clearance rate compared with weight-matched non-pregnant controls was observed (Badger et al.,
2005). Since the rate of alcohol clearance ultimately determines tissue ethanol concentrations, faster ethanol clearance may help minimize tissue concentrations and thus mitigate fetal alcohol effects (Shankar et al., 2007). The authors showed that the increased alcohol clearance was not due to changes in hepatic ADH1 activity and not due to changes in CYP2E1 activity, which was actually suppressed (Badger et al., 2005). On the other hand, although hepatic ALDH enzyme activity was also not elevated, the amount of mitochondria in the liver was increased resulting in increased overall ALDH activity on a whole-liver basis. This increase in ALDH activity may increase alcohol clearance by accelerating acetaldehyde removal. Additionally, there was an up-regulation of gastric ADH4 activity in pregnant rats compared to controls suggesting increased first-pass metabolism (Badger et al., 2005). This upregulation may be mediated by hormonal changes in pregnancy. Little work has been published on how chronic ethanol intake during pregnancy can lead to changes in maternal metabolism.

The ability of the fetus to metabolize ethanol varies during development. At birth, the rate of ethanol elimination from neonatal blood is only about half of the elimination rate from maternal blood (Idanpaaan-Heikkila et al., 1972). Studies indicate that early on, the fetus has a limited capacity for oxidative ethanol metabolism as evidenced by low hepatic ADH activity in fetuses and low expression of some ADH isozymes in first trimester fetuses (Hines and McCarver, 2002; Smith et al., 1971). Hepatic ADH activity appears to increase with gestational age, and studies report progressive expression of various ADH isoforms during development and tissue-specific changes in the relative amounts of expressed isozymes (Smith et al., 1971). With regard to CYP2E1, although some groups did not find detectable expression in the fetus (Vieira et al., 1996), others reported detectable CYP2E1 as early as 16 weeks gestation in fetal liver (Carpenter et al., 1996). It appears that CYP2E1 expression increases with gestational age, being undetectable in first trimester liver samples, but reportedly found in 37% of second trimester, and majority (80%) of third trimester fetal liver samples tested (Johnsrud et al., 2003). Although this expression is relatively low in the fetus, it gradually increases after birth, reaching 30-40% of adult hepatic levels by one year of age (Hines and
McCarver, 2002; Vieira et al., 1996). Of interest, CYP2E1 expression in fetal brain was detected as early as 7-9 weeks gestation, which, given the relatively limited expression of ADH and ALDH enzymes in this tissue, may be sufficient to generate reactive intermediates that may mediate toxicity following maternal alcohol consumption (Brzezinski et al., 1999). In another study, CYP2E1 content in the placenta was found to be variable among heavy drinkers, suggesting that induction may be taking place (Rasheed et al., 1997). The authors speculated that this inter-subject variability in induction might play a role in individual susceptibility to alcohol related defects. With respect to catalase, it has been shown that the activity of catalase in human fetal liver and kidney also increases in parallel with gestational age (DeAddya and Sengupta, 1986). However, the authors found that in the fetal brain, catalase activity is relatively low and does not show any significant changes during gestation except for a decrease in activity later in pregnancy (28 weeks and above). The significance of this finding is not clear and, overall, it is not known what role or contribution, if any, the ontogeny of catalase plays in fetal metabolism of ethanol and susceptibility to its negative effects during gestation given the minor role it plays in ethanol metabolism in adults.

With respect to non-oxidative enzymes, FAEES activity (FAEE synthesis from ethanol and free fatty acids) has been demonstrated in supernatant from tissue homogenates of human term placenta and mouse placenta (at gestational day 14), mouse embryo, primary cultures of rat fetal brain cells, fetal and postnatal rat brain, and in human fetal brain (2nd trimester) incubated with ethanol and oleic acid, demonstrating that the enzyme activity necessary for FAEE synthesis is present in fetuses and fetal brain tissue relatively early in gestation (Bearer et al., 1992, 1995). The ontogeny of various non-specific enzymes shown to possess FAEES activity, such as the different carboxylesterases and lipases, varies greatly depending on the enzyme, isoyme, and tissue of interest. The ontogeny of AEAT activity has not been studied and it is not known what specific enzymes possess this activity as of yet. The onset of hepatic UGT expression and activity (catalyze EtG formation) occurs after 20 weeks of gestation with
significant increases in the first weeks of life (Krekels et al., 2012), while some sulfotransferases (catalyze EtS production) appear to be widely expressed in the developing human, with most present at levels equivalent to or higher than the adult (Stanley et al., 2005). As with catalase, given their minimal contribution to overall ethanol metabolism in adults, it is unlikely that changes in the expression of non-oxidative metabolic enzymes during development play a significant role in overall fetal handling of ethanol. However, it is possible that the low expression of oxidative enzymes can shift ethanol metabolism towards non-oxidative pathways if they are expressed and active earlier in the gestational period, thus increasing their importance.

1.3 Fetal Alcohol Spectrum Disorders

1.3.1 History and terminology

Alcohol is the most commonly consumed teratogen worldwide, the in utero exposure to which can result in wide range of physical, cognitive, and neurobehavioural deficits (Astley and Clarren, 2000). Although several historic references suggest that the negative effects of ethanol on the developing fetus have been known for many centuries (Randall, 2001), it was not until the 1960s that a pattern of deficits in children was linked to maternal alcohol consumption in pregnancy (Lemoine, 2012; Lemoine et al., 2003). Dr. Paul Lemoine, a pediatrician from Nantes, France, was the first to describe several characteristic features observed in a group of 127 children born to mothers with histories of alcohol abuse, including craniofacial dysmorphology, growth retardation, malformations, and psychomotor deficits (Lemoine et al., 1968). Dr. Lemoine’s findings were soon corroborated by an American group who reported on a similar group of children in Seattle and who first coined the term Fetal Alcohol Syndrome (FAS) to describe the common anomalies documented in these cases (Jones and Smith, 1973, 1975). These reports led to a subsequent surge in research into the detrimental effects of ethanol on the fetus and the mechanisms, characteristics, and epidemiology of ethanol-related disabilities. Over the years, the definition of FAS has not changed significantly and the term is used to describe the most severe outcome of in utero
alcohol exposure, which is characterized by growth restriction, particular facial dysmorphology, and neurodevelopmental disabilities (Chudley et al., 2005). It has since become recognized as the leading preventable cause of mental impairment in the developed world (O’Leary, 2004). In addition to FAS, a wide range of less severe outcomes of prenatal alcohol exposure have been described and initially termed fetal alcohol effects (FAE). The entire continuum of effects was subsequently expanded to four distinct diagnoses: FAS, partial FAS (pFAS), alcohol-related birth defects (ARBD), and alcohol-related neurodevelopmental disorder (ARND) (Stratton et al., 1996). The term that was coined to encompass these four diagnoses and describe the entire spectrum of adverse fetal outcomes that result from prenatal alcohol exposure is fetal alcohol spectrum disorders (FASD) (Warren et al., 2004). The various deficits and abnormalities associated with prenatal ethanol exposure depend on factors such as timing, frequency, and amount of alcohol consumption in pregnancy, as well as on a number of maternal and fetal characteristics, which will be discussed in more detail below.

1.3.2 Clinical presentation

The teratogenic effects of alcohol can produce a spectrum of negative fetal outcomes, ranging from major malformations and serious cognitive deficits to more subtle physical, cognitive, and behavioural impairments that can, nonetheless, have significant life-long implications for the affected individual (O’Leary, 2004; Streissguth and O’Malley, 2000). The classic features of FAS (the most severe end of the spectrum) include prenatal and postnatal growth restriction; central nervous system (CNS) anomalies or dysfunction such as microcephaly, developmental delays, and intellectual and psychomotor disability; and a specific pattern of facial dysmorphology that includes short palpebral fissures, flat philtrum, thin upper lip, epicanthic folds, maxillary hypoplasia, and flattened medial midface (Jones and Smith, 1973; Lemoine et al., 2003; O’Leary, 2004; Riley and McGee, 2005). The facial dysmorphology results from ethanol-induced damage at a time when the midline of the face is forming (~3-8 weeks gestation), which is also a time of particular CNS vulnerability (Clarren et al., 1978,
As a result, the facial abnormalities and CNS dysfunction are strongly associated, with the former being a physical sign of damage to the CNS at a time of particular sensitivity (Astley and Clarren, 2001; Astley et al., 2002; Chasnoff et al., 2010). However, these physical features are present only in the most severe cases typically associated with high levels of exposure at a particular stage of pregnancy and, thus, only in a minority of individuals affected by prenatal alcohol exposure (~10% of cases) (Chudley et al., 2005). The vast majority of ethanol-affected individuals present with a highly variable and complex pattern of behavioural and cognitive abnormalities and may or may not display growth deficiencies and physical malformations. As mentioned above, these less affected individuals that have some but not all of the FAS characteristics may be classified under the pFAS, ARND, or ARBD diagnoses.

Neurological anomalies and impairment is the most common unifying feature of disorders classified under the FASD umbrella, although a high incidence of cardiac, skeletal and other malformations are also common in ethanol-affected individuals (Guerri et al., 2009). Ethanol-related neurological deficits in the fetus may result from drinking at any time during pregnancy as the CNS system is developing and remains vulnerable throughout gestation (Sulik, 2005), and no single type of CNS damage has been shown to characterize FASD (Hicks et al., 2003). Exposure in the first half of pregnancy has been shown to affect cytogenesis and cell migration of the neural crest, while exposure towards the end of pregnancy has been shown to affect brain growth, differentiation and neuronal apoptosis (Malanga and Kosofsky, 1999; Sulik, 2005). Prenatal ethanol exposure has been shown to be associated with various structural or functional changes, including an overall reduction in brain size and volumes of specific brain regions, atypical brain shape, and altered gray and white matter expression (LeBel et al., 2008; Riley and McGee, 2005). Specific brain regions that have been shown to be affected include the corpus callosum (can be absent in severe cases), hippocampus, caudate, thalamus, amygdala, globus pallidus, cerebellum, brainstem, frontal/temporal and parietal lobes, and basal ganglia (Bookstein et al., 2002, 2007; Jones and Smith, 1973; LeBel et al., 2008; McGee and Riley, 2006; Sowell et al., 2001). Animal studies
have also shown abnormalities in many neurotransmitter signaling systems (glutaminergic, dopaminergic, muscarinic, and GABAergic) (Carneiro et al., 2005; Galindo et al., 2005; Iqbal et al., 2005).

The structural and functional anomalies and changes underlie the deficits in intellectual, cognitive, motor, and behavioural impairments in individuals with FASD, which have been shown to be present even in the absence of physical features/malformations (Chudley et al., 2005; LeBel et al., 2008; Mattson and Riley, 1998; Mattson et al., 1998). These include lower overall intellectual performance, learning problems, difficulties with language acquisition, verbal learning and communication, deficits in memory, delayed motor development, gross and fine motor dysfunction, attention deficits, impaired visuospatial and motor stimuli processing, impaired executive functioning (cognitive flexibility, response inhibition, planning, organization concept formation, verbal/nonverbal fluency), failure to learn from consequence, and deficits in adaptive and social skills (Astley and Clarren, 2000; Chudley et al., 2005; Green et al., 2009; Rasmussen et al., 2008; Streissguth, 2007; Streissguth and O'Malley, 2000, 2000). Deficits fall on a continuum, with children with FAS diagnosis typically exhibiting the most impaired level of general intelligence, language-based memory, communication, and executive functioning compared with children with other FASD diagnoses (ARND and pFAS) (Chasnoff et al., 2010).

Ethanol-affected neonates and infants have been shown to have increased perinatal mortality, growth restriction, irritability, hyperactivity, feeding difficulties, self-soothing problems, and impaired habituation to sensory stimuli (Jones et al., 1974; O'Leary, 2004; Streissguth et al., 1984). Infants have been shown to have longer reaction times, be difficult to settle and have a slow-to-warm temperament (Jacobson and Jacobson, 1994; Jacobson et al., 1993, 1994; O'Malley, 2007). Children affected by prenatal alcohol exposure have been shown to be impulsive, restless, distractible, and inattentive; with many exhibiting learning problems, memory deficits, psychiatric problems, mental deficiency, motor and language difficulties, attachment insecurity,
impaired social functioning, and developmental delays (Coles et al., 2000; Fried et al., 1992; McGee et al., 2009a, 2009b; O’Connor et al., 2002a, 2002b; Streissguth et al., 1989, 1994a, 1994b; Streissguth, 1992, 2007; Streissguth et al., 1984, 1986). With respect to social functioning, children with FAS tend to be uninhibited, overly friendly, demanding of affection and physical contact, intrusive, insensitive to social cues, and lack social judgment (O’Leary, 2004). The Seattle Prospective Longitudinal Study on Alcohol and Pregnancy (SPLS) found that the intellectual impairment is observed into maturity, being worse in those with more physical manifestations (i.e. FAS diagnosis) (Streissguth et al., 1989). Not surprisingly, these impairments and deficits adversely affect academic and social functioning throughout the lifespan (Barr et al., 2006; Connor et al., 2006; Spohr and Steinhausen, 2008; Spohr et al., 2007; Streissguth, 2007; Streissguth et al., 1991).

Of interest, long-term deficits in academic achievement in alcohol-affected individuals are not necessarily due to lower IQ since the impairments in learning, executive function, and attention are also seen in affected individuals with normal IQ scores (Kerns et al., 1997; Olson et al., 1998; Streissguth et al., 1989). Heavily exposed children with and without FAS diagnoses have been shown to display deficits in concept formation even in the absence of mental retardation, likely due to impairment in problem solving skills and adaptive functioning (McGee et al., 2008). The deficits in executive function, adaptive behaviours, and social skills predispose alcohol-affected children to problem behaviours such as hyperactivity, disruptiveness, impulsivity, poor judgment and planning, and poor understanding of consequences (Jacobson and Jacobson, 2002).

Multiple neurobehavioural problems, attention problems, executive functioning deficits, and antisocial syndrome have been described in adolescents and adults prenatally exposed to ethanol (Connor et al., 2000, 2006; Howell et al., 2006; Korkman et al., 2003; McGee et al., 2008; Olson et al., 1998; Riley et al., 2003; Streissguth et al., 1991). Studies have also shown that even in the absence of mental retardation,
prenatally alcohol-exposed adolescents have substantial impairments in their abilities to solve problems in their everyday life (McGee et al., 2008), suffer from deficits in attention, verbal learning and executive function (Kerns et al., 1997), and teenagers with FAS, independent of IQ, have been shown to display social and interpersonal relationship skills equivalent to a normal 6 year old (Streissguth et al., 1994a, 1996). Furthermore, findings from the SPLS showed that even in young adulthood, those identified as having FAS or ARND had poorer motor performance, and that the odds of several psychiatric disorders and traits more than doubled for adults exposed to one or more binge alcohol episode in utero (Barr et al., 2006; Streissguth, 2007). The psychiatric disorders included Substance Dependence/Abuse Disorders (OR=2.56), Passive Aggressive Personality Disorder (OR=3.27), and Antisocial Personality Disorder (OR=3.01) (Streissguth, 2007).

The primary deficits that result from the neurological damage described above can severely impact the individual’s ability to function in society and meet expectations, thereby placing FASD-affected individuals at high risk for various detrimental outcomes that are termed “secondary disabilities”. These include disrupted school experience, unemployment, inappropriate sexual behaviours, dependent living, substance abuse, trouble with the law, and confinement (Streissguth, 1996; Streissguth et al., 2004). Disrupted school experience (suspensions, expulsion, dropping out) was reported for 14% of school children and 61% of adolescents and adults with FASD (Streissguth et al., 2004). Inappropriate sexual behaviors increase from 39% in children (exposing and inappropriate touching) to 48% in adolescents and 52% in adults (promiscuity and inappropriate advances) with FASD (Streissguth et al., 2004). Dependent living (e.g., group home, living with family or friends, or some sort of assisted living) and problems with employment were experienced by 80% of the subjects (age 21 and older) (Streissguth, 1996). Of interest, the increased rates of substance abuse (35%), including alcohol, and inappropriate sexual behavior among women with FASD makes FASD a self-perpetuating intergenerational disorder (Streissguth et al., 2004).
Trouble with the law was reported for 14% of children and 60% of adolescents and adults with FASD, the most common law violations being theft, assault, burglary, and domestic violence (Streissguth et al., 2004). Confinement was reported for 8% of children (psychiatric hospitalizations) and for 50% of adolescents and adults (Streissguth et al., 2004). In a 1999 study of 287 Canadian forensic in-patients who had been remanded for a psychiatric assessment, 1% met the criteria for FAS while 22.3% met the criteria for ARND (Fast et al., 1999). The prevalence of FASD in adult male offenders entering a federal correctional facility in Manitoba was 10% (Fraser, 2008), which was considered a conservative estimate due to difficulty in diagnosing adults with FASD (Chudley et al., 2007). In another study, a survey of 137 youths (14-19 years old) in custody in British Columbia during 2003-2004 revealed that 11.7% had been previously told by a health care professional that they had FAS/FAE (Murphy and Chittenden, 2007).

Another important observation is the very high level of co-morbidity with mental illness in the FASD-affected population (Fryer et al., 2007; O'Connor et al., 2002a; Spohr and Steinhausen, 2008; Spohr et al., 1993, 2007; Steinhausen et al., 1993; Streissguth, 1996). Among 415 individuals with FASD (6-51 years old), more than 90% had mental health problems while 23% of adults with FASD have attempted suicide (5 times higher than US average) (Streissguth, 1996). Similar high rates (87%) of psychiatric disorders were found among children with prenatal alcohol exposure, 61% of which met the criteria for mood disorders (O'Connor et al., 2002a), and in Canadian adults with FASD (92%), which had high rates of Attention-Deficit Hyperactivity Disorder (ADHD), depression and panic disorder (Clark et al., 2004; Walthall et al., 2008). In another study of 130 children admitted to psychiatry inpatient services, 30% had documented prenatal alcohol exposure, of which 26% met criteria for FAS (none for diagnosed prior admission) (O'Connor et al., 2006a). Because of such high co-morbidity, it has been suggested that fetal alcohol exposure should be considered a possible factor in the pathogenesis of childhood psychiatric disorders (Fryer et al., 2007). Even low levels of prenatal alcohol exposure (<1 drink/week) have been linked
to mental health problems in children (Sayal, 2007). There are several theories for the high co-morbidity of prenatal alcohol exposure and mental health illness. First, the mental health problems may be a result of the cognitive impairments associated with the neurological damage. Second, the susceptible genes for alcoholism and mental health problems may be the same. Third, women with mental health problems may use alcohol as self-medication for the symptoms, or, lastly, the mental health problems (e.g. ADHD) may actually be a component of FASD (Clark et al., 2004).

1.3.3 Etiology and risk factors

With regard to ethanol’s teratogenic effects on the fetus, numerous mechanisms have been suggested to mediate alcohol-induced injury, although none has been established with certainty and it is unlikely that a single mechanism can account for the multitude of outcomes and features observed in FASD (Goodlett and Horn, 2001; Goodlett et al., 2005). There are many ways in which ethanol can interact with tissues, and these interactions can have short term and long-term effects. Ethanol exposure can lead to cell death, interfere with cellular functions, and diminish cells through different mechanisms depending on the cell type, stage of development (as vulnerability of certain cell types varies) and levels of maternal drinking (Goodlett and Horn, 2001; Goodlett et al., 2005). Thus, multiple mechanisms can operate simultaneously and/or sequentially over time. Investigated mechanisms that may contribute to ethanol-induced fetal pathology (mainly studied in the CNS) include induction of apoptosis (Cartwright et al., 1998; De et al., 1994; Sulik, 2005), oxidative stress and mitochondrial dysfunction (which can lead to cell death) as a result of generation of reactive oxygen species during ethanol metabolism and reduction of antioxidant levels (Dong et al., 2010; Heaton et al., 2002; Kotch et al., 1995; Ornoy, 2007), altered cell division due to interference with growth factor signaling (Cui et al., 1997; Luo and Miller, 1998), altered protein and DNA synthesis, interference with development and activity of neurotransmitter systems including glutamate and serotonin (Costa et al., 2000; Eriksen et al., 2000), disruption of glucose transport and uptake (Hu et al., 1995; Singh et al.,
1992), reduction in cell adhesion (Ramanathan et al., 1996), and altered developmental regulation of gene expression (Goodlett and Horn, 2001; Riley and McGee, 2005).

Not all children exposed to ethanol prenatally are invariably affected or impacted to the same extent. It is estimated that among children of heavily drinking women, 40% will be adversely affected and only 4% will exhibit the full features of FAS, although this may be substantially higher in vulnerable populations (Abel, 1995). Risk for developing ethanol-induced disabilities and the severity of these disabilities is multidimensional, with a number of exposure-related and individual-related risk factors and variables. These include the timing, level, pattern, and duration of maternal drinking, as well as genetic, health, and lifestyle related factors (May and Gossage, 2011). The major contributor to variability in risk and severity of damage is considered to be the extent of alcohol exposure and the drinking pattern in pregnancy (amount, frequency, and gestational timing) (May and Gossage, 2011). Many of the dose-related effects of prenatal alcohol are well established and, in general, higher doses produce more prominent effects in the fetus (Eckstrand et al., 2012; Feldman et al., 2012; Jacobson and Jacobson, 1994; Konovalov et al., 1997; Patra et al., 2011; Sampson et al., 2000; Sood et al., 2001; Streissguth et al., 1989, 1996). Some studies showed that peak blood alcohol concentration (BAC) rather than the total amount of ethanol consumed, correlate better with adverse outcomes, suggesting that binge drinking is the most damaging form of alcohol consumption because it produces the highest BAC (Maier and West, 2001). Bailey and colleagues (2004) showed that in 7 year old children who were prenatally exposed to ethanol, binge drinking (rather than the amount of overall prenatal exposure) had significant negative association with verbal IQ and delinquent behavior (Bailey et al., 2004). Other studies have shown that drinking three or more drinks per occasion correlates with total dysmorphology and behavioral problems in children (May et al., 2007, 2008). Furthermore, populations with high rates of frequent binge drinking have the highest incidence of FASD, particularly FAS and pFAS (more severe forms) (May and Gossage, 2011; May et al., 2007; Urban et al., 2008; Viljoen et al., 2005). In a South African cohort, mothers of children diagnosed with FAS or pFAS have been
shown to drink consistently for 2 days every weekend, consuming an average of 6.6 standard drinks per evening (May and Gossage, 2011). A recent prospective cohort study from Chile showed that after exposure to heavy alcohol consumption during pregnancy, 80% of children had abnormalities associated with alcohol exposure and that the patterns of alcohol use that posed the greatest risk of adverse outcomes were binge drinking and high total weekly intake (Kuehn et al., 2012). Of interest, populations where a more moderate pattern of alcohol consumption is common (lower amounts over prolonged period of time), have fewer cases of FASD overall and more cases of less severe FASD diagnoses (May and Gossage, 2011).

Although both human and animal studies have shown that there is a great deal of variation in the traits or features of FASD produced by individual mothers, it is clear that major risk to the fetus requires chronic, daily, heavy alcohol exposure or frequent, heavy, intermittent alcohol use (Abel, 1995; O’Leary, 2004). Nonetheless, while light and moderate alcohol consumption may not be a sufficient to produce full-blown FAS, adverse affects on development (primarily cognitive and behavioral abilities) have been documented in a number of human studies (Eckstrand et al., 2012; Gusella and Fried, 1984; Hanson et al., 1978; Jacobson and Jacobson, 1994; Sood et al., 2001; Streissguth et al., 1989, 1990). Animal studies are consistent with these observations, showing adverse effects of low to moderate ethanol and even single episodes of ethanol exposure on fetal growth, CNS, development, and behaviour (Dikranian et al., 2005; Probyn et al., 2012; Rice et al., 2012; Valenzuela et al., 2012; Wozniak et al., 2004). A recent large prospective cohort study from California found that various adverse effects associated with prenatal alcohol exposure (birth weight and specific facial dysmorphology) are dose-related without evidence of a threshold, that is, there was no apparent “safe level” of alcohol consumption (Feldman et al., 2012). In contrast, a large study of the Western Australian Pregnancy Cohort did not find that light-moderate consumption of alcohol in pregnancy is a risk factor in the epidemiology of child behavioural problems (Robinson et al., 2010), and a series of recent studies utilizing the Danish National Birth Cohort did not find any serious effects on child
neurodevelopment in 5 year old children prenatally exposed to low or moderate alcohol in early pregnancy (Falgreen Eriksen et al., 2012; Kesmodel et al., 2012; Skogerbø et al., 2012; Underbjerg et al., 2012). The lack of consensus about the risks associated with low to moderate levels of prenatal alcohol exposure has generated considerable debate. It is unknown how much prenatal ethanol exposure in any particular individual is necessary to produce noticeable disabilities, and it is important to note that studies on this topic are often limited by the difficulty of gathering accurate information on drinking patterns and BACs and linking them to observed disabilities in children. Thus, at present, there is no known low dose that appears to be safe for every pregnancy and it is advised that pregnant women avoid alcohol in pregnancy, as it is the safest choice.

The timing of maternal drinking is also an important consideration since it determines which anatomical features will be affected (Feldman et al., 2012; Lipinski et al., 2012; Stratton et al., 1996; Sulik, 2005). Animal studies have shown that ethanol’s teratogenic effects depend on the fetal organs that were undergoing development at the time of exposure since it is a time of particular vulnerability (Randall, 1987, 2001). Maternal alcohol consumption during critical periods of gestation will produce a different pattern of anatomical defects and/or cognitive deficits depending on the stage of development. For example, the characteristic facial features observed in full-blown FAS occur due to exposure during 3rd to 8th week of gestation (May and Gossage, 2011). In a recent prospective study from California, it was found that smooth philtrum, thin vermillion border, and microcephaly are associated with specific gestational timing of prenatal alcohol exposure (second half of first trimester) and are dose-related without evidence of threshold (Feldman et al., 2012). Of interest, reduced birth length was increased with exposure in any trimester indicating that there is no “safe time” for alcohol exposure with respect to this outcome. Since the CNS continually develops throughout the 9 months of pregnancy, it is expected to be vulnerable to the effects of ethanol at any point in pregnancy, and a wide range of neurodevelopmental abnormalities can result depending on the timing of exposure (Randall, 1987). However, even in the brain, there are key time frames during which critical regions (e.g., the
hippocampus, regions of the frontal lobe, or corpus callosum) are particularly susceptible and ethanol exposure at those times may have especially deleterious effects (Guerri et al., 2009; Mattson et al., 2001; Riley and McGee, 2005).

The extent of ethanol exposure is not the only important factor in FASD pathogenesis since ethanol does not appear to have an equal effect on all exposed fetuses (Abel, 1995; May and Gossage, 2011). Given relatively similar reported levels and patterns of drinking across pregnancies, children may be differentially affected, with some displaying significantly more severe outcomes compared to others even if they are born to the same mother (May and Gossage, 2011). Several individual risk factors and traits have been shown to contribute to the variable nature of ethanol-induced deficits and the different degrees of damage in the offspring of individual women (or individual pregnancies) despite similar reported alcohol consumption. These include maternal age, gravidity, parity, maternal body size, nutrition, metabolism, as well as a number of social factors (May and Gossage, 2011). With respect to age, gravidity and parity, women who are higher on any of these three variables have been shown to have children with more severely adverse outcomes (Jacobson et al., 1996, 1998; May et al., 2008). In women who drink 5 or more drinks per occasion at least weekly, the risk of adverse outcomes in their children is increased 2-5 times when the mother is 30 or older (Jacobson et al., 1998). The authors suggested that the greater vulnerability of the children of older women may be due to age-related increases in maternal body fat-to-water ratio and increased alcohol metabolism in chronically drinking women (Jacobson et al., 1996).

With regard to body size, smaller mothers appear to be over-represented in epidemiological FASD studies (May and Gossage, 2011). This observation may be due to the fact that some of these women may have FASD themselves (since alcohol abuse is common in the FASD population) or because they achieve higher BAC given the same alcohol intake and therefore expose their offspring to higher levels of alcohol in utero (May and Gossage, 2011). Nutrition is another important variable in FASD.
Studies have shown that mothers of FASD children have lower intake of certain nutrients, including riboflavin, calcium, some omega-3 fatty acids, zinc, iron, copper, and B vitamins, which may be related to poor diets or impaired ability to absorb and utilize nutrients in alcoholics (May and Gossage, 2011). This suggests that nutrient deficiencies may be a particular risk factor for FASD. In support of this, recent studies have shown that adequate nutritional status with respect to some of these nutrients can be protective against the deleterious affects of prenatal alcohol exposure (Rufer et al., 2012; Summers et al., 2009).

Socioeconomic status also appears to be an important risk factor for the development of severe disabilities as alcoholic women of lower SES are at a higher risk of having a child with FAS compared to alcoholic women of higher SES (Abel, 1995; Bingol et al., 1987). This is likely due to a cumulative effect and interaction of poor living conditions, inadequate nutrition, high levels of stress, and intergenerational maternal alcoholism (Bingol et al., 1987; May and Gossage, 2011). With respect to genetic and metabolic differences, studies have shown that mothers of FAS children were more likely than control women to have the normal ADH variant ADH1B*1 than the genetic variants ADH1B*2 and ADH1B*3 associated with negative metabolism-related consequences of drinking (Arfsten et al., 2004; Jacobson et al., 2006a; Viljoen et al., 2001; Warren and Li, 2005). A recent study found that four genetic variants in alcohol metabolizing genes in 4167 children were strongly related to lower IQ at age 8, an effect that was seen only in the offspring of mothers who drank during pregnancy (Lewis et al., 2012). Of note, since this study looked at moderate drinkers, the finding that subtle changes in exposure due variable metabolism may be important for cognitive outcomes supports the idea that even lower levels of drinking can be detrimental in some pregnancies.

Another potential risk factor for developing disabilities following heavy fetal ethanol exposure is maternal use of tobacco and illicit drugs. These may alter uterine blood flow and reduce fetal oxygenation thereby leading to hypoxia and oxidative stress,
as well as affect fetal supply of nutrients; all of which may enhance the teratogenic effects of ethanol (O’Leary, 2004). However, studies that examined the interactive effect of poly-drug use in pregnancy are too few to draw any concrete conclusions (O’Leary, 2004). Lastly, women who have already had a child with FAS are at an especially high risk of having another child if they continue to drink heavily, with the rate of FAS in the offspring of a woman who already has given birth to a child with FAS estimated at 771 per 1000 (Huebert and Raftis, 1996). This may be the result of the woman’s harmful pattern of alcohol use and/or risk factors that continue to play a role in fetal outcomes. It is likely that there is interaction of many maternal risk factors and exposure-related factors that contribute to the variability observed in ethanol-induced impairments and FASD.

1.3.4 Clinical diagnosis

Given the heterogeneous nature of ethanol-induced impairments, most of which are not specific to prenatal alcohol exposure and often manifest differently across the lifespan, the diagnosis of the conditions that fall under the FASD umbrella is extremely challenging. Several criteria for diagnosis and diagnostic referral have been developed in North America, including the Institute of Medicine’s (IOM) guidelines (Hoyme et al., 2005; Stratton et al., 1996), the “4-digit Diagnostic Code” (Astley and Clarren, 2000), the CDC’s guidelines for referral and diagnosis (Bertrand et al., 2005), and the Canadian guidelines for FASD-related diagnostic referral (Chudley et al., 2005). In the IOM guidelines (Table 1.3), five disorders are outlined; 1) FAS with confirmed maternal alcohol exposure 2) FAS without confirmed maternal exposure 3) partial FAS with confirmed maternal alcohol exposure 4) Alcohol-Related-Birth Defects (ARBD) and 5) Alcohol-Related Neurodevelopmental Disorder (ARND). The 4-Digit Diagnostic Code (Table 1.4), which includes comparable diagnostic categories, is thought to provide more accurate and reproducible diagnoses due to its use of quantitative, objective measurement scales (Likert scales) that assess four key diagnostic features of FAS: growth deficiency, FAS facial features, CNS damage/dysfunction, and prenatal alcohol exposure (Hoyme et al., 2005). The Canadian Guidelines for FASD diagnosis
incorporate the elements from the IOM guidelines and the “4-digit Diagnostic Code”, suggesting that the 4-Digit approach is used to assess and objectively measure FASD characteristics, while the IOM terminology and criteria are used to describe the diagnoses (Chudley et al., 2005).

The diagnostic criteria for FAS are fairly consistent among the guidelines and remain true to the initial observations of FAS characteristics made by Jones et al. (1973). Relatively minor differences exist between the guidelines (e.g., in details and required number of features), and all require the presence of growth retardation, characteristic facial features, and evidence of CNS/neurodevelopmental dysfunction, with or without confirmed ethanol exposure. It is noted that during assessments, appropriate corrections must be made for variables like race, sex, and gestational age to ensure accurate diagnosis (Riley and McGee, 2005). Some of the outlined characteristics associated with FAS can change with age, thus complicating the diagnosis of older children (Bertrand et al., 2005). Specifically, growth deficits and facial features may become less apparent as the individual matures, particularly after puberty, making it difficult to diagnose FAS in adolescents or adults (Bertrand et al., 2005).

Furthermore, although the presence of the above mentioned characteristics (growth retardation, characteristic facial features, and CNS anomalies/dysfunction) exclude most other syndromes, a history of heavy maternal alcohol use in pregnancy is important for confirmation of the diagnosis (O’Leary, 2004). However, unlike other disorders under the FASD umbrella, FAS can be diagnosed even if a reliable maternal drinking history is not available if the abnormalities are consistent with the syndrome and other conditions have been excluded. Essentially, FAS is a diagnosis of exclusion, particularly when a history of maternal alcohol use is not available (Riley and McGee, 2005).

Individuals who have some but not all characteristics of the full-blown syndrome may be diagnosed with pFAS, ARND, and ARBD. Generally, diagnosis of these less severe outcomes of prenatal ethanol exposure requires evidence of some alcohol-related abnormalities (or a combination of features) such as growth retardation,
craniofacial characteristics (in the case of pFAS), congenital anomalies (in the case of ARBD), and CNS neurodevelopmental abnormalities and a pattern of cognitive or behavioral abnormalities that is inconsistent with developmental level and familial/environmental factors (ARND). In all these cases, other potential syndromes and factors must be excluded and a confirmation of maternal alcohol consumption is necessary to make a diagnosis. At present, clinicians are more likely to diagnose children with FAS (only about 10% of cases) or pFAS that display the pathognomonic craniofacial features than the less severe cases of FASD such as ARND (Hoyme et al., 2005; Stratton et al., 1996). The confirmation of maternal alcohol exposure required to make these diagnoses is a particular challenge (as will be discussed in detail below), limiting the ability of physicians to diagnose the majority of FASD cases (Chudley et al., 2005; O’Leary, 2004).

Table 1.3 IOM Guidelines for FASD Diagnosis (Stratton et al., 1996).

<table>
<thead>
<tr>
<th>FAS with confirmed maternal alcohol exposure</th>
<th>FAS without confirmed maternal alcohol exposure</th>
<th>pFAS with confirmed maternal alcohol exposure</th>
<th>ARND</th>
<th>ARBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth retardation.</td>
<td>CNS neurodevelopmental abnormalities.</td>
<td>AND:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNS neurodevelopmental abnormalities.</td>
<td></td>
<td>CNS neurodevelopmental abnormalities.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OR</td>
<td>OR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OR</td>
<td>Behavioral/ cognitive abnormalities that are inconsistent with developmental level and cannot be explained by other factors.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Confirmed maternal alcohol exposure.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Congenital anomalies, including malformations and dysplasias.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.4 4-Digit Code Criteria for FASD (Astely and Clarren, 1999)

<table>
<thead>
<tr>
<th>RANK</th>
<th>Growth Deficiency</th>
<th>FAS facial phenotype</th>
<th>CNS damage/dysfunction</th>
<th>Prenatal alcohol exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Significant</td>
<td>Severe</td>
<td>Definite</td>
<td>High risk</td>
</tr>
<tr>
<td></td>
<td>(height AND weight &lt;3rd percentile)</td>
<td>Short palpebral fissures (≥2 SD below mean)</td>
<td>Structural/neurological evidence</td>
<td>Confirmed heavy exposure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thin upper lip</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Indistinct philtrum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Probable</td>
<td>Some risk</td>
</tr>
<tr>
<td></td>
<td>(height AND weight &lt;10th percentile)</td>
<td>2-3 of the above features</td>
<td>Significant dysfunction in 3+ domains</td>
<td>Confirmed exposure (level may be unknown)</td>
</tr>
<tr>
<td>2</td>
<td>Mild</td>
<td>Mild</td>
<td>Possible</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>(height OR weight &lt;10th percentile)</td>
<td>1 of the above features</td>
<td>Evidence of some dysfunction</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>Absent</td>
<td>Unlikely</td>
<td>No risk</td>
</tr>
<tr>
<td></td>
<td>(height AND weight ≥10th percentile)</td>
<td>Absent</td>
<td>No evidence of impairment</td>
<td>Confirmed abstinence during gestation</td>
</tr>
</tbody>
</table>

1.3.5 Incidence/prevalence and economic burden

At present, it is not known for certain how many people are affected by FASD as epidemiologic research in this area is severely limited by the difficulty in identifying and diagnosing affected individuals. The rates of both FAS and FASD are believed to be underestimated because of lack of physical features in the majority of cases, underreporting of maternal alcohol consumption in pregnancy (which is vital for diagnosis in the absence of physical features), and the lack of medical expertise in diagnosing affected individuals (Clarren and Lutke, 2008).

The current estimates of incidence of full-blown FAS and FASD vary between studies depending on the methodology and population assessed. Studies have reported the rates of FAS to be in the range of 1-7 cases per 1,000 live births in various
populations of the United States (Abel, 1995; Abel and Sokol, 1987; Clarren et al., 2001; May and Gossage, 2001; May et al., 2009; Sampson et al., 1997; Stratton et al., 1996). However, substantially higher rates of FAS are reported among some high-risk populations. For example, rates of FAS reached almost 1% in some Native American populations (Plains and Plateau culture tribes) (May and Gossage, 2001). In some Canadian First Nations communities, the rates of FAS and pFAS range from 2.5-10% (May et al., 2009; Square, 1997). Rates of FAS exceeded 5% in one South African study (May et al., 2000), while the combined rate of FAS and pFAS in more recent studies of Cape provinces of South Africa have been 88 to 89 per 1,000 children (8.8-8.9%) (May et al., 2007; Urban et al., 2008). This estimate is from a population with a generally low SES, a long history of wine production, and a high prevalence of binge drinking.

The estimated prevalence of FASD is even less exact but considerably higher than that of the full-blown syndrome, as FAS is the least common form of FASD. The less severe alcohol-related conditions such as partial FAS, ARND, and ARBD are believed to occur about three times as often as FAS (Sampson et al., 1997). Conservative estimates of FASD rates have reported 9-10 per 1000 live births (~1%) in North America (May and Gossage, 2001; Sampson et al., 1997). A recent estimate of the current prevalence of FASD in populations of younger school children suggest it may be as high as 2-5% in the US and some Western European countries (May et al., 2009). These estimations indicate the severity of FASD as a public health concern and indicate that FASD affects far more neonates than other neonatally screened disorders such as congenital hypothyroidism or phenylketonuria (Klett, 1997; Williams et al., 2008). Prenatal alcohol exposure is recognized as the leading, non-genetic cause of mental retardation and developmental disabilities in the Western World (Abel and Sokol, 1987), accounting for significant educational and public health expenditures (Stade et al., 2009; Stratton et al., 1996).
Although the literature on the economic burden of FASD is scarce, the limited number of Canadian and US studies indicate that the cost burden of FASD is profound creating a significant economic impact on the individual, family, and society (Popova et al., 2011). Estimates of lifetime costs of FAS alone suggest these were around $2 million in 2002 for each affected individual, 1.6 million (80%) of which were associated with health care, special education and residential care, and $0.4 million (20%) with productivity losses (Lupton et al., 2004). The median adjusted annual cost of FAS alone to the US economy was estimated at $3.6 billion, with the costs associated with the entire fetal alcohol spectrum surely being much higher (Lupton et al., 2004). In another study, the estimated annual costs of FAS in US were closer to $4.15 billion in 1998, which rose to $5.4 billion by 2003 (Harwood, 2000). In Canada, the adjusted annual cost of one individual with FASD (FAS, pFAS or ARND) for 2007 was estimated at $21,642, while at the population level, the estimated adjusted annual cost was $5.3 billion for persons 0–53 years of age (Stade et al., 2009). A large proportion of these costs are associated with the debilitating ‘secondary’ disabilities of FASD that lead to productivity losses and burden health care, education, social services and criminal justice systems. The health care burden alone of just the full blown FAS in Canada in 2008–2009 was about $6.7 million in direct costs of acute care, psychiatric care, day surgery, and emergency department services (Popova et al., 2011). If extrapolated to include less severe forms and other countries, the global economic burden is enormous, exemplifying the need to establish FASD management and prevention strategies globally.

1.3.6 Management and treatment

Although the primary alcohol-induced damage is thought to be, for the most part, permanent, studies have shown that factors such as early diagnosis (specifically before the age of 6), living in a stable and nurturing home, never having experienced violence, having been found eligible for developmental disability services, and having basic needs met are protective in that they are associated with a decreased risk of developing
secondary disabilities such as disrupted school experience, unemployment, institutionalization, and trouble with the law (Streissguth, 1996; Streissguth et al., 2004). Early diagnosis is associated with improved life outcomes in FASD-affected individuals likely because it permits early intervention and access to specialized support and resources, all of which may help affected individuals lead more productive lives and lessen the impact that FASD has on the individual and society. Of interest, Streissguth et al. (2004) noted that individuals who did not meet the criteria for a FAS diagnosis or those with an IQ >70 were at higher risk for delinquency, alcohol and drug problems, and school problems. This may be due to the fact that these individuals were less likely to be identified and qualify for services. Because the younger the age at which an affected child is identified, the lower the frequency of secondary disabilities (Streissguth, 1996), early diagnosis is of paramount importance for managing FASD in our society and in reducing the tremendous economic burden associated with it.

In a systematic review of animal studies that examined interventions for the detrimental effects of prenatal alcohol exposure, Sussman and Koren (2006) reported that in rodents, postnatal environmental enrichment, postnatal handling, exercise, and therapeutic motor training may attenuate and ameliorate ethanol-induced abnormalities and deficits such as learning, motor function, and planning (Sussman and Koren, 2006). For example, one study showed that motor and spatial impairments in rat pups exposed to ethanol can be ameliorated by providing them with various objects to manipulate and play with and housing them with other pups (Hannigan and Berman, 2000). Another study showed that providing ethanol-exposed rat pups with opportunities for voluntary exercise improved spatial memory (performance on Morris water maze test) (Thomas et al., 2008). In another series of studies, it was shown that postnatal choline supplementation attenuated the severity of ethanol-induced learning deficits and behavioural changes like hyperactivity in rats prenatally exposed to ethanol (Thomas et al., 2000, 2004, & 2007). These findings are encouraging and require further research in humans to determine if such interventions could improve neurological performance and reduce the severity of fetal alcohol effects.
In humans, a number of interventions have been shown to improve outcomes in FASD-affected individuals, as well as to provide the necessary supports to aid the development of necessary skills that will help the child become an independent adult. In terms of pharmacotherapy, Snyder et al., (1997) found that stimulants (methylphenidate, pemoline, or dexedrine) improved hyperactivity but not attention compared to placebo in a group of 12 children (6-16 years of age) with FAS and ADHD. In a retrospective study where data were extracted from the medical records of 27 children with FASD who had been referred to an ADHD medication service, stimulants were found to improve hyperactivity/impulsivity and opposition/defiance but were not as effective at improving inattention (Doig et al., 2008). The authors speculated that these findings suggest that inattention may be less responsive to ADHD medication.

In a recent systematic review of school-based interventions, Peadon et al. (2009) evaluated and discussed interventions designed to improve the developmental outcomes of individuals with FASD, reduce secondary disabilities, and improve the lives of families of individuals affected by FASD. Since a diagnosis of FASD in and of itself does not provide enough information on the appropriate treatment, a careful assessment of each individual is essential (Peadon et al., 2009). Different interventions must be specifically designed to target deficits in areas like memory, information processing, academic skills, social skills, attention, motor skills, and executive functioning, as well as address various behavioural and mental health issues. It is important to note that the majority of the individuals with FASD do not have low IQ and thus may not qualify for many support services. Since they may, nevertheless, have impaired mental functioning (McGee et al., 2008), individual in-depth assessments are needed to identify the specific deficits and providing these individuals with critical services that would otherwise be unavailable to them (Peadon et al., 2009).

Numerous human studies have been carried out in recent years on the effectiveness of interventions focusing on math skills, behavioural regulation, peer relations and social communication, executive function, compliance, learning readiness, and challenging behavior of clinical concern in individuals affected by FASD (Bertrand,
Many of these were randomized controlled trials. Interventions and treatment typically focus on adapting the FASD-affected children to their environment by addressing their neurodevelopmental issues. The common technique used in these interventions is teaching by explicit instruction rather than relying on observational learning (Bertrand, 2009; Paley and O'Connor, 2009; Peadon et al., 2009). Interventions to support emotional and behavioural regulation focus on modifying the environment and providing structure and consistency through daily routines and rules. For example, minimizing visual and auditory distractions in classrooms and use of clearly organized material can aid learning in children with FASD (Paley and O’Connor, 2009). Researchers have also suggested that to be effective, interventions must not only aim to address the range of deficits exhibited by individuals with FASD, but also focus on educating, training and providing resources and services to their caregivers (Paley and O’Connor, 2009). Additionally, biological parents of these children may be dealing with ongoing substance abuse problems and stigmatization, which must be addressed since it could have a very detrimental effect on the child and intervention efforts.

Overall, there is increasing evidence in the literature that although incurable, FASD can be managed, and individuals with prenatal alcohol exposure can lead productive lives if they can be identified and referred to intervention and support programs. Unfortunately, as discussed previously, conditions that fall under the FASD umbrella are vastly under-diagnosed and many individuals affected by in utero alcohol exposure never receive specialized support and interventions that have been shown to be beneficial. For this reason, screening tools that may identify individuals at risk for FASD such that they can be referred for further assessments and diagnostic clinic need to be developed.
### Table 1.5 Intervention studies (adapted from Peadon et al., 2009; Bertrand 2009; Paley and O'Connor, 2009).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Intervention</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young US women with FASD (n=19, 14-36 yo).</td>
<td>Community intervention model of targeted education and collaboration with service providers (~12 month pilot)</td>
<td>Decreased alcohol and drug use, increased use of contraceptives and health care services, stable housing.</td>
<td>(Grant et al., 2004)</td>
</tr>
<tr>
<td>US children with FAS, pFAS or ARND and social skills deficit (n=100, 6-12 yo).</td>
<td>Parent assisted child friendship training (CFT) vs. delayed treatment + parent info sessions on FASD and social skills (12 90-min sessions over 12 weeks)</td>
<td>CFT group showed significant improvement in social skills knowledge, social skills and problem behavior (parent-reported).</td>
<td>(O'Connor et al., 2006b)</td>
</tr>
<tr>
<td>US children with FAS or other alcohol-related disorders (n=58, 3-7 yo).</td>
<td>Parent-child interaction therapy vs. parenting support and management (14 weekly 90-min sessions)</td>
<td>Both groups showed decreased parental stress and child behavior problems.</td>
<td>Gurwitch et al., 2009 (in Bertrand, 2009)</td>
</tr>
<tr>
<td>US children with FASD (n=52, 5-11 yo).</td>
<td>Families moving forward (FMF) model of supportive behavioural consultation for families with children affected by FASD vs. community standard of care (16 in-home sessions every other week over 9-11 months).</td>
<td>FMF group showed significant improvement in sense of parenting efficacy and child behavior problems.</td>
<td>Olson et al., 2009 (in Bertrand, 2009)</td>
</tr>
<tr>
<td>Canadian children with ARND or static encephalopathy (n=33, 4-11 yo).</td>
<td>Rehearsal training to improve working memory.</td>
<td>Intervention group had significantly improved memory for numbers (working memory span) compared to control at follow up (6-21 days after intervention).</td>
<td>(Loomes et al., 2008)</td>
</tr>
<tr>
<td><strong>Subjects</strong></td>
<td><strong>Intervention</strong></td>
<td><strong>Results</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>US children with FAS or FASD (n=54-61, 3-10 yo).</td>
<td>Mathematics intervention vs. standard psycho-education (6 weeks)</td>
<td>Intervention group had significant improvement in mathematical knowledge immediately after completion and in math skill 6 months later (as well as in behavior).</td>
<td>(Coles et al., 2009; Kable et al., 2007)</td>
</tr>
<tr>
<td>US children with confirmed prenatal alcohol exposure (n=78, 6-11 yo).</td>
<td>Neurocognitive habilitation vs. community standard (12 weekly 75-min neurocognitive habilitation group therapy)</td>
<td>Significant improvement in executive functioning skills and emotional problem solving.</td>
<td>(Wells et al., 2012)</td>
</tr>
<tr>
<td>South African children with FAS and pFAS (n=65, 9-10 yo).</td>
<td>Language and literacy intervention (1 hr/week for 9 months).</td>
<td>Intervention group had significantly greater improvement compared to control group in early literacy and specific categories of language (syllable manipulation, letter sound knowledge, written letters, word and non-word reading and spelling).</td>
<td>(Adnams et al., 2007)</td>
</tr>
<tr>
<td>US children with and without prenatal alcohol exposure (n=85, 6-12 yo).</td>
<td>Children’s friendship training (CFT) vs. standard of care intervention for social skill of children seeking treatment in a community mental health center (12 90-min sessions over 12 weeks).</td>
<td>Children in CFT group with or without prenatal alcohol exposure showed significantly improved knowledge of appropriate social skills, improved self-concept, and parent-reported social skills.</td>
<td>(O’Connor et al., 2012)</td>
</tr>
</tbody>
</table>

### 1.4 Screening for Prenatal Alcohol Exposure

As discussed in the previous sections, early recognition and diagnosis of individuals affected by prenatal alcohol exposure is critical to reducing the risk of deleterious outcomes and secondary disabilities (Streissguth et al., 2004). Consequently, it would also significantly lower the substantial economic burden of FASD to society. However, an important obstacle to early recognition and diagnosis is
the need to establish a history of maternal alcohol consumption in pregnancy since a reliable maternal drinking history is essential to establishing an accurate FASD diagnosis in the vast majority of FASD cases where the characteristic facial features are absent (Chudley et al., 2005). Since accurate prenatal exposure history is often unavailable at the time of diagnosis (the majority of children with FASD are in foster care or adopted) or simply inaccurate due to maternal underreporting, implementing screening strategies for identifying prenatal alcohol exposure and thus the individuals at risk for alcohol-related disabilities may be of paramount importance in addressing this issue. Such screening could identify those at risk and direct potentially alcohol-affected children into diagnostic follow-up programs. Furthermore, screening for prenatal alcohol exposure may present an opportunity for primary prevention by identifying women with alcohol abuse problems and offering treatment and help, thereby preventing future alcohol-exposed pregnancies (Riley and McGee, 2005).

An additional benefit of screening for prenatal alcohol exposure is that it would facilitate the gathering of more accurate estimates of incidence and prevalence of prenatal alcohol exposure and FASD and of the associated economic costs, and would thus aid efforts to allocate sufficient health care, educational, and social support to affected individuals (Stratton et al., 1996). Furthermore, by providing an accurate maternal drinking history, screening can help researchers link specific risk factors to particular adverse outcomes within the continuum of adverse alcohol effects. Thus, overall, obtaining an accurate exposure history by screening for prenatal alcohol exposure has the potential to facilitate FASD research, diagnosis, intervention, and prevention. An additional consideration is that screening may have a significant impact beyond FASD in addressing issues of poverty, child protection, mental health, as well as substance abuse and physical/sexual abuse (Garey, 2006; Leonardson and Loudenburg, 2003).

It should be stressed that screening is not the same as diagnosis. Identifying an individual with prenatal alcohol exposure is identifying those who are at risk for alcohol-related disabilities (Chudley et al., 2005). To be of benefit, screening for prenatal alcohol
exposure should be complemented by a long-term follow up program involving periodical neurobehavioural assessments and referrals to diagnostic clinics and intervention programs. Appropriate follow-up should ideally involve a multi-disciplinary team with appropriate FASD-training and include physicians, psychologists, occupational therapists, speech-language pathologists, and additional mental health specialists (Chudley et al., 2005). Existing screening methods for prenatal alcohol exposure include maternal questionnaires, indirect maternal markers of chronic alcohol consumption, and direct measurements of ethanol and ethanol metabolites in maternal and fetal matrices. These are discussed in more detail in the sections below.

1.4.1 Self-report and questionnaires

The traditional method of screening for gestational alcohol use in standard practice is maternal self-report. Simply asking pregnant women about their alcohol consumption often results in denial or severely underestimated values due to widespread stigma associated with alcohol consumption in pregnancy, guilt, shame, denial of the problem by the women and those close to them, and fears of child apprehension and reprisal (Russell et al., 1996). Additionally, because most high-risk substance-addicted women do not engage in regular prenatal care with a physician, the lack of familiarity and trust may further deter women from being truthful at the time of delivery. In a recent study, Hannigan and colleagues (2010) found that retrospective reports 14 years postpartum identified 10.8 times more women as at risk than antenatal reports for the same women (Hannigan et al., 2010). Furthermore, the rates of prenatal alcohol exposure are several fold higher when assessed using ethanol biomarkers (described below) than when assessed by self-report on antenatal forms (Gareri et al., 2008).

A number of brief screening questionnaires have been devised to identify individuals at risk for problem drinking (Chang, 2001) (Table 1.6). These include the CAGE (Mayfield et al., 1974), T-ACE (Sokol et al., 1989), MAST, AUDIT and TWEAK (Russell et al., 1996). These questionnaires typically ask a series of questions, and the
answers are awarded points that add to the overall score. For example, TWEAK is an acronym for 5 questions: Tolerance ("How many drinks can you hold/need to feel a high?"), Worried ("Have close friends or relatives worried or complained about your drinking in the past year?"), Eye-opener ("Do you sometimes take a drink in the morning when you first get up?"), Amnesia ("Has a friend or family member ever told you about things you said or did while you were drinking that you could not remember?"), and K/cut down ("Do you sometimes feel the need to cut down on your drinking?") (Chang, 2001). These questionnaires can be administered and scored in less than 5 minutes and many have been validated in women and pregnant women (Bradley et al., 1998; Chang et al., 1998; McNamara et al., 2005; Sokol et al., 1989). The TWEAK and T-ACE, specifically, have been found to be highly sensitive and more reliable than clinician interview for detecting alcohol use in pregnancy (Chang, 2001; Chang et al., 1998), and are generally recommended in Canada for screening women, whereas the CAGE is considered less suitable (Koren et al., 2003). However, since screening questionnaires by nature are a form of self-report, underreporting is still common (Bradley et al., 1998; Russell et al., 1996). Wurst et al. (2008) found that 8.7% of women in antenatal clinics reported drinking on the AUDIT questionnaire, yet 25.2% tested positive for alcohol metabolites (FAEE and EtG, discussed below) in urine and hair samples indicating recent consumption (Wurst et al., 2008). This indicates that the present questionnaires require improvement and that more effective questionnaire designs should be developed and administered in appropriate settings.
### Table 1.6 Screening questionnaires for identifying risk drinking.

<table>
<thead>
<tr>
<th><strong>TWEAK</strong>(^1) (<em>Russel et al., 1996</em>)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tolerance:</strong> How many drinks can you hold? OR how many drinks does it take to feel a high?</td>
<td></td>
</tr>
<tr>
<td><strong>Worry:</strong> Do close friends/family ever worry or complain about your drinking?</td>
<td></td>
</tr>
<tr>
<td><strong>Eye opener:</strong> Do you sometimes have a drink first thing in the morning to steady your nerves or get rid of a hangover?</td>
<td></td>
</tr>
<tr>
<td><strong>Amnesia:</strong> Have you ever awakened in the morning after drinking the night before and found that you could not remember a part of the evening before?</td>
<td></td>
</tr>
<tr>
<td><strong>(K)cut down:</strong> Do you sometimes feel the need to (K)cut down on your drinking?</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>CAGE</strong>(^2) (<em>Mayfield et al., 1974</em>)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cut down:</strong> Have you ever felt you should cut down on your drinking?</td>
<td></td>
</tr>
<tr>
<td><strong>Annoyed:</strong> Have people annoyed you by criticizing your drinking?</td>
<td></td>
</tr>
<tr>
<td><strong>Guilty:</strong> Have you ever felt bad or guilty about your drinking?</td>
<td></td>
</tr>
<tr>
<td><strong>Eye opener:</strong> Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover?</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>T-ACE</strong>(^3) (<em>Sokol et al., 1989</em>)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tolerance:</strong> How many drinks can you hold? OR how many drinks does it take to feel a high?</td>
<td></td>
</tr>
<tr>
<td><strong>Annoyed:</strong> Have people annoyed you by complaining about your drinking?</td>
<td></td>
</tr>
<tr>
<td><strong>Cut down:</strong> Have you ever felt you should cut down on your drinking?</td>
<td></td>
</tr>
<tr>
<td><strong>Eye opener:</strong> Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover?</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) 2 points for YES to tolerance and worry questions; 1 point each for the rest.

\(^2\) 1 point for each YES.

\(^3\) 2 points for YES to tolerance question; 1 point each for the rest.

#### 1.4.2 Indirect maternal markers

Traditional maternal blood markers used to identify women who consume alcohol during pregnancy are generally indirect physiological indicators of alcohol exposure. These include carbohydrate-deficient transferring (CDT), gamma-glutamyltransferase (GGT), aspartate and alanine aminotransferases (AST and ALT, respectively), and mean corpuscular volume (MCV) (*Littner and Bearer, 2007*). In essence, these are
biomarkers of effect (indicators of altered structure/ function) since they detect the toxic effects of alcohol on body organ-systems. For example, CDT is an alteration of target protein, whereas GGT, AST, ALT, MCV are early indicators of organ damage (Bearer, 2001). Of these, CDT (a modified form of iron-transporting protein transferrin that increases in concentration after ethanol intake) generally has a higher sensitivity and specificity than the others and is a recommended clinical diagnostic test for heavy ethanol consumption (Hannuksela et al., 2007; Niemelä, 2007). However, levels tend to be higher in women than men regardless of drinking history, and it has been shown to be less sensitive in women, having a median sensitivity of 51% across 16 studies in women (Allen et al., 2000). Studies have also shown that pregnancy may also elevate CDT levels, further limiting its suitability as a marker of drinking in pregnancy (Littner and Bearer, 2007). Furthermore, CDT has a relatively short half-life and the levels usually return to normal within 4 weeks of abstinence (Szabo et al., 2011).

The markers GGT and MCV are established indicators of early organ damage. Elevated blood levels of GGT (a liver enzyme involved in glutathione metabolism) indicate long-term chronic alcohol exposure and it possesses similar sensitivities and specificities in women as CDT (Allen et al., 2000). The main limitation of GGT is the fact that it is a non-specific marker of hepatic injury, can be elevated by certain drugs (e.g. phenytoin, nonsteroidal anti-inflammatory drugs), and may be elevated in several conditions common in pregnancy such as obesity, hypertension, and type-II diabetes (Littner and Bearer, 2007; Whitfield, 2001). MCV is a measure of red blood cell size, which increases from heavy alcohol intake. In women, MCV has been found to be superior to CDT and GGT in detecting heavy alcohol consumption (Mundle et al., 2000), however, it can be elevated in the presence of haematological disease (macrocytic anemia), certain vitamin deficiencies (B12 and folic acid) (Neumann and Spies, 2003), and is normally elevated in the second and third trimester of pregnancy (Szabo et al., 2011). Some studies have shown that using a combination of maternal blood markers may be superior to the use of any single marker alone. For example, the combination of GGT and CDT has a higher diagnostic sensitivity, specificity, and a stronger correlation.
with the actual amounts of alcohol consumption (Hannuksela et al., 2007; Hietala et al., 2006; Niemelä, 2007).

It should be noted that the level of alcohol consumption that will produce elevated maternal blood markers is quite high. For example, elevated CDT levels are seen after consumption of 50-80 g of ethanol per day (4-6 standard drinks) for 2-3 weeks; consumption of 60 g of ethanol per day (5 standard drinks) is required for GGT to reach a 30% sensitivity in women (Sharpe, 2001); and daily intake of 60 g of ethanol for a month is required for MCV to reach 40% sensitivity in women (Szabo et al., 2011). Thus, a significant proportion of individuals would not be identified by these traditional markers due to their lack of sensitivity in detecting lower but potentially teratogenic levels of alcohol consumption (Neumann and Spies, 2003). Overall, while the traditional biomarkers are useful in specific clinical contexts related to alcoholism, the use of any single or multiple maternal blood markers of ethanol use may be ineffective in identifying many drinking mothers (Bearer, 2001; Stoler et al., 1998). Ideally, a biomarker for detecting prenatal ethanol exposure should be sensitive and specific enough to detect various degrees of fetal ethanol exposure and not be affected by other conditions that may be observed in pregnancy.

1.4.3 Direct markers of ethanol exposure

Direct alcohol biomarkers are generally biomarkers of exposure and include ethanol itself and its oxidative and non-oxidative metabolites. Because ethanol distributes throughout total body water, it can be measured in essentially any body fluid, most commonly blood, urine, and breath (Swift, 2003). Ethanol in blood, urine, or breath, is a marker of very recent alcohol intake (Das et al., 2008). In the context of prenatal alcohol exposure, maternal or neonatal blood and urine, as well as cord blood, can be analyzed for ethanol. However, due to ethanol’s rapid elimination, positive urine or blood alcohol screens in the neonate can only occur if the mother has used ethanol shortly prior to the onset of labour (Bearer, 2001). This rapid elimination severely limits the utility of blood and urine ethanol concentrations for screening.
The oxidative metabolite of ethanol, acetaldehyde, is not a useful marker of alcohol consumption since it is rapidly metabolized to acetic acid (Swift, 2003). However, since it is extremely reactive, it can bind to macromolecules (tissue proteins) to produce acetaldehyde adducts that can persist for days after alcohol has been eliminated (Allen et al., 2003; Swift, 2003). In essence, these acetaldehyde-protein adducts are biomarkers of early biological effects and have been used experimentally as a marker of past alcohol consumption (Swift, 2003). Acetaldehyde adducts include those with hemoglobin, albumin, CYP450 2E1, red blood cell membrane proteins, and a few other enzymes (Allen et al., 2003). Haemoglobin-acetaldehyde adducts have been used most commonly and shown to have a higher sensitivity in determining heavy drinking as compared to GGT, ALT, AST, and MCV (Hazelett et al., 1998). However, a single dose of alcohol was found to significantly increase acetaldehyde-hemoglobin adducts in controls, suggesting that the test cannot discern chronic versus acute alcohol exposure (Hazelett et al., 1998). An important consideration for the use of acetaldehyde adducts is the variability among individuals in acetaldehyde synthesis and metabolism (and thus adduct formation) as a result of genetic polymorphisms. For example, individuals with the ALDH2*1/*2 polymorphism accumulate more acetaldehyde and have higher levels of acetaldehyde adducts (Takeshita and Morimoto, 2000). Due to this variability and the difficulty of measuring acetaldehyde adducts, they have limited potential as a biomarkers of prenatal alcohol exposure. With regard to acetate (acetic acid), the major final product of oxidative ethanol metabolism, the relatively short persistence of increased blood acetate following alcohol consumption and the lack of correlation with ethanol concentrations limits its utility as a marker (Swift, 2003). It should be noted that expression/activity of CYP2E1, which also participates in oxidative alcohol metabolism, may potentially serve as biomarker for alcohol use because it is induced by alcohol (Bearer, 2001). However, little work has been conducted on validating these in humans and no studies of pregnant women or newborns have been reported.
Non-oxidative metabolism, which constitutes a minor pathway of overall ethanol metabolism, results in the enzymatic production of conjugation products of ethanol and endogenous substrates such as fatty acids, phosphatidylcholine, sulfate, or glucuronic acid. The derivatives of non-oxidative ethanol metabolism include FAEEs, PEth, EtS, and EtG, respectively. These metabolites have been measured in various matrices and investigated as biomarkers of ethanol exposure since they persist in the body longer than ethanol (Szabo et al., 2011). Ethyl glucuronide, one of the better studied markers, is present in urine for up to several days after ethanol consumption and has a high sensitivity for the identification of recent alcohol consumption (Wurst et al., 1999, 2000, 2005). It can also be detected in the blood, hair, and meconium, and studies evaluating the validity of EtG for the identification of alcohol abuse and/or social drinking among pregnant women are underway (Szabo et al., 2011; Wurst et al., 2005). One limitation of EtG is the potential for false positive results due to sample contamination with alcohol-containing products (e.g. hand sanitizers, mouthwashes, some lotions) (Kissack et al., 2008). Furthermore, false positive and false negative results can also be obtained in urine due to synthesis and hydrolysis of EtG by bacteria in patients with urinary tract infections or due to postcollection sample contamination (Szabo et al., 2011; Helander et al., 2007).

Ethyl sulfate is another ethanol metabolite that can be measured in urine. While not as sensitive to bacterial hydrolysis as EtG, it is present in urine for a shorter period of time after alcohol ingestion (~30 hours) (Helander and Dahl, 2005; Kissack et al., 2008). The utility of using EtS in pregnant women is largely unknown but likely limited by the relatively short time period of detection. With regard to PEth, studies have demonstrated strong correlation of blood PEth with ethanol intake and high sensitivity in detecting heavy ethanol exposure (higher than for CDT and GGT) in actively drinking men and women (Aradottir et al., 2006). However, as with EtG and EtS, the utility of PEth as marker of prenatal ethanol exposure requires further validation. Since pregnancy is a unique physical condition that can significantly affect the disposition of ethanol in the body through changes in total blood volume, renal function, hepatic
metabolism, enzyme expression, and volume of distribution, the use of some of these markers for ascertaining fetal ethanol exposure and gestational ethanol use/abuse require independent validation as these can affect their sensitivity and specificity.

FAEEs (the conjugation products of ethanol and fatty acids) are the best-studied non-oxidative metabolites to date and have been investigated in various tissues, species, and populations (including pregnant women and newborns). Different FAEE species are synthesized in the body depending on the fatty acid that is esterified to ethanol. Owing to their lipophilic nature, FAEEs can accumulate in various tissues such as the heart, adipose, liver, pancreas, and brain allowing them to serve as markers of past and chronic alcohol exposure (Laposata, 1998; Laposata and Lange, 1986). Studies that analyzed FAEEs in post-mortem tissues consistently found higher levels of FAEEs in samples from alcoholics compared to non-drinkers, and validated tissue FAEEs concentrations (liver and adipose) as biomarkers of pre-mortem ethanol intake (Bjorntorp et al., 1990; Depergola et al., 1991; Refaai et al., 2002; Salem et al., 2001). Although using tissue FAEE levels for ascertaining prenatal alcohol exposure is obviously not a viable option as it requires sampling of internal organs, studies have shown FAEEs to be present in blood following ethanol ingestion with their levels correlating with BACs (Doyle et al., 1994; Laposata et al., 1995). Measuring FAEEs in blood as markers of acute and chronic ethanol intake in humans has been investigated by several groups (Best et al., 2003; Bisaga et al., 2005; Soderberg et al., 1999). These studies have shown that blood FAEE levels are higher in chronic alcohol abusers as compared to acute drinkers (Kaphalia et al., 2004), and that they remain elevated for a prolonged period of time (up to 99 hours), likely due to a slow reflux of FAEEs from storage compartments (e.g. liver, pancreas, adipose) (Borucki et al., 2004, 2005, 2007). Since this is still a rather limited detection period, maternal serum FAEEs are unlikely to be useful as a biomarker of prenatal ethanol exposure and have not been studied in pregnant women.
One matrix in which FAEEs can be indicative of past and chronic alcohol intake is hair since these esters are deposited in sebum and are incorporated into the hair shaft (Pragst et al., 2001). A number of studies investigated FAEE levels in hair of individuals with known histories of ethanol exposure or in ethanol-exposed animals to assess their utility as biomarkers of chronic ethanol exposure (Auwarter et al., 2001; Caprara et al., 2005; Kulaga et al., 2006, 2009; Pragst et al., 2000, 2001; Wurst et al., 2004). Human studies found that FAEEs were present at greater concentrations in hair of individuals with a history of heavy alcohol consumption (fatalities and alcoholics in treatment) as compared to social drinkers and teetotalers. Hair FAEE analysis demonstrated high sensitivity and specificity in non-pregnant alcoholic patients in a detoxification program and among heavy drinkers (Pragst and Yegles, 2008; Wurst et al., 2004). With regard to the utility of this test in detecting prenatal alcohol exposure, it has been reported that maternal hair FAEE concentrations can be used to identify heavy drinking during pregnancy and that mild infrequent maternal drinking does not elevate FAEEs in neonatal scalp hair (Caprara et al., 2005; Kulaga et al., 2007). Hair analysis for FAEEs does have certain limitations, however. For example, collection of hair, particularly from the neonate, can be problematic as many infants are not born with a sufficient quantity of hair. Furthermore, women may be hesitant to consent for aesthetic or cultural reasons, and differences in hair growth and hair care can affect results (Pragst et al., 2001).

With respect to ascertaining prenatal alcohol exposure, the matrix that has received the most attention and is deemed to hold the most promise in neonatal screening is meconium. As a cumulative matrix into which FAEEs deposit, it can potentially provide a record of in utero alcohol exposure over the last two trimesters of pregnancy. The validation, advantages, and limitations of meconium FAEE analysis for the determination of prenatal ethanol exposure are discussed in the following section.
1.5 Meconium FAEEs as a biomarker

1.5.1 Meconium as an analytical matrix

FAEEs have been detected and can be measured in meconium - a matrix unique to the fetus. It is a complex matrix that begins to form in the fetal GI tract with the commencement of fetal swallowing around 12-15 weeks of gestation, accumulates during pregnancy, and is normally discharged shortly after birth in the neonate’s first few bowel movements (Kwong and Ryan, 1997). Meconium consists of substances ingested by the fetus during gestation, such as amniotic fluid, intestinal secretions, water, epithelial cells, mucus, carbohydrates, proteins, lipids, and other cellular debris (Righetti et al., 2003). This cumulative matrix can serve as a record of fetal exposure to xenobiotics throughout pregnancy since substances that reach fetal circulation can deposit directly in meconium by being excreted in fetal bile and/or through fetal swallowing of amniotic fluid containing xenobiotics excreted in fetal urine (Ostrea Jr., 1999). The recurrent cycle of fetal elimination into amniotic fluid and the swallowing of amniotic fluid increase the chances of drug detection in this matrix (Ostrea Jr., 1999). Theoretically, xenobiotics begin to deposit in meconium when it begins to form and, as such, meconium could be used to detect exposures beginning at 12-15 weeks of gestation and up until delivery and discharge of meconium by the neonate. Indeed, in utero exposure to cocaine in a fetus as young as 17 weeks of gestation was detected through meconium analysis (Ostrea Jr. et al., 1994). Meconium testing has been used for detection of in utero exposure to a variety of illicit and therapeutic drugs, cotinine (as a measure of tobacco exposure), and, of course, FAEEs as a measure of alcohol exposure (Bearer et al., 1992; Mac et al., 1994; Ostrea Jr. et al., 2006).

Meconium has several important advantages as compared to conventional analytical matrices like urine and blood (Table 1.7). First and foremost, being a cumulative matrix, it is representative of a long period of fetal exposure, thereby providing a wide window of detection (spanning the second half of pregnancy) (Ostrea Jr. et al., 2006). Secondly, since meconium is discarded material that can be taken
directly from the diaper, its collection is simple, quick, and non-invasive, requiring no contact with the neonate. Meconium is typically passed within 48 hours of birth in the vast majority of healthy neonates and can thus be collected by hospital staff caring for the neonate in the postpartum period. However, if meconium is missed during this time, so is the opportunity for screening. Another limitation is the fact that it is a complex matrix typically requiring more extensive sample processing prior to analysis. Lastly, it is still not clear how different doses, timing, and patterns of fetal drug and alcohol exposure translate to meconium concentrations of the analytes of interest, and how long various compounds persist in meconium.

**Table 1.7 Matrices for measuring direct biomarkers of prenatal alcohol exposure** (adapted from Bearer, 2001).

<table>
<thead>
<tr>
<th><strong>Matrix</strong></th>
<th><strong>Time frame of detection</strong></th>
<th><strong>Advantages</strong></th>
<th><strong>Limitations</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal or neonatal blood</td>
<td>Recent alcohol exposure (few hours-days depending on marker and level of drinking).</td>
<td>A number of direct and indirect ethanol markers can be measured.</td>
<td>Invasive.</td>
</tr>
<tr>
<td>Cord blood</td>
<td>Recent alcohol exposure (few hours-days depending on marker and level of drinking).</td>
<td>Non-invasive, large sample quantity.</td>
<td>Narrow window of opportunity for collection.</td>
</tr>
<tr>
<td>Maternal or neonatal urine</td>
<td>Recent alcohol exposure (few hours-days depending on marker and level of drinking).</td>
<td>Large sample quantity.</td>
<td>Difficult to collect neonatal, cooperation required for maternal.</td>
</tr>
<tr>
<td>Maternal hair</td>
<td>Long-term, chronic alcohol exposure.</td>
<td>Sectioning of hair possible, long window for collection.</td>
<td>May not be available or acceptable to mother. Differences in hair growth/care may affect results.</td>
</tr>
<tr>
<td>Neonatal hair</td>
<td>Third trimester but depends on hair growth.</td>
<td>Long window for collection</td>
<td>May not be available or acceptable to parents</td>
</tr>
</tbody>
</table>
1.5.2 Fatty acid ethyl esters in fetal tissues and meconium

FAEEs in meconium have been investigated as biomarkers of fetal ethanol exposure by several groups (Bearer et al., 1999, 2003, 2005; Brien et al., 2006; Chan et al., 2004a; Klein et al., 1999; Littner et al., 2008; Ostrea Jr. et al., 2006). Rodent placentae, maternal tissue, and fetuses have been shown to accumulate significant levels of FAEEs one hour after maternal ethanol administration, and they persist for at least 14 days in fetal rat organs (Bearer et al., 1992; Hungund and Gokhale, 1994). Since placental perfusion studies have shown that FAEEs do not cross the placenta, FAEEs found in fetal tissues including meconium are a product of fetal ethanol metabolism rather than of maternal origin (Chan et al., 2004b). Although FAEE synthesis has not been completely characterized in the fetus, FAEES activity has been demonstrated in human term placenta and mouse placenta (at gestational day 14), mouse embryo, primary cultures of rat fetal brain cells, fetal and postnatal rat brain, and in human fetal brain (2nd trimester), demonstrating that the enzyme activity necessary for FAEE synthesis is present in fetuses and fetal brain tissue relatively early in gestation (Bearer et al., 1992, 1995). As a side note, some suggest that FAEE may play a role in mediating organ damage in alcoholics (Best and Laposata, 2003; Werner et al., 1997) as well as in ethanol teratogenesis (Bearer et al., 1992). They have been shown to uncouple oxidative phosphorylation, decrease the rate of cell replication, decrease protein synthesis, increase lysosomal fragility, and accumulate in lipid droplets (Best and Laposata, 2003).

1.5.3 Development of meconium FAEE as a biomarker of prenatal alcohol exposure

The presence of FAEEs in meconium was first documented by Mac et al. (1994) and subsequently by Klein et al. (1999) and Bearer et al. (1999). These early studies found elevated meconium FAEE levels in infants born to heavily drinking women. Specifically, Klein et al. reported much higher concentrations of FAEEs (13,126 ng/g) in the meconium of a neonate born to a mother who reported drinking throughout
pregnancy as compared to meconium from three controls (Klein et al., 1999). They reported that palmitic, linoleic, and stearic ethyl esters were found in the alcohol-exposed infant's meconium but were absent from that of unexposed. Bearer and colleagues investigated the presence of ethyl linoleate in meconium of infants born to US women (n=248) who self-reported varying amounts of alcohol use during pregnancy and found that this FAEE was associated with higher self-reported weekly alcohol use in the month prior to pregnancy, in the first trimester, and overall (Bearer et al., 1999). Presence of ethyl linoleate had 72% sensitivity and 51% specificity for distinguishing women who consumed at least one drink per week in the third trimester from those who had abstained in that cohort. In a subsequent study by that group in a different population (South African), ethyl oleate was found to be a superior marker for ethanol exposure and correlated most strongly with self-reported maternal drinking in pregnancy as compared to other FAEE species (Bearer et al., 2003). The optimal positive cutoff value of 32 ng/g of ethyl oleate had a sensitivity of 84.2% and specificity of 83.3% for identifying women reporting drinking ≥1.5 oz. absolute alcohol per drinking day.

In another study by the same authors where meconium was collected from 30 unexposed infants from Jordan and 248 infants from Cleveland with varying exposure status, 6 of the 7 measured FAEEs were significantly different between the non-abstainers and abstainers (Bearer et al., 2005). FAEEs best predicted drinks per drinking day, and ethyl linoleate had the highest sensitivity and specificity for detecting maternal drinking (88% and 64%, respectively for positive cutoff of 32 ng/g) (Bearer et al., 2005). A study by another group also found that the incidence of ethyl linoleate in meconium was significantly higher in alcohol-exposed groups than the control groups (US cohort, n = 124) and that only alcohol-exposed infants were found in the group with the highest ethyl linoleate concentrations (Ostrea Jr. et al., 2006). Although the sensitivity of ethyl linoleate (cutoff of 0.25 µg/g) in detecting prenatal alcohol exposure was only 26.9%, its specificity and positive predictive value were 96.8 and 96.2%, respectively (Ostrea Jr. et al., 2006). There was also some evidence that the
occurrence and levels of ethyl arachidonate were significantly higher in the alcohol-exposed groups than the control groups.

The results from these studies sparked some debate on whether meconium should be tested for all commonly occurring FAEE species or one particular species. The observations that ethyl linoleate was the most sensitive and specific marker in some populations whereas ethyl oleate in others suggested that the most prevalent FAEE species in meconium may differ between population and ethnic groups as a result of genetic variations in FAEE-synthetic enzymes or the amount of specific fatty acids in different diets. Thus, the use of the cumulative sum of several FAEE species in meconium, as employed by other groups (Chan et al., 2003, 2004a; Moore et al., 2003; Noland et al., 2003) may be more reliable as it provides some redundancy for variability in fatty acid profiles between populations and individuals. The most common FAEE species measured in human matrices (including meconium) are presented in Table 1.8.

Table 1.8 FAEE species commonly measured/detectable in humans.

<table>
<thead>
<tr>
<th>FAEE Species</th>
<th>Configuration</th>
<th>Mw (g/mol)</th>
<th>Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Palmitate</td>
<td>E16:0</td>
<td>284.5</td>
<td>C\textsubscript{18}H\textsubscript{36}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>E18:1</td>
<td>310.5</td>
<td>C\textsubscript{20}H\textsubscript{38}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Linoleate</td>
<td>E18:2</td>
<td>308.5</td>
<td>C\textsubscript{20}H\textsubscript{36}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>E18:0</td>
<td>312.5</td>
<td>C\textsubscript{20}H\textsubscript{40}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Linolenate</td>
<td>E18:3</td>
<td>306.5</td>
<td>C\textsubscript{20}H\textsubscript{34}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Arachidonate</td>
<td>E20:4</td>
<td>332.5</td>
<td>C\textsubscript{22}H\textsubscript{36}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Myristate</td>
<td>E14:0</td>
<td>256.4</td>
<td>C\textsubscript{16}H\textsubscript{32}O\textsubscript{2}</td>
</tr>
<tr>
<td>Ethyl Laurate</td>
<td>E12:0</td>
<td>228.4</td>
<td>C\textsubscript{14}H\textsubscript{28}O\textsubscript{2}</td>
</tr>
</tbody>
</table>

From the above studies it became apparent that low concentrations of FAEEs are found in meconium of infants without prenatal alcohol exposure, which is likely due to endogenous ethanol production in the gut and/or due to small quantities of alcohol that may also be present in certain medications and food additives (Chan et al., 2003). Therefore, studies were needed to standardize FAEE levels to account for their endogenous presence, and to validate a positive screening cutoff to distinguish between
ethanol-exposed and non-exposed neonates. To address this issue, a meconium FAEE baseline study was conducted where FAEE concentrations were measured in infants born to 183 non-drinking women (Chan et al., 2003). The meconium FAEE levels in the abstaining population were compared against FAEE concentrations in meconium of infants born to 6 heavy drinkers (i.e. chronic drinkers; ≥2 drinks/day; binge drinkers, ≥ 5 drinks/occasion) and 17 social drinkers (1 drink on 1 occasion to 1 drink/month). They found an average 5-fold difference between the mean total FAEE concentrations between the heavy drinking groups as compared to abstainers and social drinkers, and that the social drinking group was indistinguishable from the confirmed non-drinking group. They established a positive cutoff of 2.00 nmol total FAEE per gram meconium with a sensitivity of 100% and specificity of 98.4% using the sum of four individual FAEEs (ethyl palmitate, stearate, oleate, and linoleate) for detecting heavy drinking. Of interest, in another study by the group in a clinic based sample, all of the positive cases that were classified as positive using only ethyl oleate (32 g/ng as positive cutoff) as suggested by Bearer et al., (2003) were identified using the 2 nmol/g cumulative FAEE cutoff, while 5 samples were detected as positive only by the cumulative screening cutoff (Chan et al., 2004c).

In recent years, studies have also been conducted on the association between meconium FAEE levels and ethanol-related adverse effects in the offspring. One animal study in fetal guinea pigs has demonstrated a negative correlation between meconium FAEE levels and fetal body and brain weight in fetuses chronically exposed to ethanol (Brien et al., 2006). Several human studies have also reported on the association of elevated meconium FAEE and adverse outcomes, including lower APGAR scores (Derauf et al., 2003), growth restriction (Derauf et al., 2003; Noland et al., 2003), lower scores on executive functioning task at age 4 (Noland et al., 2003), decreased psychomotor performance in children at age 2 (Peterson et al., 2008), a diagnosis of FAS or pFAS at age 5 (Jacobson et al., 2006b), and correlation between ethyl oleate with recognition memory, processing speed, and complexity of symbolic play (Jacobson
et al., 2006b). These studies have been described and summarized in a review article (Koren et al., 2008).

An important advantage of using FAEEs in meconium as markers of fetal alcohol exposure is that they are products of fetal ethanol metabolism since placental perfusion studies have demonstrated that FAEE do not cross the human placenta (Chan et al., 2004b). This means that meconium FAEE concentrations are a direct marker of ethanol that has been transferred to and metabolized by the fetus and are therefore indicative of fetal ethanol load (Chan et al., 2004b). Several unknowns and limitations do exist. For instance, exposure occurring in the first trimester of pregnancy will not be captured since meconium does not begin to form in fetal intestines until the second trimester. The half-life of FAEEs in meconium is also unknown so it is not clear whether heavy drinking will be captured if the mother had largely abstained towards the end of pregnancy. Additionally, it is unclear how timing, dose and pattern of drinking affect the FAEE profile in meconium or what role maternal diet, life style, and genetics play in determining concentrations and types of FAEE in meconium. Although dose–response relationships between FAEEs in meconium and maternal self-reports have been reported in a few studies (Bearer et al., 2003; Ostrea Jr. et al., 2006), it is clear that the concentration of FAEEs in meconium does not definitively translate into the quantity of ethanol consumed by the mother. This could be due to vast variability in patterns of drinking, in individual metabolism of ethanol, genetic influences, and possible differences in fatty acid profiles. However, the currently established positive cutoff utilizing the sum of several FAEE species is expected to adequately rule out neonates born to women who did not drink heavily in pregnancy (Chan et al., 2003).
2 Thesis Scope

2.1 Statement of the Problem and Overall Aim

It is evident that biological markers of prenatal alcohol exposure may be of key clinical value since, by providing an accurate history of fetal alcohol exposure, they can facilitate early recognition and diagnosis of alcohol-affected individuals. Although the primary alcohol-induced damage is thought to be for the most part permanent, early diagnosis is beneficial and associated with a decreased risk of secondary disabilities such as disrupted school experience, unemployment, institutionalization, and trouble with the law; likely because it permits early intervention and specialized support (Streissguth et al., 2004). Thus, screening programs for prenatal alcohol exposure using objective methods like biological markers may be an important strategy for the management of the immense global concern that is FASD. As demonstrated in both human and animal studies, meconium analysis for FAEE holds much promise as a potential screening tool for the identification of alcohol-exposed newborns. Although currently limited to research, meconium testing for FAEE could potentially be implemented as a universal screen or targeted to high-risk populations to aid in the identification of newborns at risk for FASD (Goh et al., 2008).

Before meconium FAEE testing can be used clinically for the identification of newborns at risk for FASD, several important knowledge gaps need to be addressed. First, although meconium FAEEs have been shown to detect heavy prenatal alcohol exposure, it is unknown whether the levels of these esters in meconium are predictive of ethanol-induced fetal pathology. Secondly, potential confounding factors need to be investigated that can influence the accuracy of the test results, specifically, whether contamination of meconium with postnatal stool that commonly occurs with delayed sample collection can affect the results. Thirdly, although meconium FAEE testing has great potential in clinical practice, to date, the application of such testing has been limited to anonymous studies aimed at estimating the prevalence of prenatal ethanol exposure in select populations. Thus, it is important to explore the potential utility and
logistics of meconium testing in clinical practice, and to explore the types of screening program that would be acceptable and beneficial. Lastly, the enzymology of FAEE synthesis remains murky at best and requires greater understanding if these esters are to be used as biomarkers since this knowledge can be important for accurate interpretation of FAEE concentrations in analytical matrices. Thus, the overall aim of this thesis is to further investigate, validate, and assess the clinical utility of meconium FAEE analysis as a screening tool for the identification of infants at-risk for FASD by addressing the knowledge gaps outlined above. We hypothesized that this research would reveal some important benefits as well as potential limitations of meconium testing for FAEE, and that this new knowledge would bring meconium FAEE testing closer to the clinical arena.

2.2 Objectives & Hypotheses

This thesis is divided into four separate studies, which were guided by the four primary objectives and hypotheses outlined below. These studies are presented in detail in their respective chapters.

PRIMARY OBJECTIVES:

I. To assess the utility of meconium FAEE concentration in identifying individuals at risk for alcohol-related impairments by determining the relationship between ethanol-induced organ-system injury and FAEE concentrations in meconium of fetal sheep (Chapter 2).

In humans, FAEE in neonatal meconium constitute sensitive and specific biomarker of heavy maternal drinking in the latter half of pregnancy (Bearer et al., 2003; Koren et al., 2008). However, the ability of meconium FAEE concentrations to identify individuals with subtle manifestations of organ or system injury is not known. This study aimed to measure meconium FAEE concentrations in fetal sheep following daily, relatively moderate-dose ethanol exposure in late gestation, and to assess their relationship with
resultant organ-system injury. The results are meant to determine whether meconium FAEE concentrations can potentially be used to identify neonates at risk for dysfunction of major organs following in utero exposure to ethanol that does not result in overt physical signs of ethanol teratogenicity.

II. To determine how delayed meconium collection leading to contamination with postnatal stool can affect meconium analysis for FAEEs (Chapter 3).

Before meconium FAEE testing can be used as a screening tool for the identification of newborns prenatally exposed to ethanol, confounders that can influence the accuracy of the results need to be investigated since false positive test results could have serious legal, social, and clinical implications for the child and family in question; while false negative results would fail to identify an at-risk child. One potential confounder is sample contamination with postnatally produced stool, which can occur as a result of delayed sample collection. Meconium is passed in the newborn’s first few bowel movements typically within 48 hours of birth (Kwong and Ryan, 1997), during which time it is collected for analysis. As neonates typically begin feeding within a few hours of birth, a potential problem that arises if samples are collected later in the postpartum period, particularly after the first meconium has been passed, is risk of intra-intestinal contamination with ingested dietary components, developing gut flora, various products of digestion, and other constituents of postnatal stool, and this may have analytical ramifications. This study investigated whether delayed sample collection (leading to collection of samples potentially contaminated with postnatal stool) can result in false positive test results for FAEEs. The results shed light on an important limitation of meconium analysis for FAEEs and have important implications with regard to proper sample collection protocols—information that is imperative for ensuring that the results of FAEE testing are representative of in utero ethanol exposure.
III. To assess the potential clinical utility of voluntary meconium screening for prenatal alcohol exposure, in which meconium analysis is coupled to long-term follow-up and interventions in a high-risk obstetric unit (Chapter 4).

Meconium FAEE testing can potentially be used as a neonatal screen by health-care professionals to identify neonates at-risk for FASD, thereby permitting diagnostic follow-up of these children and early intervention in those who develop disabilities. Such screening would not only provide accurate exposure history required for diagnosis, but if implemented along with a comprehensive follow-up program and interventions, could facilitate early recognition and treatment of FASD (Gifford et al., 2010; Goh et al., 2008; Hopkins et al., 2008). As an added value, it may identify and allow for intervention in problem-drinking mothers, which, in turn, may prevent future alcohol-exposed pregnancies (Koren et al., 2008). However, since informed consent from a competent patient or appointed guardian prior to treatment or testing is an ethical and legal component of medical practice (Etchells et al., 1996; Flagler et al., 1997), a screening program of this nature could require consent of the child’s legal guardian (typically the parent), which may diminish the benefits of such testing in practice. This study assessed whether women would willingly partake in a screening program of this nature by launching a pilot screening program for prenatal alcohol exposure in a high-risk obstetric population previously shown to have a high prevalence of FAEE-positive meconium via anonymous meconium testing. The results provide insight into the type of screening program that would be required if FAEE testing is to be implemented in clinical practice for population-based screening.

IV. To examine the capacity for FAEE synthesis and the enzymology of this non-oxidative metabolic pathway of ethanol in mammalian organs and tissues (Chapter 5).

The capacity for non-oxidative ethanol metabolism to FAEEs varies greatly among tissues and organs, which is likely related to differential expression of enzymes with
FAEE synthetic activity and presence or absence of competing metabolic pathways (e.g., oxidative), among other factors. It is important to gain a greater insight into the enzymology behind FAEE-synthesis and the capacity of different tissues to synthesize these esters for several reasons. In relation to the aim of this thesis, the concentration of FAEEs in a particular tissue or matrix will depend on the capacity of that tissue to synthesize, retain, and degrade these esters. As such, understanding the origins of FAEEs in a tissue and the enzymology behind FAEE synthesis may aid with the interpretation of tissue levels of FAEEs and help with selection of appropriate matrices for analysis.

**PRIMARY HYPOTHESES AND RATIONALE:**

I. **Higher concentrations of FAEEs will be present in meconium of ethanol-exposed fetuses; and meconium FAEE concentrations would be predictive of ethanol-induced organ-system injury in the fetus.**

Rationale: As direct ethanol metabolites, it is expected that meconium FAEE concentrations will be elevated in fetal sheep exposed to relatively moderate-dose ethanol in late gestation as compared to non-exposed controls. If meconium FAEE concentrations are confirmed to have high sensitivity and specificity for identifying the ethanol exposure regimen used in this study, a positive cutoff could be established that will reliably identify ethanol-exposed fetal sheep, and meconium FAEE concentrations should be predictive of fetal ethanol-induced organ damage.

II. **Collection of samples excreted later in the postpartum period can lead to false positive test results for FAEEs, which could be because of contamination with dietary components of postnatally produced stool and ethanol-producing microorganisms.**

Rationale: Aside from analytical ramifications like decreased sensitivity and introduction of contaminants interfering with analysis, intra intestinal contamination of meconium with elements of postnatal stool, specifically ingested dietary components and acquired
microorganisms, may produce misleading test results. Although the gut is sterile at birth, gut colonization begins at delivery and the numbers of microorganisms rapidly increase over the first few days. Since ethanol is a byproduct of normal microbial metabolism, its local production from ingested milk or formula in the gastrointestinal tract by the developing gut flora and subsequent conjugation to fatty acids may elevate FAEE levels in stool and contaminated meconium, resulting in a positive test result that is not due to in utero alcohol exposure.

III. Many women who consumed alcohol in pregnancy would refuse to participate in an open meconium screening program for prenatal alcohol exposure, not wishing to be identified despite the potential value to child health.

Rationale: Despite the potential benefits of neonatal screening for prenatal alcohol exposure, requirement of consent of the child’s legal guardian (typically the parent) may diminish the value of meconium screening in a clinical setting. This may be due to embarrassment, guilt, and fears of stigma associated with drinking in pregnancy, as well as due to fears of child apprehension and/or investigation by children’s aid societies. All these factors are likely to deter women who consumed alcohol from consenting to testing.

IV. FAEE-synthetic activity in various mammalian tissues is expressed by enzymes with other primary physiological functions, and the contribution of any particular enzyme to FAEE synthesis is tissue-specific.

Rationale: Given that the conjugation reaction is a common mechanism by which exogenous and endogenous agents are detoxified, it seems unlikely that ethanol conjugation to fatty acids is mediated by a specific enzyme. Since some studies have implicated well-known enzymes, it is likely that FAEE-synthetic activity in mammalian tissues is mediated by enzymes with wide substrate specificity, likely those that participate in lipid metabolism. Furthermore, since many such enzymes exist, different
enzymes may mediate FAEE-synthesis in different tissues depending on tissue-specific expression of these and competing enzymes.

3 References


Bertrand J, Floyd LR, and Weber M. (2005). Guidelines for Identifying and Referring Persons with Fetal Alcohol Syndrome (Centers for Disease Control and Prevention (CDC)). MMWR 54(No. RR-11)


Pragst F, Auwaerter V, Sporkert F, and Spiegel K. (2001). Analysis of fatty acid ethyl esters in hair as possible markers of chronically elevated alcohol consumption by headspace solid-phase


Streissguth AP. (1996). Understanding the occurrence of secondary disabilities in clients with Fetal Alcohol Syndrome (FAS) and Fetal Alcohol Effects (FAE) (Seattle, WA: University of Washington School of Medicine).


Substance Abuse and Mental Health Services Administration (2011). Results from the 2010 Summary of National Findings (Rockville, MD: Substance Abuse and Mental Health Services Administration). Available at: http://www.samhsa.gov/data/NSDUH/2k10NSDUH/2k10Results.pdf


Chapter 2  Meconium Fatty Acid Ethyl Esters as Biomarkers of Late Gestational Ethanol Exposure and Indicator of Ethanol-Induced Multi-Organ Injury in Fetal Sheep

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[IZ performed all FAEE analyses, data analysis, and prepared the manuscript for submission].
1 Abstract

**Background:** Meconium fatty acid ethyl esters (FAEE) constitute a biomarker of heavy fetal ethanol exposure. Our objective was to measure meconium FAEE in fetal sheep following daily, relatively moderate-dose ethanol exposure in late gestation, and to evaluate their utility in identifying fetal organ-system injury. **Methods:** Pregnant ewes received ethanol (0.75 g/kg; \( n = 14 \)) or saline (\( n = 8 \)) via 1-h IV infusion daily during the third trimester equivalent, while additional pregnant sheep served as untreated controls (\( n = 6 \)). The daily ethanol regimen produced similar maximal maternal and fetal plasma ethanol concentrations of 0.11–0.12 g/dL. Ewes and fetuses were euthanized shortly before term, and meconium was collected and analyzed for FAEE (ethyl palmitate, stearate, linoleate, and oleate). **Results:** Meconium total FAEE concentration was significantly higher in ethanol-exposed fetuses compared with controls, and a positive cutoff of 0.0285 nmol total FAEE/g meconium had 93.3% sensitivity and specificity for detecting fetal ethanol exposure. When the studied animals (ethanol-exposed and controls) were classified according to meconium FAEE concentration, FAEE-positive and FAEE-negative groups frequently differed with respect to previously examined pathological endpoints, including nephron endowment, lung collagen deposition, cardiomyocyte maturation, and tropoelastin gene expression in cerebral vessels. Furthermore, in all studied animals as a group (ethanol-exposed and controls combined), meconium FAEE concentration was correlated with many of these pathological endpoints in fetal organs. **Conclusions:** We conclude that, in fetal sheep, meconium FAEE could serve as a biomarker of daily ethanol exposure in late gestation and could identify fetuses with subtle ethanol-induced toxic effects in various organs. This study illustrates the potential for using meconium FAEE to identify neonates at risk for dysfunction of major organs following in utero ethanol exposure that does not result in overt physical signs of ethanol teratogenicity.
2 Introduction

Alcohol (ethanol) is a well-established human teratogen that can result in a range of physical defects, and cognitive and behavioral deficits, known collectively as fetal alcohol spectrum disorders (FASD) (Riley et al., 2011). The effects of prenatal alcohol exposure can range in severity from mild to debilitating, with the Fetal Alcohol Syndrome (the most severe end of the spectrum) having the principal features of growth restriction, characteristic craniofacial dysmorphology, and central nervous system dysfunction, including intellectual, neurological and behavioural deficits (Jones and Smith, 1973), (Burd, 2004; Jones and Smith, 1975). Dosage regimen, gestational timing of alcohol exposure, as well as maternal and fetal characteristics, contribute to the multiplicity of the FASD phenotype that presents in postnatal life. Prenatal alcohol exposure is considered a leading known cause of developmental disability, with FASD affecting as many as 1–5% of children in North America and Western European countries (Abel and Sokol, 1987; May et al., 2009).

A major challenge in diagnosing milder forms of FASD is the absence of clear external markers of ethanol teratogenicity such as the craniofacial characteristics, which are present only in the most severe cases. In the absence of such overt dysmorphology, it is necessary to confirm prenatal alcohol exposure to make a diagnosis (Chudley et al., 2005). Because of the poor sensitivity and reliability of maternal self-reporting of alcohol consumption (Russell et al., 1996), this is often problematic and can lead to misdiagnosis and/or late recognition of FASD. Since early diagnosis and intervention are associated with improved outcomes and decreased secondary disabilities in individuals with a FASD (Streissguth et al., 2004), the development of a reliable biomarker of fetal ethanol exposure is therefore of great clinical importance as it can enable the early recognition of at-risk individuals, timely diagnosis, and implementation of interventions.

Fatty acid ethyl esters (FAEE) are produced by non-oxidative ethanol biotransformation involving the conjugation of ethanol to endogenous free fatty acids or
fatty-acyl-CoA (Best and Laposata, 2003). Unlike ethanol and its proximate metabolite, acetaldehyde, FAEE persist for a prolonged period of time in the body and accumulate in various tissues and fluids. One matrix in which FAEE accumulate and can be measured is fetal meconium, which begins to form in the fetal intestine with the emergence of fetal swallowing of amniotic fluid around 15 weeks of gestation (Arvedson, 2006). In humans, FAEE in neonatal meconium have been validated as sensitive and specific biomarkers of heavy maternal drinking in the latter half of pregnancy (Bearer et al., 2003, 2005; Koren et al., 2008), and a recent Canadian FASD steering committee has concluded that analysis of FAEE in meconium may be a useful screening tool for the identification of newborns at risk for FASD (Goh et al., 2008). However, the sensitivity and specificity of meconium FAEE for detecting lower levels of fetal ethanol exposure, and their ability to identify individuals with subtle manifestations of organ or system injury are not clear.

Recently, a comprehensive study was conducted on the effects of repeated maternal administration of relatively moderate-dose ethanol in pregnant sheep during the third-trimester-equivalent on several fetal organs. In this study, pregnant ewes received one-hour daily ethanol infusion during the third-trimester-equivalent that produced maximal maternal and fetal plasma ethanol concentrations (PEC) of 0.11–0.12 g/dL, which approximated blood ethanol concentration measured in non-pregnant women drinking socially (equivalent to a 55–70 kg woman consuming 3–4 standard drinks over one hour) (Moore et al., 2007). This maternal ethanol exposure regimen did not result in overt fetal dysmorphology, but ethanol-induced changes were found in several fetal organs examined, including the heart (Goh et al., 2011), kidney (Gray et al., 2008), lung (Sozo et al., 2009), brain and placenta (Kenna et al., 2011). As meconium was collected from these fetuses, our objective was to determine whether meconium FAEE concentration is a biomarker of repeated fetal exposure to this ethanol regimen in the third-trimester-equivalent of ovine pregnancy. Furthermore, we also assessed the relationship between meconium FAEE concentration (biomarker of exposure) and fetal organ injury (effect) in the studied animals. Our results suggest that
meconium FAEE concentrations could serve as a biomarker of daily ethanol exposure in late gestation, and could be used to identify fetuses with subtle ethanol-induced multi-organ pathology, thereby supporting the potential utility of meconium testing as a screening tool for the identification of newborns at risk for FASD.

3 Materials and Methods

3.1 Ethics Statement

All animal experiments were approved by the Animal Ethics Committee of Monash University where the animal experimentation was performed (permit number: PHYS/2005/31) in accordance with the National Health and Medical Research Council of Australia guidelines. All surgery was performed under anesthesia as described below, and all efforts were made to minimize suffering.

3.2 Research Plan

Experimental animal preparation, ethanol dosing, and study design have been described previously in detail (Goh et al., 2011; Gray et al., 2008; Kenna et al., 2011; Sozo et al., 2009). The treatment groups are presented in Table 2.1. Briefly, at 90–91 days gestational age (DGA; full term ~147 DGA), 22 pregnant Merino X Border-Leicester ewes were anesthetized with intravenous thiopental sodium (1 g) and maintained by inhalation of 1–2% halothane in O₂-N₂O (50:50 vol/vol). Catheters were aseptically inserted into a maternal carotid artery for blood sampling and into a jugular vein for ethanol or saline infusion. Six additional sheep served as untouched controls. From 95–124 DGA, 14 randomly chosen surgically-instrumented pregnant ewes received daily, 1-h intravenous infusion of 0.75 g ethanol/kg maternal body weight of 40% (v/v) ethanol-saline solution, and 8 pregnant ewes received 1-h intravenous infusion of an equivalent volume of saline. Pregnant ewes had free access to food and water, and animals in the saline cohort were provided with about 120 g (~250 calories) of additional food to compensate for calories derived from ethanol metabolism. At 126 DGA, pregnant ewes (n = 8 ethanol and n = 8 saline group) were anesthetized, and
catheters were inserted aseptically into the fetal brachial artery for fetal blood collection. During fetal surgery, twins were observed in one ethanol and one control animal, and only one of the twins was catheterized. After recovery from surgery, daily maternal ethanol infusion resumed on 128 DGA and continued until 133 DGA. Paired fetal and maternal arterial blood samples were collected from 131–134 DGA and it was determined that this ethanol regimen produced maximal maternal and fetal PEC of 0.12 ± 0.01 g/dL and 0.11 ± 0.01 g/dL (mean +/- SEM), respectively, at the end of the 1-h ethanol infusion (Goh et al., 2011; Gray et al., 2008; Kenna et al., 2011; Sozo et al., 2009).

### Table 2.1 Animal treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Maternal Surgery</th>
<th>Maternal Treatment</th>
<th>Fetal Surgery</th>
<th>Ewe (n)</th>
<th>Fetuses (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>Ethanol</td>
<td>Yes</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>Saline</td>
<td>Yes</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>Ethanol</td>
<td>No</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>No</td>
<td>None</td>
<td>No</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

a Surgical implantation of catheters into a maternal carotid artery for blood sampling and into a jugular vein for ethanol or saline infusion at 90-91 days gestational age (DGA).

b Daily 1-h infusion of ethanol (0.75 g/kg) or saline from 95-133 DGA.

c Surgical implantation of catheter into fetal brachial artery for the collection of fetal blood at 126 DGA.

### 3.3 Postmortem Examination and Meconium Collection

Pregnant ewes and their fetuses were euthanized with an intravenous dose of pentobarbital sodium (130 mg/kg maternal body weight) administered to the ewe at 134 DGA. Each fetus was immediately removed from the uterus and weighed, and then major fetal organs (kidneys, lungs, heart and brain) were collected and weighed. Several (2–3) placental cotyledons were also collected. Fetal large intestine was removed, cut into proximal (colonic) and distal (rectal) segments, and meconium was collected by squeezing the intestine to push out the contents from one end. The samples were then frozen at −80°C, until shipping (on dry ice) to the Hospital for Sick
Children, Toronto, Canada for analysis. The subsequent investigations of ethanol-induced pathology in the kidneys (Gray et al., 2008), lungs (Sozo et al., 2009), heart (Goh et al., 2011), and placenta and brain (Kenna et al., 2011) were conducted in surgically-instrumented animals that received ethanol and their saline controls (groups 1 and 2 in Table 2.1), and the findings have been previously reported in the referenced publications.

The vascular findings in these fetuses are pending publication. In brief, cerebral vessels from the 1st branch of the middle cerebral artery were isolated and frozen at −60°C. Cerebral vascular tissue was homogenized and RNA extracted using a RNeasy mini kit (Qiagen, Australia). The mRNA was converted to cDNA, and collagen I α1 and tropoelastin mRNA levels quantified using real-time PCR (Realplex Real-Time Multiplexing System, Eppendorf, Germany). Samples were measured in triplicate and a negative control sample which contained no mRNA was included in each PCR run. Fetal mRNA expression levels were normalized against the housekeeping gene 18S rRNA for each sample and analysed using the CT (cycle threshold) method. Values were expressed relative to the mean mRNA expression level in control animals for each gene. This protocol was previously used and reported by our group (Sozo et al., 2009). Primer sequences, cDNA concentrations, and annealing temperatures that are specific to the vascular tissue are summarized in the supplement (Table S2.1).

3.4 Meconium Analysis for FAEE

Four FAEE (ethyl palmitate, linoleate, oleate, and stearate) were extracted from the individual meconium samples (two per fetus; one colonic and one rectal) and quantified by headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME GC-MS) using an established methodology (Hutson et al., 2011). Briefly, FAEE were isolated from 0.5 g meconium via liquid-liquid extraction with heptane:acetone (5:2, vol/vol), and the extract was concentrated to dryness with a stream of N₂ gas. The extracted FAEE were quantified using HS-SPME GC-MS with their respective deuterated ethyl esters used as internal standards (prepared as
described previously) (Hutson et al., 2011). A gas chromatograph with a mass selective detector GC-MS QP-2010 PLUS equipped with an AOC-5000 autosampler and GCMSsolutions Software (Shimadzu, Columbia, MD) was used for analysis. A 65-µm polydimethylsiloxane/divinylbenzene fiber (Supelco, Bellefonte, PA) and a FactorFour capillary column (Varian, Palo Alto, CA) were used as per established protocol. The calibration curve of each FAEE was linear, and the coefficients of determination ($r^2$) ranged from 0.98 to 1.00. The limit of detection (LOD) and limit of quantification (LOQ) of this method for each ester in sheep meconium were determined by construction of a calibration curve in the low concentration range (2.5–40 ng FAEE). The LOD was calculated as three times the ratio between the SD of the linear regression line and the slope of the calibration curve (LOD = 3·SD/slope) and the LOQ was determined as ten times the same ratio (LOQ = 10·SD/slope). The LOD for the four quantified FAEE ranged from 3.682 to 4.941 ng FAEE/g meconium, and the LOQ was in the range of 12.274 to 16.470 ng FAEE/g meconium. The intra-day coefficient of variation (CV) ranged from 0.5–10.4%, and the inter-day CV ranged from 2–11.3%. The FAEE concentration data were expressed as nanomoles FAEE per gram meconium.

3.5 Data Analysis

All FAEE concentrations that fell below the LOQ could not be reliably quantified, and were assigned a value of zero in all quantitative and statistical analyses. Data analyses were performed with GraphPad Prism 5.0 (GraphPad Prism; San Diego, CA), where more information on the statistical tests used can be found. Since there was no evidence that meconium FAEE concentrations differed between colonic and rectal meconium samples as assessed by the Wilcoxon signed rank test for matched pairs (data shown in Figure S2.1 in supplement), the average meconium FAEE concentrations (determined for each fetus by averaging the rectal and colonic FAEE concentrations) were used in all subsequent analyses. Meconium FAEE concentrations were compared between treatment groups using the Kruskal-Wallis test with Dunn’s multiple comparison test, and between all ethanol-exposed (groups 1 and 3 in Table 2.1 combined) and non-exposed fetuses (groups 2 and 4 in Table 2.1 combined) with the
Mann-Whitney U-test. Concentrations of different FAEE species in meconium of ethanol-exposed fetuses were compared using Friedman test with Dunn’s multiple comparison test. A receiver operating characteristic (ROC) curve was constructed, and the area under the ROC curve (AUC) for each FAEE was calculated to assess the ability of meconium FAEE concentration to differentiate between ethanol-exposed and non-exposed fetuses and to identify an optimal positive cutoff value. Sensitivity, specificity, and positive and negative FAEE predictive values were determined for various cutoff values.

The utility of meconium FAEE in identifying fetuses with ethanol-induced pathology was evaluated in surgically-instrumented fetuses exposed to ethanol or saline (groups 1 and 2 in Table 2.1) by determining the relationship between meconium total FAEE concentration and previously determined specific physiological and/or anatomical endpoints in the organs of these animals. This relationship was assessed as a continuous variable using Spearman correlation analysis, and as a dichotomous variable (positive/negative groupings) by applying the optimal positive cutoff for meconium FAEE concentration and evaluating whether FAEE-positive and FAEE-negative fetuses displayed differences in pathological endpoints using an unpaired Student’s t-test or Mann-Whitney U test. Relationship between meconium FAEE concentration and fetal PEC in instrumented fetuses exposed to ethanol (group 1 in Table 2.1) was assessed using Spearman correlation analysis. Two groups of data were considered to be statistically different when $p < 0.05$.

4 Results

4.1 Fetal Growth and Organ Pathology

Ethanol treatment did not significantly alter fetal body weight or absolute organ weights (Goh et al., 2011; Gray et al., 2008; Kenna et al., 2011; Sozo et al., 2009). However, when adjusted for body weight, heart weight was greater in ethanol exposed fetuses compared to controls (Goh et al., 2011). There were no significant differences in
the weights of any other organs when adjusted for body weight. Although ethanol-exposed fetuses did not exhibit overt morphological signs of injury, ethanol-induced changes were found in several organs examined as previously described (Goh et al., 2011; Gray et al., 2008; Kenna et al., 2011; Sozo et al., 2009). These changes will be discussed below in relation to meconium FAEE concentration.

4.2 FAEE in Meconium Samples

In control fetuses (saline and untouched controls), trace levels (above LOD but below LOQ) of mostly one or two measured FAEE species were frequently detectable in at least one of the two collected meconium samples (colonic and/or rectal) from each fetus, but only several fetuses had meconium with quantifiable amounts of any FAEE species (>LOQ), and this was ethyl oleate in all cases (Table 2.2 and Table 2.3). In contrast, meconium of all ethanol-exposed fetuses (instrumented and non-instrumented) contained detectable levels of at least three different FAEE species (Table 2.3). Ethyl oleate, palmitate, and stearate were always detectable (>LOD) and often quantifiable (>LOQ) in at least one of the two samples from each ethanol-exposed fetus (Table 2.2). In contrast, trace amounts of ethyl linoleate were found in meconium samples of both ethanol-exposed and control fetuses, but were quantifiable (>LOQ) in only one ethanol-exposed fetus.
Table 2.2 Number of offspring with undetectable (<LOD), trace (between LOD and LOQ), and quantifiable (>LOQ) FAEE concentrations in meconium (in at least one of two collected samples).

<table>
<thead>
<tr>
<th>Ethanol-exposed groups\textsuperscript{a}</th>
<th>Undetectable (&lt;LOD)</th>
<th>Trace (LOD-LOQ)</th>
<th>Quantifiable (&gt;LOQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Palmitate (E16:0)</td>
<td>0 (n=9) 3 (n=6)</td>
<td>1 (n=9) 3 (n=6)</td>
<td>1 (n=9) 3 (n=6)</td>
</tr>
<tr>
<td>Ethyl Linoleate (E18:2)</td>
<td>1 (n=9) 3 (n=6)</td>
<td>7 (46.7%)</td>
<td>3 (53.3%)</td>
</tr>
<tr>
<td>Ethyl Oleate (E18:1)</td>
<td>0 (n=9) 3 (n=6)</td>
<td>1 (n=9) 3 (n=6)</td>
<td>1 (6.7%)</td>
</tr>
<tr>
<td>Ethyl Stearate (E18:0)</td>
<td>0 (n=9) 3 (n=6)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Control groups\textsuperscript{b}</td>
<td>2 (n=9) 4 (n=6)</td>
<td>2 (n=9) 4 (n=6)</td>
<td>2 (n=9) 4 (n=6)</td>
</tr>
<tr>
<td>Ethyl Palmitate (E16:0)</td>
<td>9 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Ethyl Linoleate (E18:2)</td>
<td>4 (40%)</td>
<td>5 (90%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Ethyl Oleate (E18:1)</td>
<td>1 (33.3%)</td>
<td>6 (46.7%)</td>
<td>1 (20.0%)</td>
</tr>
<tr>
<td>Ethyl Stearate (E18:0)</td>
<td>8 (80%)</td>
<td>1 (20%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Surgically-instrumented and non-surgically-instrumented fetuses of surgically-instrumented pregnant ewes that received ethanol treatment (groups 1 & 3, respectively).

\textsuperscript{b} Surgically-instrumented fetuses of surgically-instrumented ewes that received saline treatment and non-surgically-instrumented fetuses of untouched ewes (groups 2 & 4, respectively).
Table 2.3 Number of offspring with detectable (>LOD) and quantifiable (>LOQ) number of different FAEE species in meconium (in at least one of two collected samples).

<table>
<thead>
<tr>
<th># of FAEE</th>
<th>Detectable (&gt;LOD)</th>
<th>Quantifiable (&gt;LOQ)</th>
<th>Detectable (&gt;LOD)</th>
<th>Quantifiable (&gt;LOQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol Groups&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Control Groups&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ethanol Groups&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Control Groups&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 (n=9) 3 (n=6) All (n=15)</td>
<td>1 (n=9) 3 (n=6) All (n=15)</td>
<td>1 (n=9) 3 (n=6) All (n=15)</td>
<td>1 (n=9) 3 (n=6) All (n=15)</td>
</tr>
<tr>
<td>0</td>
<td>0 0 0 (0%)</td>
<td>0 0 0 (0%)</td>
<td>0 1 1 (6.7%)</td>
<td>7 5 12 (80.0%)</td>
</tr>
<tr>
<td>1</td>
<td>0 0 0 (0%)</td>
<td>4 1 5 (33.3%)</td>
<td>5 3 8 (53.3%)</td>
<td>2 1 3 (20.0%)</td>
</tr>
<tr>
<td>2</td>
<td>0 0 0 (0%)</td>
<td>2 0 2 (13.3%)</td>
<td>3 1 4 (26.7%)</td>
<td>0 0 0 (0%)</td>
</tr>
<tr>
<td>3</td>
<td>1 3 4 (26.7%)</td>
<td>2 5 7 (46.7%)</td>
<td>1 1 2 (13.3%)</td>
<td>0 0 0 (0%)</td>
</tr>
<tr>
<td>4</td>
<td>8 3 11 (73.3%)</td>
<td>1 0 1 (6.7%)</td>
<td>0 0 0 (0%)</td>
<td>0 0 0 (0%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Surgically-instrumented and non-surgically-instrumented fetuses of surgically-instrumented pregnant ewes that received ethanol treatment (groups 1 & 3, respectively).

<sup>b</sup>Surgically-instrumented fetuses of surgically-instrumented ewes that received saline treatment and non-surgically-instrumented fetuses of untouched ewes (groups 2 & 4, respectively).
As mentioned above, since there was no statistically significant difference between FAEE concentrations in colonic and rectal meconium samples (Figure S2.1 in supplement), average meconium concentrations for each fetus were used in comparisons of meconium FAEE content between treatment groups. Meconium FAEE concentrations were significantly higher in the two ethanol-exposed groups (1 and 3) compared to the two control (non-exposed) groups (2 and 4), and there were no statistically significant differences in FAEE concentrations between untouched and saline controls (groups 2 and 4) or between surgically instrumented and non-instrumented fetuses exposed to ethanol (groups 1 and 3) (Figure 2.1). Since instrumentation did not appear to affect meconium FAEE concentration (and was not expected to, biologically), the two ethanol groups and the two control groups were combined and compared based on treatment received (ethanol vs. no ethanol), which again showed significantly higher meconium FAEE concentrations in the ethanol-exposed fetuses compared to the non-exposed controls (p<0.0001) (Figure 2.1). Mean meconium FAEE concentration (average of rectal and colonic meconium FAEE concentrations of each fetus) in ethanol-exposed fetuses was 0.179 nmol/g (median: 0.108, range: 0.020–0.561) compared with 0.004 nmol/g (median: 0, range: 0–0.034) in control fetuses. In meconium of ethanol-exposed fetuses, ethyl oleate was generally found at higher concentrations than other measured FAEE species, followed by stearate and palmitate (Figure 2.2).
Figure 2.1 Effect of daily ethanol exposure in late gestation on FAEE concentration in fetal meconium. Bars depict FAEE concentration (sum of four) in meconium (averaged rectal and colonic concentrations for each fetus) of ethanol-exposed (groups 1 and 3) and non-exposed fetuses (groups 2 and 4). Data are presented as mean +/- SEM, n = 6–9 per treatment group, n = 15 for combined ethanol and control groups. Kruskal-Wallis test with Dunn’s multiple comparison test was used to compare FAEE concentrations between the four different treatment groups. No statistically significant differences in FAEE concentrations were found between untouched and saline controls (groups 2 and 4) or between surgically instrumented and non-instrumented fetuses exposed to ethanol (groups 1 and 3) (p>0.05). FAEE concentrations were significantly higher in the two ethanol-exposed groups (1 and 3) compared to the two control groups (2 and 4) (**p<0.01 compared to saline, ‡p<0.05 and ‡‡p<0.01 compared to untouched control). Mann-Whitney U test was used to compare the average concentration between all ethanol-exposed (groups 1 and 3 combined) and non-exposed fetuses (groups 2 and 4 combined). FAEE concentrations were significantly higher in ethanol-exposed fetuses compared to unexposed controls (†††p<0.0001).
Figure 2.2 Concentration of different FAEE species in meconium from ethanol-exposed and control fetuses. Data are presented as mean +/- SEM, n = 15 per treatment group. FAEE concentrations were compared using Friedman test with Dunn’s multiple comparison test (\( **p<0.01, ***p<0.001 \)).

4.3 ROC Analysis: Sensitivity and Specificity

The ability of meconium FAEE concentration to identify the ethanol exposure regimen used in this study was determined by ROC analysis using data from all ethanol-exposed and control (saline and untouched) fetuses and using data from instrumented fetuses only (groups 1 and 2 in Table 2.1). The AUCs of the ROC curves for each FAEE are presented in Table 2.4). The sum of four FAEE had the highest AUC value of >0.98 in both cases, which was significantly different from 0.5, indicating excellent ability of the test to discriminate between ethanol-exposed and non-exposed offspring. No single FAEE species and no other combination of FAEE had a higher AUC value, and thus, the sum of the four FAEE was used in all subsequent analyses. The ROC curves for sum of four FAEEs along with the cutoff values associated with the highest sensitivity and specificity for detecting fetal ethanol exposure are depicted in Figure 2.3. Using data from all ethanol exposed animals or limiting it to instrumented fetuses receiving ethanol or saline (groups 1 and 2, respectively) yielded virtually identical ROC curves and results (Figure 2.3). The positive cutoff value of 0.0285 nmol
FAEE/g meconium had 93% sensitivity and specificity for detecting fetal ethanol exposure in the studied animals. Since this positive cutoff value offered the optimum balance between sensitivity and specificity, it was chosen to be used in subsequent analyses assessing the relationship between meconium FAEE and fetal organ abnormalities.

Table 2.4 ROC curve analysis of meconium FAEE.

<table>
<thead>
<tr>
<th></th>
<th>Data from all ethanol exposed(^a) and non-exposed(^b) fetuses</th>
<th>Data from instrumented fetuses receiving ethanol or saline (groups 1 and 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>95% CI</td>
</tr>
<tr>
<td>Ethyl Palmitate</td>
<td>0.8333</td>
<td>0.6768 to 0.9899</td>
</tr>
<tr>
<td>(E16:0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>0.9356</td>
<td>0.8429 to 1.0280</td>
</tr>
<tr>
<td>(E18:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>0.9333</td>
<td>0.8285 to 1.0380</td>
</tr>
<tr>
<td>(E18:0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl Linoleate</td>
<td>0.5333</td>
<td>0.3237 to 0.7429</td>
</tr>
<tr>
<td>(E18:2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>0.9867</td>
<td>0.9575 to 1.0160</td>
</tr>
</tbody>
</table>

\(^a\) Surgically-instrumented and non-surgically-instrumented fetuses of surgically-instrumented pregnant ewes that received ethanol treatment (groups 1 & 3, respectively).

\(^b\) Surgically-instrumented fetuses of surgically-instrumented ewes that received saline treatment and non-surgically-instrumented fetuses of untouched ewes (groups 2 & 4, respectively).
4.4 Relationship between Meconium FAEE and Fetal Organ Abnormalities

Kidney.

In the fetal kidneys, ethanol exposure resulted in an 11% decrease in nephron endowment with no apparent change in overall kidney growth (Gray et al., 2008). Accordingly, when the studied animals (instrumented ethanol-exposed fetuses and saline controls) were classified using their meconium FAEE concentration, those testing above the chosen positive cutoff for meconium FAEE (>0.0285 nmol FAEE/g)
meconium), as a group, had a lower nephron number compared with fetuses testing below the positive cutoff value for FAEE. Furthermore, in these animals as a group (ethanol-exposed and saline controls combined), there was a negative correlation between meconium FAEE concentration and nephron number in fetal kidneys (Table 2.5).

**Lung.**

Ethanol exposure led to a 75% increase in collagen I α1 mRNA level and significantly increased collagen deposition in fetal lungs (Sozo et al., 2009). Similarly, we found that when the studied animals (instrumented ethanol-exposed fetuses and saline controls) were divided into meconium FAEE-positive and FAEE-negative groups using the positive cutoff of 0.0285 nmol/g, fetuses that tested above this value had significantly more collagen deposition (Table 2.5). When meconium FAEE concentration was considered as a continuous variable in these animals (ethanol-exposed and saline controls combined), there was a positive correlation between meconium FAEE concentration and increased collagen deposition in fetal lungs. Significantly decreased mRNA levels of surfactant protein (SP)-A and SP-B, and proinflammatory cytokines interleukin (IL)-1β and IL-8 in the lungs of ethanol-exposed fetuses were also reported (Sozo et al., 2009). Accordingly, a significant negative correlation was observed between meconium FAEE concentration and SP-B mRNA expression in these animals as a group. This was not the case for SP-A or interleukin mRNA expression.

**Heart.**

In the fetal heart, ethanol exposure resulted in an increase in relative heart weight and left ventricle wall volume (Goh et al., 2011). When FAEE concentration was considered as a continuous variable in these animals (instrumented ethanol-exposed fetuses and saline controls combined), there was a significant positive correlation between meconium FAEE concentration and relative heart weight (Table 2.5). However, when the studied animals were classified according to their meconium FAEE
concentration, the difference in relative heart weight and left ventricle wall volume in FAEE-positive fetuses compared with FAEE-negative fetuses did not reach statistical significance (p = 0.073 and 0.069, respectively) (Table 2.5). Ethanol exposure was also shown to advance cardiomyocyte maturation in the left ventricle of these fetal sheep (Goh et al., 2011), and, accordingly, there was a significantly higher proportion of binucleated cardiomyocytes and a lower proportion of mononucleated cardiomyocytes within the left ventricle and septum in FAEE-positive compared with FAEE-negative fetuses. Furthermore, there was a significant positive correlation of meconium FAEE concentration with binucleated cardiomyocytes and a negative correlation with mononucleated cardiomyocytes within the left ventricle and septum in the studied animals as a group (Table 2.5).

**Brain.**

In the fetal brain, ethanol exposure increased mRNA levels of tropoelastin and collagen I α1 in small cerebral vessels (unpublished observations), and three out of eight ethanol-exposed fetuses examined for brain pathology exhibited small subarachnoid hemorrhages in the cerebrum and/or cerebellar parenchyma associated with focal cortical neuronal cell death and gliosis (Kenna et al., 2011). Accordingly, FAEE-positive fetuses (>0.0285 nmol/g) had significantly elevated level of tropoelastin mRNA compared with FAEE-negative fetuses, although the collagen I α1 mRNA levels in FAEE-positive fetuses were not significantly different from the FAEE-negative fetuses (Table 2.5). Furthermore, in the studied animals as a group (exposed and control fetuses combined), meconium FAEE concentration correlated positively with both tropoelastin and collagen I α1 mRNA levels in cerebral vessels (Table 2.5). The three fetuses in the ethanol group with histologically identified hemorrhages in the cerebrum and/or cerebellum had meconium FAEE concentrations ranging from 0.041 to 0.094 nmol/g and, therefore, were identifiable as FAEE-positive based on the established cutoff.
In the placenta, ethanol exposure elevated mRNA level of tumor necrosis factor (TNF)-α, suggesting a persistent inflammatory response in this tissue (Kenna et al., 2011). Although there was a positive, statistically significant correlation between meconium FAEE concentration and mRNA expression of TNF-α in the placentas of ethanol-exposed fetuses and controls as a group, when animals were divided into meconium FAEE-positive and FAEE-negative groups using the 0.0285 nmol FAEE/g meconium cutoff value, there was no statistical difference between the TNF-α mRNA expression values for the two groups (Table 2.5).

4.5 Relationship between Meconium FAEE and Fetal PEC

In instrumented fetuses exposed to ethanol (group 1 in Table 2.1), there was no statistically significant correlation between the total meconium concentration of FAEE and maximal PEC achieved in individual fetuses at the end of the 1-hr maternal ethanol infusion, or with AUC of the ethanol-concentration-time-curve in individual fetuses measured on 131–134 DGA (Spearman r = 0.028, p = 1.000).
Table 2.5 Relationship between meconium FAEE concentration and ethanol-induced changes in fetal organs (endpoint).

<table>
<thead>
<tr>
<th>Organ endpoint</th>
<th>Meconium group assignment</th>
<th>Correlation with meconium FAEE content†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FAEE-negative (&lt;0.0285 nmol/g)</td>
<td>FAEE-positive (&gt;0.0285 nmol/g)</td>
</tr>
<tr>
<td>Kidney&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Nephron number</td>
<td>427706 ± 8012</td>
</tr>
<tr>
<td>Lung&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Collagen deposition (% collagen/ total area)</td>
<td>21.42 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>COLIα1 mRNA level</td>
<td>1.05 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>SP-A mRNA level</td>
<td>0.87 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>SP-B mRNA level</td>
<td>1.00 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>IL-1β mRNA level</td>
<td>0.72 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>IL-8 mRNA level</td>
<td>0.67 ± 0.39</td>
</tr>
<tr>
<td>Heart&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Heart weight/ body weight (g/kg)</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>LV+S wall volume/ body weight (mm³/kg)</td>
<td>3.29 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>% mononucleated cardiomyocytes within LV+S</td>
<td>35.8 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>% binucleated cardiomyocytes within LV+S</td>
<td>64.2 ± 1.8</td>
</tr>
<tr>
<td>Cerebral vessels</td>
<td>Tropoelastin mRNA level</td>
<td>1.08 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>COLIα1 mRNA level</td>
<td>1.07 ± 0.14</td>
</tr>
<tr>
<td>Placenta&lt;sup&gt;d&lt;/sup&gt;</td>
<td>TNF-α mRNA level</td>
<td>1.17 ± 0.19</td>
</tr>
</tbody>
</table>
Note: Pathology analyses were conducted in instrumented animals receiving ethanol and their appropriate instrumented controls that received saline (groups 1 and 2 listed in Table 2.1). Organ pathology findings based on treatment group assignment (ethanol vs. control) have been previously reported in their respective publications, which are cited for each organ of interest. Herein, the animals are grouped according to their meconium FAEE concentrations, rather than treatment received. All animal pathology data is displayed as mean ± SEM, with an n value of 4-9 fetuses per group depending on the outcome in question. The mRNA data are presented as fold change relative to control. Lung mRNA data were log-transformed prior to statistical analysis. Spearman correlation analysis between meconium FAEE concentration and anatomical/physiological endpoints in individual animals was conducted using data from ethanol-exposed fetuses and saline controls, with an n of 9-15 fetuses depending on the endpoint in question.

Abbreviations: Collagen I α1 (COLIα1), surfactant protein (SP), interleukin (IL), left ventricle plus septum (LV+S), tumour necrosis factor alpha (TNF-α).

a For detailed methodology and results based on treatment received, see Gray et al., 2008.

b For detailed methodology and results based on treatment received, see Sozo et al., 2009.

c For detailed methodology and results based on treatment received, see Goh et al., 2011.

d For detailed methodology and results based on treatment received, see Kenna et al., 2011.

* Unpaired Student’s t-test unless otherwise specified.

‡ Mann-Whitney U test.

† Spearman correlation
5 Discussion

The present study evaluated the usefulness of measuring meconium FAEE concentrations following ethanol exposure of fetal sheep in late gestation that does not cause abnormal physical development, but produces subtle, important pathology in major organs. Of specific interest was the relationship between this biomarker of fetal ethanol exposure and organ pathology (effect) as an assessment of its ability to identify ethanol-affected newborns. The late gestation ethanol regimen is relevant because it has been shown that women may be more likely to drink in the third trimester than throughout pregnancy in response to the commonly-held misconception that alcohol consumption is less harmful to the fetus later in pregnancy (Chang et al., 2006; Drews et al., 2003). However, the third trimester is a period of structural and functional maturation of many organs, including the brain, which undergoes a growth spurt peaking at parturition in humans, and at approximately 133 DGA in sheep (Cudd, 2005). The observed alterations in fetal organs induced by ethanol exposure during this gestational time period may lead to sub-optimal organ function and susceptibility to dysfunction, especially if challenged by other risk factors commonly seen in ethanol-exposed neonates (e.g., prematurity, low birth weight, poor nutritional status). For example, decreased nephron endowment and altered maturation of the heart may have implications for renal and cardiovascular function postnatally; increased collagen deposition and decreased expression of surfactants and interleukins in the lungs may decrease distensibility and predispose to infection; and increased expression of tropoelastin and collagen in cerebral vasculature may increase risk of hemorrhage. Furthermore, the effect that these ethanol-induced system-wide changes can have on the brain may be of developmental and functional significance.

The fetal sheep is a suitable model for studying ethanol teratogenicity and validation of biomarkers because of similarities in organ development and comparable ethanol disposition with the human fetus. In both sheep and humans, ethanol readily distributes across the placenta and the pharmacokinetics of maternal-fetal distribution
and elimination are similar (Brien and Smith, 1991; Smith et al., 1991), with ethanol clearance predominantly regulated by its oxidative metabolism in the maternal liver (Clarke et al., 1989). Furthermore, the ability of the ovine fetus to synthesize FAEE is likely comparable to the human fetus since a previous study in fetal sheep demonstrated that high level/binge exposure to ethanol in late gestation resulted in FAEE concentration of comparable magnitude to that observed in heavily ethanol-exposed human neonates (Littner et al., 2008).

The present study demonstrated that, in sheep, fetal meconium FAEE concentration can serve as a biomarker of chronic ethanol exposure in the third trimester that results in peak PEC frequently observed in non-pregnant women drinking socially (Bearer et al., 2005). Furthermore, a positive cutoff could be established to identify ethanol-exposed fetuses with high sensitivity and specificity, and thereby detect those with ethanol-induced pathological abnormalities. As expected, the total concentration of FAEE (sum of 4) found in meconium of ethanol-exposed sheep was relatively low; ~10 to 100-fold lower than the 2 nmol/g positive cutoff proposed for identifying heavy ethanol exposure in human newborns (Chan et al., 2003). When the studied animals (regardless of exposure status) were divided into FAEE-positive and FAEE-negative groups, these differed in numerous pathological endpoints in fetal organs, including nephron endowment, lung collagen deposition, cardiomyocyte maturation, and tropoelastin gene expression in cerebral vasculature. Furthermore, meconium FAEE concentration correlated with many of the examined pathological endpoints when assessed as a continuous variable in the studied animals as a group, and we cannot exclude that the small number of animals studied for organ toxicity may have precluded statistical significance in some analyses (potential type II error). These results suggest that meconium FAEE may be useful in identifying newborns at risk for ethanol-induced organ and system dysfunction, who may not exhibit overt signs of organ malformations or injury that result only from heavy exposure during critical windows of development.
In the present study, we evaluated the four FAEE species the sum of which has been shown to have the highest sensitivity and specificity for detecting prenatal ethanol exposure in humans (Chan et al., 2003). We found that using a combination of the four FAEE species was advantageous over the use of any single FAEE in identifying ethanol-exposed sheep fetuses, and possessed the highest area under the curve in ROC analysis. Of the different FAEE species, ethyl oleate and stearate possessed comparably high specificities and sensitivities in identifying ethanol exposure, but the combination of FAEE was still superior to any single FAEE alone. These results are in agreement with several studies that have reported combined FAEE concentration as a better biomarker of prenatal ethanol exposure (Brien et al., 2006; Caprara et al., 2005; Chan et al., 2003), but are in contrast to other studies that suggest the use of one particular FAEE (e.g. ethyl oleate or linoleate) (Bearer et al., 2003, 2005; Littner et al., 2008; Ostrea Jr. et al., 2006). Interestingly, we found mostly trace amounts of ethyl linoleate in meconium of ethanol-exposed sheep and some controls, and this ester had the lowest area under the curve in ROC analysis of the four measured FAEE. This contrasts with some previous animal and human studies that have found this FAEE to be a prominent in meconium of ethanol-exposed newborns (Bearer et al., 2003; Littner et al., 2008). This may be due to species/breed and diet differences, as well as different ethanol exposure patterns, which can all potentially affect formation of specific FAEE species. Moreover, ethyl linoleate may be less stable in meconium than other FAEE due to its polyunsaturated chemical structure, which could make it more susceptible to degradation during shipping and storage, similarly to ethyl arachidonate (Moore et al., 2003).

One previous study investigated meconium FAEE concentration in fetal sheep prenatally exposed to ethanol (Littner et al., 2008). Pregnant ewes were infused with ethanol in late gestation, but higher doses of ethanol were used (1.25–2.00 g/kg maternal body weight; unreported PEC). As in the present study, concentrations of several FAEE species were significantly higher in ethanol-exposed animals compared with controls, but the concentrations were several-fold higher than in the present study,
as would be expected due to reporting of FAEE concentrations per dry meconium weight rather than wet weight, and the higher ethanol dosage used. In contrast to our study, however, significant FAEE concentrations in some control sheep were found, which may be due to animal breed (Suffolk vs. Merino x Border Leicester) and/or diet differences leading to variation in endogenous ethanol levels and thus, variable baseline FAEE concentration. Differences also could be due to the use of different analytical procedures; the less selective GC-flame ionization detector method used by Littner and colleagues may be more prone to “false positives” compared with the GC-MS procedure used in the present study (Burd and Hofer, 2008; Gareri et al., 2006).

Maternal and fetal instrumentation, which was necessary for ethanol/saline administration and PEC measurements in this study, was not a likely confounding factor with regard to meconium FAEE concentrations. Since groups 1 and 3 were exposed to the same ethanol dosing regimen, we did not expect and did not find differences in meconium FAEE concentrations between the two groups. Similarly, we did not find differences in baseline FAEE concentrations between groups 2 and 4 (instrumented animals receiving saline and untouched controls), none of which received ethanol—the primary determinant of FAEE levels in tissues. In contrast, the difference in meconium FAEE concentrations between groups that received ethanol and those that did not, was significant. Additionally, we confirmed that inclusion of non-instrumented fetuses did not affect the results of ROC curve sensitivity/specificity analysis and yielded essentially the same optimal cutoff values, thus further confirming that instrumentation was not a likely confounding factor with respect to meconium FAEE concentrations.

Using meconium FAEE as a biomarker has numerous advantages. Meconium is a cumulative matrix that provides a record of xenobiotic exposure over a relatively long time; it can be collected non-invasively; and sensitive methods have been developed to measure FAEE in meconium (Bearer et al., 2005; Hutson et al., 2011; Roehsig et al., 2010). Additionally, human studies exist that have reported association between elevated meconium FAEE concentration and lower APGAR scores (Derauf et al., 2003),
growth restriction and decreased executive functioning (Noland et al., 2003), and poor mental and psychomotor development (Peterson et al., 2008). Thus, the results of this and previous studies are highly encouraging that meconium FAEE may be well-suited for identifying newborns at risk for disabilities due to prenatal ethanol exposure. Another advantage is that meconium FAEE are a product of fetal ethanol burden because maternally produced FAEE do not cross the placenta (Chan et al., 2004). Of interest, we found no correlations between meconium FAEE concentration in individual fetuses and peal PEC or AUC of the fetal PEC-time curve. Thus, the observed variability in meconium FAEE among fetuses in the ethanol group may reflect individual differences in ethanol metabolism, FAEE baseline concentration, content of fatty acids, abundance and kinetics of enzymes with FAEE-synthetic activity, and the rate of FAEE breakdown.

There are some challenges to using meconium FAEE as a biomarker of fetal ethanol exposure. Since meconium begins to form in the human fetal intestine in the second trimester (15–20 weeks gestation) and accumulates mostly in the latter part of pregnancy (Burd and Hofer, 2008), prenatal alcohol exposure earlier in pregnancy may not be detectable if the mother later abstained from drinking. The finding that there was no significant difference in total FAEE concentration between colonic and rectal meconium may indicate that, similarly to humans, a large proportion of meconium is formed and deposited towards the end of pregnancy (when ethanol exposure occurred in the current study). There are also some potential limitations to using sheep as an animal model. Firstly, although in humans it is known that FAEE do not cross the placenta and are thus indicative of fetal ethanol load, such studies have not been conducted in sheep placentas. Secondly, humans and sheep have different diets which could affect the fatty acid composition in fetal blood and thus the FAEE profiles. Thirdly, sheep are ruminant animals capable of producing small amounts of ethanol from glycerol fermentation (Jarvis et al., 1997), which could explain the presence of FAEE in controls. However, how much this differs from endogenous ethanol production in the human gut is unknown. Thus, although we were able to establish a highly sensitive and specific FAEE cutoff that could detect relatively moderate fetal ethanol exposure in this
animal study, large population-based studies are required to determine the sensitivity and specificity of meconium FAEE for detecting various patterns of alcohol use in human pregnancy.

We conclude that meconium FAEE concentration can serve as a reliable biomarker of fetal ethanol exposure in late ovine gestation, and can be used as an indicator of subtle abnormalities in fetal organ development. These findings are encouraging as they suggest that meconium FAEE may be used to identify newborns at risk of multi-organ dysfunction, who may not be displaying overt physical signs of ethanol teratogenicity typically observed only after heavy exposures during critical points of development.

6 Acknowledgments

We thank the authors of the cited studies involving these fetal sheep for providing the anatomical/physiological data of individual animals.
7 Supporting Information

Figure S2.1 Effect of daily ethanol exposure in late gestation on FAEE concentration in fetal rectal and colonic meconium. Bars depict FAEE concentration (sum of four) in meconium collected from the rectal and colonic portions of the large intestine, as well as their average, in ethanol-exposed (groups 1 and 3) and control (groups 2 and 4) fetuses. Data are presented as mean +/- SEM, n = 15 per treatment group. There was no statistically significant difference in meconium FAEE concentration between colonic and rectal meconium samples as assessed by the Wilcoxon signed rank test for matched pairs (p>0.05). Kruskal-Wallis test with Dunn’s multiple comparison test, which was used to compare FAEE concentrations in colonic and rectal samples between ethanol exposed and non-exposed fetuses, showed significantly higher FAEE concentrations in both colonic and rectal meconium of ethanol exposed fetuses compared to controls (**p<0.01; ***p<0.001). Mann-Whitney U test was used to compare the average meconium FAEE concentration (colonic and rectal concentrations averaged for each fetus) between the two treatment groups and yielded the same result (†††p<0.001).

Table S2.1 Forward and reverse primer sequences (5’-3’) used for qPCR to amplify Collagen Iα1 and tropoelastin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Primer conc.</th>
<th>cDNA conc.</th>
<th>Annealing temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Iα1 Forward</td>
<td>aagacatccccactcacc</td>
<td>10μM</td>
<td>500ng</td>
<td>60°C</td>
</tr>
<tr>
<td>Reverse</td>
<td>cagatcgcagcagccaca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropoelastin Forward</td>
<td>atctctcagcgcagcagcag</td>
<td>10μM</td>
<td>1000ng</td>
<td>58°C</td>
</tr>
<tr>
<td>Reverse</td>
<td>gtttgtgtggaagaaagca</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
8 References


Chapter 3 False Positive Meconium Test Results for Fatty Acid Ethyl Esters Secondary to Delayed Sample Collection

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[IZ performed subject recruitment and sample collection at MSH, meconium experiments and sample analysis for FAEE and lipase activity, and prepared the manuscript for submission. BK analyzed samples for ethanol.]
1 Abstract

**Background:** Meconium analysis for fatty acid ethyl esters (FAEEs) is a validated method for identifying heavy prenatal ethanol exposure. This study investigated whether delayed sample collection can result in false positive test results for FAEEs because of collection of samples potentially contaminated with postnatally produced stool.

**Methods:** Serial excretions were prospectively collected from neonates born to nondrinking mothers to capture the transition from meconium to postnatal stool. These were analyzed for FAEEs using headspace-solid phase microextraction and gas chromatography–mass spectrometry. Experiments involving incubation of samples with glucose or ethanol were performed to explore a potential mechanism of FAEE elevation.

**Results:** A total of 136 samples were collected from 30 neonates during their first few days of life (median of 4 samples/baby over a mean period of 68.5 hours postpartum). Although the first-collected meconium sample tested negative for FAEEs in all babies, later samples tested above the 2 nmol/g positive cutoff in 19 of 30 babies. Median time to appearance of FAEE-positive samples was 59.2 hours postpartum. *In vitro* experiments demonstrated that FAEE levels can be further increased in late samples (likely containing postnatal stool) after incubation with glucose, and that FAEEs are readily formed in meconium in the presence of ethanol. **Conclusions:** Collection of samples excreted later in the postpartum period can lead to false positive test results for FAEEs, which could be because of contamination with dietary components of postnatally produced stool and ethanol-producing microorganisms. Clinically, it is critical to collect the earliest possible excretion for determination of FAEEs to ensure that the FAEE content is representative of in utero ethanol exposure.
2 Introduction

Fatty acid ethyl esters (FAEEs) are nonoxidative metabolites of ethanol formed when ethanol is esterified to endogenous free fatty acids or fatty acyl-CoA, a reaction attributed to FAEE synthase and acyl-CoA: ethanol O-acyltransferase enzyme activities (Laposata, 1998). Several nonspecific mammalian enzymes have been shown to possess such activity, including a number of carboxylesterases and lipases (Best and Laposata, 2003). Because FAEEs tend to persist in the body and accumulate in various tissues and matrices, these esters may be used as markers of past and chronic ethanol exposure (Best and Laposata, 2003). One such matrix is fetal meconium, in which levels of FAEEs have been shown to reflect in utero ethanol exposure (Koren et al., 2008). Currently, meconium FAEE analysis is primarily conducted in the context of child protection and to objectively assess the prevalence of prenatal alcohol exposure in select populations. It has also been proposed as a useful screening tool for identifying newborns at risk for alcohol-related disabilities clinically (Goh et al., 2008).

Meconium begins to accumulate in the fetal intestine at 12 to 16 weeks of pregnancy with the emergence of fetal swallowing, and is comprised of materials ingested in utero (Moore et al., 1998). It is excreted in the newborn's first few bowel movements shortly after birth, which in healthy full-term infants typically occurs within 48 hours (Griffin and Beattie, 2001), but may be delayed in premature, unhealthy, or low birth weight neonates (Kumar and Dhanireddy, 1995; Verma and Dhanireddy, 1993). The neonate’s excretions gradually transition from the black, tarry, and odorless meconium to postnatally produced milk stool over the first few days of life.

In practice, samples collected for FAEE analysis may not be the very first meconium excreted by the neonate for several reasons. The first meconium may be passed while in utero, and thus, only later samples are available for analysis; the very first meconium may simply be missed, and so subsequent samples are collected instead; or, several consecutive samples may be collected and pooled to achieve sufficient quantity for analysis. As neonates typically begin feeding within a few hours of
birth, a potential problem that arises if samples are collected later in the postpartum period, particularly after the first meconium has been passed, is risk of intra intestinal contamination with ingested dietary components, developing gut flora, various products of digestion, and other constituents of postnatal stool as the transition to milk stool begins.

Aside from analytical ramifications like decreased sensitivity and introduction of contaminants interfering with analysis, we hypothesized that such intra intestinal contamination with elements of postnatal stool, specifically ingested dietary components and acquired microorganisms, may produce misleading test results. As ethanol is a byproduct of normal microbial metabolism, its local production from ingested milk or formula in the gastrointestinal (GI) tract by the developing gut flora and subsequent conjugation to fatty acids may elevate FAEEs in stool and contaminated meconium, resulting in a positive test result in the absence of in utero alcohol exposure. As will be discussed and investigated in this article, it is possible, or even likely, that the neonate’s GI tract can acquire sufficient intestinal flora and possesses the metabolic capacity to form FAEEs in the first few days of life.

This study examined whether delayed sample collection can result in false positive test results for FAEEs because of potential intra intestinal contamination of sample with elements of postnaturally produced stool. To determine this, we collected and analyzed serial samples from newborns born to nondrinking women over their first few days of life to capture the transition from meconium to postnatal stool. As a biologically plausible mechanism, we investigated whether FAEEs can be produced in vitro in meconium and postnatal stool as a result of microbial fermentation of carbohydrates.

3 Materials and Methods

3.1 Subjects and Sample Collection

Self-reported nondrinking women delivering at Mt. Sinai Hospital (Toronto, Canada) were recruited between February 15, 2010 and March 31, 2010. Participants
and their nurses were instructed to collect all soiled diapers from their newborns while on the maternity ward (the first few days postpartum). Collection involved placing diapers into provided specimen collection bags labeled with their subject number, recording the time of diaper collection, and storing these in a provided container with dry ice. It was assumed that collection occurred shortly after the baby’s bowel movement, and thus, the time of diaper collection was assumed to be a proxy for time of sample passage. Collected diapers were transported to the Motherisk laboratory at the Hospital for Sick Children in Toronto twice-daily, where samples of sufficient quantity for analysis (≥ 0.5 g) were transferred into labeled specimen collection containers (50-ml screw cap conical polypropylene tubes; Sarstedt AG & Co., Numbrecht, Germany) and stored at -20°C until analysis.

Additionally, we had the first few serial samples available from a group of neonates born in a high-risk obstetric unit at St. Joseph’s Hospital (London, Ontario, Canada) between July 1, 2009 and May 31, 2010 that were collected as part of another, unrelated meconium study. These serial samples were also assigned subject numbers and analyzed for FAEEs. Many of the neonates delivered at this high-risk site were premature or otherwise unhealthy and were thus expected to have delayed passage of meconium. They were included to investigate whether such delay may be associated with elevated FAEEs even in the very first excretion.

Maternal and neonatal characteristics were obtained from charts. Babies born to mothers who indicated drinking in pregnancy, those from whom <2 sufficient samples were collected, and those whose samples were inappropriately labeled (e.g., no timing of collection) were excluded.

3.2 Analysis for FAEEs

All collected samples of sufficient quantity from each neonate were analyzed for FAEEs using headspace-solid phase microextraction followed by gas chromatography–mass spectrometry (HS-SPME GC–MS). The method was developed and validated in
our laboratory, and data on linearity, precision, recovery, analytical sensitivity, as well as
detection and quantification limits have been previously described elsewhere (Hutson et
al., 2011). Briefly, 4 FAEEs were quantified (ethyl palmitate, linoleate, oleate, and
stearate) using their corresponding $d_{5}$-ethyl esters as internal standards. A GC–MS QP-
2010 PLUS equipped with AOC-5000 autosampler (Shimadzu, Columbia, MD), a
FactorFour Capillary column (30 m X 0.25 mm X 0.25 μm; Varian, Palo Alto, CA), and
Lab- Solutions GCMSsolutions Software version 2.50SU1 (Shimadzu) were used. A
cumulative sum of $\geq 2.00$ nmol FAEE/g meconium was considered positive, suggesting
heavy prenatal ethanol exposure (Chan et al., 2003).

### 3.3 Glucose Spiking Experiments

To investigate whether microorganisms may be involved in FAEE production,
serial samples from babies whose late samples showed FAEE elevations were
incubated with and without glucose. Briefly, suspensions of 0.25 g sample (early
meconium or late samples) in 1-ml of double-distilled water with and without glucose
(100 μmol/ml) (Sigma-Aldrich, St. Louis, MO) were capped and incubated at 37°C for 24
or 48 hours prior to FAEE analysis. Early samples were defined as the first-collected
meconium from neonates and were all passed $\leq 15$ hours postpartum, while the late
samples in these experiments were typically a pooled sample of several non-first
excretions from the same baby collected between 20 hours postpartum and the baby’s
discharge from the hospital (mean time of collection ranged from 20 to 85 hours
postpartum) and thus likely contained postnatal stool. Samples that showed increases
in FAEEs after incubation were re-analyzed after a 24-hour pretreatment with an
antibiotic cocktail containing vancomycin (50 μg/ml), cefoxitin (240 μg/ml), gentamicin
(80 μg/ml), or the antifungal agent amphotericin B (25 μg/ml) prior to 48-hour incubation
with glucose. The chosen antibiotic concentrations and pretreatment conditions (24
hours at 37°C prior to glucose addition) were based on a protocol used for transplant
organ decontamination (Germain et al., 2010). All drugs were obtained from Sigma-
Aldrich.
Ethanol was measured in the above late samples (quantity permitting) before and after a 48-hour incubation with and without glucose. Briefly, fecal suspensions (0.25 g in 1-ml double-distilled water) were centrifuged for 5 minutes at 18,300 X g in an IEC Micromax centrifuge (Thermo Fisher Scientific, Waltham, MA) to isolate the water portion (supernatant), which was then analyzed for ethanol using n-propanol as an internal standard. Analysis was performed using a Thermo Scientific (Mississauga, ON, Canada) DSQ II GC–MS equipped with a TriPlus autosampler. An Agilent (Palo Alto, CA) DB-ALC-1 column (30 m x 0.32 mm ID x 1.80 µm) was used for analyte separation. The GC conditions were as follows: oven temperature isothermal at 40°C and injector temperature was 175°C. Helium was used as the carrier gas at a flow rate of 27 ml/min. Headspace conditions were as follows: incubation for 15 minutes at 60°C, agitator on and off every 30 seconds, syringe temperature 70°C, and injection vol 1.00 ml of headspace. MS ions 27, 31, 45, and 59 were monitored. The change in ethanol concentration in the supernatant (fecal water) seen with glucose incubation compared with incubation without glucose was then correlated against change in FAEE concentration in the same sample under the same conditions.

3.4 Ethanol Spiking Experiment

To determine whether meconium contains enzymes capable of conjugating ethanol to fatty acids, meconium suspensions (0.25 g blank meconium in 1-ml of double-distilled water) were spiked with increasing concentrations of ethanol (0.5 to 500 µmol/ml) (Commercial Alcohols; Brampton, ON, Canada) and analyzed for FAEEs after 2 hours. Effect of incubation time (0 to 4 hours), temperature (25 and 37°C), meconium boiling (10 minutes at 100° C), as well as of the lipase inhibitor tetrahydrolipstatin (30 µg/ml) (Orlistat®; Sigma-Aldrich) on FAEE formation from added ethanol was also investigated. Chosen tetrahydrolipstatin concentration was based on a published protocol for inhibiting postsampling lipolysis in human plasma (Beysen et al., 2003). Blank meconium used in these experiments was a pooled, homogenized sample of ten early meconium samples (first-collected) previously determined to have a concentration of each of the 4 FAEEs below the limit of detection.
3.5 Lipase Assay

To determine whether lipases (many of which are known to possess FAEE synthetic activity) are present in meconium and postnatal stool, lipase activity was measured in early meconium and late samples (likely containing postnatal stool) using a commercially available colorimetric lipase activity assay, QuantiChrom™ Lipase Assay Kit (DLPS-100; BioAssay Systems, Hayward, CA). The solid and opaque consistency of meconium and stool precluded direct analysis; hence, 1 ml of double-distilled water was added to 0.25 g of early sample (10 pooled samples of first-collected meconium) and late sample (5 pooled samples, non-first excretions passed >20 hours postpartum). Suspensions were vortexed until homogenous consistency (approximately 2 minutes) and centrifuged for 5 minutes at 18,300 X g in an IEC Micromax centrifuge to isolate the supernatant (fecal water). Lipase activity was measured in the supernatant according to manufacturer's instructions. Absorbance at 412 nm was measured at 10 and 20 minutes using a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT) and analyzed using Gen5™ Data Analysis Software (BioTek Instruments). Enzyme activity was calculated as follows: Activity (Units/L)=(OD_{20\text{min}}-OD_{10\text{min}})/(OD_{\text{calibrator}}-OD_{\text{H2O}})\times735, where OD is the optical density. A unit was defined as the amount of enzyme that catalyzes the cleavage of 1 µmol of substrate per minute under the assay conditions (pH = 8.5).

3.6 Ethics

The study was approved by the research ethics board of Mt. Sinai Hospital. Samples from St. Joseph's Hospital were collected as part of another meconium study approved by the research ethics boards of the University of Western Ontario and the Hospital for Sick Children.

3.7 Statistical Analysis

Data analyses were performed with GraphPad Prism 5.0 (Graph-Pad Prism, San Diego, CA). Serial samples data were analyzed with Spearman correlation, Kaplan–
Meier analysis, and the Mann–Whitney test. Pearson correlation, linear regression, paired t-test, and one-way analysis of variance (ANOVA) followed by Tukey’s or Dunnett’s tests were used to analyze experiments on \textit{in vitro} FAEE formation and lipase activity. Concentrations below the limit of quantification were considered to be zero in statistical analyses. Differences were considered statistically significant at $p < 0.05$.

4 Results

4.1 Subjects and Sample Collection

Subject characteristics and details of sample collection are presented in Table 3.1. In total, 136 samples from 30 babies were successfully collected and analyzed for FAEEs. Samples were collected over a mean period of 68.5 hours postpartum (median of 48.2). On average, 4 to 5 sufficient samples were collected ($\geq 0.5$ g) and analyzed from each baby (median 4; range 2 to 10).

All women in this study denied use of alcohol, illicit drugs, and cigarettes on antenatal forms. All 17 babies from Mt. Sinai Hospital were healthy, full-term neonates. Babies from St. Joseph’s Health Care were either preterm (6 of 13), unhealthy, or born to women with health conditions and high-risk pregnancies. All neonates except one were breastfed or fed a combination of breast milk and formula. One neonate had meconium-stained amniotic fluid.
Table 3.1 Neonatal characteristics and details of samples collection.

<table>
<thead>
<tr>
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<th>Mt. Sinai Hospital</th>
<th>St. Joseph’s Health Care</th>
<th>Total study pop.</th>
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<tr>
<td>Number of mothers</td>
<td>15</td>
<td>12</td>
<td>27</td>
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<tr>
<td>Maternal age (years) ± SD</td>
<td>32.08 ± 3.50</td>
<td>27.40 ± 4.43</td>
<td>30.04 ± 4.51</td>
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<tr>
<td>% Cesarean sections</td>
<td>46.67</td>
<td>30.00b</td>
<td>40.00</td>
</tr>
<tr>
<td>Number of neonates</td>
<td>17</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>Mean gestational age (weeks) ± SD</td>
<td>38.96 ± 1.08</td>
<td>35.14 ± 4.55d</td>
<td>37.55 ± 3.37</td>
</tr>
<tr>
<td>Mean birth weight (g) ± SD</td>
<td>3,363 ± 550.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Time to 1st meconium (hours postpartum)</td>
<td>Mean: 14.61</td>
<td>Mean: 29.59</td>
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</tr>
<tr>
<td></td>
<td>Median: 12.87</td>
<td>Median: 22.53</td>
<td>Median: 16.22</td>
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<tr>
<td>Duration of sample collection (hrs postpartum)</td>
<td>Mean: 57.68</td>
<td>Mean: 82.74</td>
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<td></td>
<td>Median: 49.62</td>
<td>Median: 45.25</td>
<td>Median: 48.19</td>
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<tr>
<td></td>
<td>Range: 21.45 to 126.77</td>
<td>Range: 19.83 to 250.00</td>
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<td>Number of samples collected per baby ± SD</td>
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<td>Mean: 3.69 ± 0.48</td>
<td>Mean: 4.53 ± 1.73</td>
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<tr>
<td></td>
<td>Median: 5</td>
<td>Median: 4</td>
<td>Median: 4</td>
</tr>
</tbody>
</table>

a Maternal age absent from charts of 4 women (2 MSH and 2 SJH).
b Delivery method information missing from charts of 2 women.
c Three sets of twins (2 in MSH, 1 in SJH).
d Gestational age was missing from charts of 3 neonates.
e Samples of sufficient quantity for analysis (≥ 0.5 g).
4.2 Meconium FAEE Levels

In all 30 babies (from both birthing sites), the first-collected meconium sample tested negative for FAEEs (< 2 nmol/g); however, in most cases, later samples exhibited increasing FAEE levels, and 19 of the 30 neonates (63%) excreted FAEE-positive samples by the time of their discharge from the hospital (≥ 2 nmol/g) (Figure 3.1 shows representative chromatograms). A significant positive correlation between time of sample collection (hours after birth) and FAEE levels was observed (p < 0.0001) (Figure 3.2A).

![Figure 3.1 Gas chromatography–mass spectrometry chromatograms of 2 samples from a single neonate showing a discrepancy in FAEE levels. Four FAEEs were quantified using their corresponding d5-FAEEs as internal standards. The m/z values and abundance factors are given on the right. Peaks corresponding to ethyl esters are identified to the left of each peak. A meconium sample collected 14 hours postpartum had no detectable FAEEs, whereas a subsequent sample collected 22.3 hours postpartum contained 4.05 nmol/g FAEEs.]
On average, the 11 babies who did not excrete FAEE-positive samples spent less time in the hospital, and thus, samples were collected over a shorter period of time; median of 31.3 versus 57.7 hours (mean of 40.83 vs. 84.6 hours, respectively; Mann–Whitney test, p = 0.02).

Median time to appearance of FAEE-positive samples (2 nmol/g) was 59.2 hours postpartum (as assessed by a Kaplan–Meier curve) (Figure 3.2B). Four of the 30 babies (approximately 13%) excreted FAEE-positive meconium <24 hours postpartum, with the earliest positive sample collected 18.9 hours postpartum. Increase in FAEEs was seen in both full-term healthy neonates from Mt. Sinai Hospital and neonates from the high-risk obstetric unit. There was no significant difference in time to positivity between the 2 groups (log-rank test, p > 0.05).
Figure 3.2. FAEE levels and timing of sample collection. (A) Relationship between FAEE levels in neonatal excretions and timing of sample collection. FAEEs were measured in 136 samples collected from 30 neonates. Of these, 10 samples were not plotted because FAEE levels could not be reliably quantified. The best-fit line is shown along with the Spearman correlation coefficient. First samples collected postpartum are indicated as white squares. (B) Kaplan–Meier graphs depicting time to sample positivity (FAEE ≥ 2 nmol/g) in 30 neonates. Censored cases are neonates who were discharged before passing FAEE-positive sample.
4.3 Glucose Spiking Experiments

Net change in FAEEs for a particular specimen after incubation was expressed as the difference between FAEE concentration after incubation and baseline FAEE concentration in the sample with no incubation (time 0). No substantial increase in FAEEs after incubation with or without additional glucose (100 µmol/ml) was seen in 9 early meconium samples collected ≤ 15 hours postpartum (first-collected samples; all negative for FAEE) (Figure 3.3A). A marked increase in FAEEs was seen in 1 of 11 late samples collected ≥ 20 hours postpartum (non-first excretions; most FAEE-positive) after incubation without added glucose, while a decrease in FAEE levels was seen in remaining samples (Figure 3.3A) as expected from degradation (Moore et al., 2003). However, when these late samples were incubated with added glucose (100 µmol/ml), a substantial increase in FAEEs was observed in 5 of 11 samples (Figure 3.3A).

Elevations in FAEEs seen in late samples after incubation with glucose were still observed in samples pretreated with amphotericin B prior to glucose incubation, but not when the same samples were pretreated with an antibiotic cocktail consisting of vancomycin, cefoxitin, and gentamicin (Figure 3.3B).

Ethanol was detected in the supernatant of 6 of 7 late samples (ethanol concentrations ranged 10 to 69.40 µM), and these levels further increased in 3 samples after incubation for 48 hours at 37°C and in 6 of 7 samples after incubation with added glucose (100 µmol/ml) (ethanol concentration ranged 22.2 to 297.2 µM), which was statistically significant when compared with unincubated samples (p < 0.05, paired t-test). Ethanol concentrations were higher in 7 of 9 samples incubated with glucose as compared to incubation without glucose, bordering on statistical significance (p = 0.06). Importantly, the change in ethanol levels after incubation with added glucose, compared with incubation without glucose, significantly correlated with the change in FAEEs in these samples under the same experimental conditions (Figure 3.3C).
Figure 3.3 Effect of incubation with and without added glucose on FAEE levels in serial samples.

(A) Net change in FAEE levels in 9 early samples (first meconium, collected ≤15 hours postpartum) and in 11 late samples (non-first excretion, collected ≥20 hours postpartum) after incubation at 37°C with or without added glucose (100 µmol/ml). (B) Effect of antimicrobials on FAEE increases seen with glucose incubation. Four late samples that displayed elevation in FAEEs after incubation with glucose were pretreated with amphotericin B (AmB; 25 µg/ml) or an antibiotic cocktail (ABX; vancomycin 50 µg/ml, cefoxitin 240 µg/ml, gentamicin 80 µg/ml) for 24 hours at 37°C prior to incubation (48 hours at 37°C) with and without glucose (100 µmol/ml). Net change is the difference in FAEE levels in glucose-incubated samples compared with incubation without glucose. *p < 0.05 versus untreated controls compared by repeated-measures ANOVA, followed by Dunnett’s test. Results are displayed as mean ± SEM. (C) Correlation between change in FAEE levels and change in ethanol levels in late samples after incubation with glucose compared with incubation without glucose. Best-fit line is shown along with the Pearson correlation coefficient.
4.4 Ethanol Spiking Experiments and Lipase Activity

Spiking blank meconium with increasing concentrations of ethanol led to a proportional increase in FAEE levels (Figure 3.4A), which was temperature and time dependent (Figure 3.4B, C). As FAEEs were not detected in meconium that was boiled prior to ethanol addition (results not shown), enzymatic involvement was suspected. Tetrahydrolipstatin (a lipase inhibitor) significantly decreased FAEE production from added ethanol in blank meconium suggesting that lipases may be involved in the conjugation of ethanol to fatty acids in meconium (Figure 3.4D). A colorimetric assay confirmed that lipase activity was present in early meconium and in late samples likely contaminated with postnatal stool and was several orders of magnitude higher in the latter (Figure 3.5). Accordingly, tetrahydrolipstatin significantly decreased lipase activity in both early meconium and late samples.
Figure 3.4 FAEE formation from added ethanol (EtOH) in blank meconium. All experiments were conducted in triplicate. Results are displayed as means ± SE. (A) FAEE levels in blank meconium spiked with increasing concentrations of EtOH. Samples were analyzed for FAEEs 2 hours after EtOH addition. Linear regression line and associated $r^2$ value are shown. (B) Effect of incubation temperature on FAEE formation in blank meconium spiked with 50 µmol/ml EtOH and 4-hour incubation. *$p < 0.001$ compared by one-way ANOVA, followed by Tukey’s test. (C) Effect of incubation time on FAEE formation in blank meconium spiked with 50 µmol/ml EtOH and incubated at 37°C. Linear regression line and associated $r^2$ value are shown. (D) FAEE levels in blank meconium spiked with EtOH (50 µmol/ml) in the absence or presence of tetrahydrolipstatin (THL; 30 µg/ml). Samples were analyzed after 4 hours at 37°C. *$p < 0.001$ compared by one-way ANOVA, followed by Tukey’s test.
Discussion

To our knowledge, this is the first report that collection of samples excreted later in the postpartum period and failure to collect the earliest excretions may lead to falsely elevated test results for FAEEs, which could be because of meconium contamination with elements of postnatal stool. In our study, despite all first meconium samples being negative for FAEEs, later collected samples became positive in 63% of neonates early in the postpartum period as the transition to postnatal stool began. Mean time to appearance of positive samples was approximately 60 hours postpartum, with the
earliest observed at 19 hours. This finding has important implications for the use and interpretation of meconium analysis for FAEEs in research, clinical, and legal settings.

We believe these were false positive results for several reasons. First, all women denied alcohol use in pregnancy, had no mention of concerns regarding alcohol or drug use in their charts, and volunteered to participate knowing that meconium would be tested for FAEEs. Second, their neonates' first few meconium samples tested negative for FAEEs, but a large proportion (more than half) of the neonates passed FAEE-positive samples later in the postpartum period. It is highly unlikely that such a large proportion of these women drank heavily in late pregnancy. Last, a positive correlation was observed between timing of sample collection and FAEE content in the sample, raising serious concerns regarding the validity of the results.

As a possible mechanism for FAEE elevation in later collected samples, we propose that postnatal stool and stool-contaminated meconium may contain ingested carbohydrates and acquired gut flora capable of fermenting these to ethanol, and that ethanol can subsequently be conjugated to form FAEEs. This is supported by our fermentation experiments where incubation of later collected specimens (likely containing postnatal stool) with added glucose resulted in further increases in FAEEs, an effect obliterated by antibiotics, suggesting microbial involvement. Ethanol levels in these samples also increased after incubation with glucose, and this increase correlated with increases in FAEEs. Furthermore, adding ethanol to blank meconium resulted in a proportionate increase in FAEEs, a reaction that may be mediated by lipases as it was inhibited by tetrahydrolipstatin. This suggests that even early meconium contains the enzymatic machinery capable of conjugating ethanol to FAEEs, and thus, false positivity may occur early in the transition between stool and meconium if fermenting microorganisms and substrates are present, and not just in late stool samples that can typically be distinguished visually. However, other sources of FAEEs that were not investigated in this study are plausible and may play a role in FAEE elevations. For example, FAEEs may be present in small amounts in breast milk or formula and thus be
directly ingested, or they may be elevated because of external contamination of the sample with ethanol- or FAEE-containing cleansing products used during diaper changes (baby wipes, diaper creams, etc.).

With regard to our proposed mechanism, the ability to produce ethanol by fermentation is widespread among microorganisms, many of which reside in the human gut (Corry, 1978), which contains >400 different species (Mackie et al., 1999). Ethanol formed by microbial fermentation in the GI tract by facultative or obligatory anaerobic microorganisms is a well-known physiological phenomenon (Boumba et al., 2008; Krebs and Perkins, 1970). Endogenously formed ethanol is eliminated mainly in the liver by alcohol dehydrogenase (ADH), the inhibition of which was shown to result in significant elevation of endogenous plasma ethanol levels in humans (up to approximately 30 µM after carbohydrate intake) (Sarkola and Eriksson, 2001). Furthermore, microbial ethanol fermentation was shown to result in elevated blood ethanol levels in patients with short bowel syndrome (Dahshan and Donovan, 2001; Jansson-Nettelbladt et al., 2006), elevated breath alcohol levels in obese patients (Nair et al., 2001), and may even contribute to pathology associated with small-intestinal bacterial overgrowth and nonalcoholic fatty liver disease (Abu-Shanab and Quigley, 2010).

Of interest, risk of false positive ethanol results because of microbial fermentation is a well-known complication of ethanol analysis in forensic cases (O’Neal and Poklis, 1996). Numerous studies have demonstrated that significant amounts of ethanol can be produced in human corpses and in isolated tissues contaminated by microorganisms (Appenzeller et al., 2008; Collison, 2005; Helander et al., 2009; Kuhlman et al., 1991; Lewis et al., 2004). Intestinal microorganisms play an important role in postmortem ethanol production as they penetrate the intestinal walls and distribute to blood and tissues after death (Boumba et al., 2008). The amount of ethanol produced in such cases depends on the microorganisms present, substrate availability, and environmental factors such as pH, presence of oxygen, temperature, and time (Corry, 1978). If, as hypothesized, ethanol production by intestinal microorganisms is the main
mechanism behind FAEE elevation in stool and contaminated meconium, many of these factors would affect FAEE levels and thus the risk of false positive test results. In the neonatal GI tract, the presence of microorganisms would depend on the dynamics of gut colonization, while FAEE formation would also depend on the presence of conjugating enzymes.

Although the GI tract (and thus meconium) of a normal fetus is sterile, intestinal colonization begins at delivery when newborns are exposed to maternal vaginal flora, fecal bacteria, environment, and particularly diet, as breast milk contains up to $10^9$ microbes/l in healthy mothers (Mackie et al., 1999). Colonization is extremely rapid, and microorganisms have been found in upper GI tract and nasopharynxes of babies immediately after birth (Bettelheim et al., 1974; MacGregor and Tunnessen, 1973) and in the gastric content of 5- to 10-minute-old babies (Brook et al., 1979). Neonates are colonized by numerous bacterial species capable of fermentation including *Escherichia coli* and streptococci that reach $10^8$ to $10^{10}$ cells/g feces within 3 days of life, as well as anaerobic species (*Bacteroides*, *Bifidobacterium*, and *Clostridium*) that dominate by days 4 to 7 (Mackie et al., 1999). Fermentation products such as ethanol, lactate, acetate, and other short-chain fatty acids (SCFA) are found in neonatal feces and are produced during carbohydrate fermentation by neonatal fecal suspensions (Edwards and Parrett, 2002; Lifschitz et al., 1990; Stansbridge et al., 1993; Wolin et al., 1998). The extent of microbial ethanol production in infant colon requires further study, but our results suggest that ethanol produced by gut microflora is not only metabolized oxidatively via ADH but also through conjugation to fatty acids (i.e., FAEE formation). The dynamics of colonization are highly variable among infants and are affected by factors like delivery method, antibiotic use, environment, and diet, which may thus, theoretically, also affect the risk of false positive FAEE results and time to passage of FAEE-positive samples.

As several digestive enzymes can synthesize FAEEs in the presence of ethanol, including several lipases (carboxyl ester lipase and pancreatic triglyceride lipase), we
hypothesized that they may mediate FAEE formation from ethanol in meconium and stool. Accordingly, we demonstrated lipase activity in meconium and in later samples likely contaminated with postnatal stool (in which it is higher as would be consistent with commencement of digestion), and our experiments with tetrahydrolipstatin showed that this lipase inhibitor significantly decreased FAEE formation from ethanol in meconium. As expression of pancreatic triglyceride lipase is low at birth, lipase activity in meconium and stool samples may be attributed to carboxylester lipase (cholesterol esterase/bile salt-dependent lipase), gastric lipase, and/or the pancreatic lipase-related protein 2, all of which are expressed at birth (Lindquist and Hernell, 2010). Furthermore, bile salt stimulated lipase, a product of the lactating mammary gland, is an equivalent enzyme to carboxylester lipase found in breast milk (Lindquist and Hernell, 2010) and may account for the high lipase activity observed in late samples. However, it cannot be excluded that tetrahydrolipstatin inhibits related enzymes capable of FAEE synthesis. Moreover, microbial lipases may also mediate fatty acid conjugation to ethanol, and their contribution to total FAEE formation may be especially important in late samples abundant in microorganisms.

Strict guidelines regarding sample collection are needed to reduce the risk of false positive results. We advise that all efforts are made to collect the earliest neonatal excretion to ensure accuracy of results, and suggest that the possibility of contamination is considered when interpreting FAEE-positive samples collected after the first day of life and/or if several bowel movements had already occurred. In our study, none of the first meconium samples collected were FAEE-positive, not even in neonates from the high-risk obstetric unit, some of whom passed first meconium more than 24 hours postpartum. Because of small sample size, however, the possibility that first meconium can become positive if it remains in the GI tract long enough to be affected by gut colonization and digestion requires further investigation.

Some indicators of false positivity exist and should be noted during analysis and interpretation. For example, sample contamination with postnatally produced stool may
often be visually evident as milk stool differs from the black, tarry, and odorless meconium in color, texture, and frequently present smell. However, distinguishing samples at risk for false positivity by visual inspection alone may be problematic as the commencement of transition is not always visually or otherwise obvious over time or within specimen. Since even first meconium has the enzymatic capacity to conjugate ethanol to fatty acids, false positivity will be an issue very early in the transition if gut flora capable of fermentation is present. Indeed, we encountered FAEE-positive samples without overt signs of contamination. Testing for presence of microbes is unlikely to be useful as even first meconium samples passed only a few hours after birth may contain microorganisms because of rapid gut colonization (Hall and O'Toole, 1934; Palmer et al., 2007). However, the types and abundance of both microorganisms and substrates in meconium may differ from later stool, and this should be investigated further. Other indicators may be the presence of other volatiles in the sample that are common products of bacterial fermentation (SCFAs), as well as, high levels of fats (from diet) (Righetti et al., 2003).

This study has several limitations. First, it was assumed that collection occurred shortly after the baby's bowel movement, and so, the time of collection was used as proxy for time of sample passage. Although every effort was made to ensure that diapers were collected and frozen promptly, it is possible, or even likely that some babies were not changed immediately after passage of meconium, and so, the samples remained in the diaper at room temperature for some period of time. This could have altered FAEE levels in the sample as some FAEE degradation and possibly even synthesis may have occurred post sample passage. Second, we cannot exclude that some soiled diapers were accidently discarded by parents or nurses, but this is unlikely to have affected the evident trend of FAEE elevation observed over time. Third, we did not conduct microbiological analyses, and presence of microbes in samples was inferred from in vitro fermentation experiments with and without antibiotics. It should be noted that the conditions during these fermentation experiments were different from the neonatal GI tract. Specifically, samples were not incubated under anaerobic conditions
optimal for fermentation, although because they were capped, anaerobic conditions were likely achieved once oxygen was consumed by aerobic species. Furthermore, microbial content of our samples may have differed from the neonatal GI tract as some may have been introduced into the meconium during handling while others may not have survived the passage, exposure to oxygen, and storage conditions (-20°C frozen up to 6 months). This may also explain why FAEE levels did not increase in all “late” FAEE-positive samples after incubation. With regard to ethanol spiking experiments, a wide range of ethanol concentrations was used, including the maximum that could be produced by common intestinal bacteria from the amount of glucose added (50 to 200 µmol/ml) (Corry, 1978). Whether these levels resemble in vivo is not known, although ethanol levels of approximately 40 µmol/g excreted feces in infants have been reported (Wolin et al., 1998). Overall, our in vitro experiments support a biologically plausible mechanism by which falsely elevated FAEEs may be obtained, but to what extent this occurs in vivo is unknown, and, as mentioned previously, other mechanisms may be at play.

In summary, this study sheds light on a considerable artifact that may affect the validity of meconium test results for FAEEs, which may have severe legal and social consequences. We showed that elevated FAEE levels can be observed in samples excreted later in the postpartum period as compared to earlier samples from the same baby, and thus, delayed sample collection may lead to false positive FAEE test results. We propose that meconium FAEE levels may be elevated in later samples because of intra intestinal contamination with dietary components of postnatally produced stool and ethanol-producing microorganisms that are acquired in the first few days of life. We suggest that all efforts are made to collect the earliest excretions, preferably the very first meconium passed by the neonate early in the postpartum period, and urge that caution is exercised when interpreting FAEE-positive results in samples passed after the first day of life, those collected after the first meconium was passed, and in samples with signs of contamination with postnatal stool.
6 Acknowledgements

We thank Lisa Lum, Kirsten Eisinga, and the rest of the nursing staff at St. Joseph’s Health Care for their help with sample collection. The study was supported by a CIHR operating grant (GK). GK is supported by the Ivey Chair in Molecular Toxicology, Department of Medicine, University of Western Ontario. IZ is supported by Ontario Graduate Scholarship and through the University of Toronto Open Fellowship.

7 Supplementary figures

(Not included in published article)

Figure S3.1 FAEE levels in first and last collected samples from each neonate. The first sample in all 30 babies tested negative for FAEEs (<2nmol/g meconium), however, the last collected sample in 19/30 neonates tested positive for FAEEs. The difference in FAEE levels between the first meconium sample collected and the last collected sample from the same baby (that could be reliably quantified) was statistically significant as assessed by Wilcoxon signed rank test (*p < 0.0001).
**Figure S3.2 Effect of boiling on FAEE formation from ethanol in blank meconium.** Ethanol (350 µmol/mL) was added to non-boiled and boiled (100°C for 10 min) blank meconium samples. In contrast to non-boiled meconium, adding ethanol to boiled meconium did not result in any detectable FAEE formation after one hour at room temperature. Experiments were conducted in triplicate and results are displayed as means +/- SE. * p < 0.001 compared to non-boiled meconium with added ethanol (One-way ANOVA with Tukey’s multiple comparison test).
Figure S3.3 Effect of tetrahydrolipstatin on FAEE formation from ethanol in blank meconium.

Ethanol (350 µmol/mL) was added to blank meconium samples in the presence or absence of increasing concentration of the lipase inhibitor tetrahydrolipstatin (THL) (0.3, 3 and 30 µg/mL). Presence of THL resulted in a significant, concentration-dependent decrease in FAEE production from ethanol; *p < 0.001 compared to vehicle control, §p < 0.001 compared to 0.3 µg/mL THL (One-way ANOVA with Tukey’s multiple comparison test). Experiments were conducted in triplicate and results are displayed as means +/- SE.
8 References


Chapter 4 Voluntary Neonatal Screening for Prenatal Alcohol Exposure: A Pilot Study in a High-Risk Obstetric Unit

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This work is published:


[IZ coordinated the study, performed all meconium analyses, data analysis, and prepared the manuscript for submission. LL and KE performed subject recruitment.]
1 Neonatal Screening for Prenatal Alcohol Exposure: Assessment of Voluntary Maternal Participation in an Open Meconium Screening Program.

1.1 Abstract

Meconium fatty acid ethyl esters (FAEEs) are validated biomarkers of fetal alcohol exposure. Meconium FAEE testing can potentially be used as a screen by health-care professionals to identify neonates at-risk for fetal alcohol spectrum disorders, thereby permitting diagnostic follow-up of these children and early intervention in those who develop disabilities. The purpose of this study was to assess whether women would willingly partake in a screening program of this nature. This was determined by launching a pilot screening program for prenatal alcohol exposure in a high-risk obstetric unit previously shown to have a high prevalence of FAEE-positive meconium via anonymous meconium testing. The program involved voluntary testing of meconium for FAEEs and long-term developmental follow-up of positive cases through an existing public health program. The participation rate in the screening program was significantly lower than when testing was conducted anonymously (78% vs. 95%, respectively; p < 0.05), and the positivity rate was 3% in contrast to 30% observed under anonymous conditions (p < 0.001). These low rates suggest that the majority of mothers who consumed alcohol in pregnancy refused to participate. We conclude that despite the potential benefits of such screening programs, maternal unwillingness to consent, likely due to fear, embarrassment, and guilt, may limit the effectiveness of meconium testing for population-based open screening, highlighting the need for public education and social marketing efforts for such programs to be of benefit.

1.2 Introduction

Fetal Alcohol Spectrum Disorder (FASD) is a term that describes the broad range of physical, cognitive, and behavioral disabilities that can arise due to prenatal alcohol
exposure (Chudley et al., 2005; Streissguth and O’Malley, 2000). Estimated to affect 9.1/1000 live births, FASD is a leading preventable cause of mental retardation in the western world and a substantial economic burden (Sampson et al., 1997). The annual costs in Canada alone exceed $5 billion in productivity losses, medical costs, special education, social services, and externalizing behaviors (Stade et al., 2009).

Although the primary alcohol-induced damage is permanent, early diagnosis is beneficial and associated with a decreased risk of secondary disabilities such as disrupted school experience, unemployment, institutionalization, and trouble with the law; likely because it permits early intervention and specialized support (Streissguth et al., 2004). Unfortunately, recognizing FASD is extremely challenging, and diagnosis is often contingent upon establishing a history of significant in utero alcohol exposure (Chudley et al., 2005). Since maternal reports are unreliable in supplying this information due to recall bias and common under-reporting (McNamara et al., 2005; Russell et al., 1994), objective methods and biomarkers for ascertaining prenatal alcohol exposure have been investigated.

Fatty acid ethyl esters (FAEEs) are non-oxidative metabolites of ethanol formed by the esterification of ethanol to endogenous fatty acids or fatty acyl-CoA, which deposit and accumulate in fetal meconium (Best and Laposata, 2003; Koren et al., 2008). Numerous studies have established that elevated meconium FAEE concentration can serve as a biomarker of heavy prenatal alcohol exposure occurring in the last two trimesters of pregnancy (Bearer et al., 1992, 1999, 2003, 2005; Brien et al., 2006; Chan et al., 2003; Klein, et al., 1999; Littner et al., 2008; Moore et al., 2003). Furthermore, agreement between meconium FAEEs and various alcohol-related outcomes has been demonstrated (Brien et al., 2006; Hutson et al., 2010; Jacobson, 2006; Noland et al., 2003; Peterson et al., 2008), and this test has been used anonymously to obtain epidemiological data on prenatal alcohol exposure in select populations (Garcia-Algar et al., 2008; Gareri et al., 2008; Goh et al., 2010; Hutson et al., 2010).
It has been recognized that meconium analysis for FAEEs may serve as a neonatal screening tool for the identification of alcohol-exposed newborns, which could potentially be implemented as a universal screen or targeted to high-risk populations (Goh et al., 2008). Such screening would not only provide accurate exposure history required for diagnosis, but if implemented along with a comprehensive follow-up program and interventions, could facilitate early recognition and treatment of FASD (Gifford et al., 2010; Goh et al., 2008; Hopkins et al., 2008). As an added value, it may identify and allow for intervention in problem-drinking mothers, which, in turn, may prevent future alcohol-exposed pregnancies (Koren et al., 2008). However, since informed consent from a competent patient or appointed guardian prior to treatment or testing is an ethical and legal component of medical practice (Etchells et al., 1996; Flagler et al., 1997), a screening program of this nature may require consent of the child’s legal guardian (typically the parent). This could diminish the value of meconium screening in a clinical setting since embarrassment, guilt, and fears of stigma and child apprehension may deter women who consumed alcohol from consenting to testing despite the potential value to child health.

To determine if women would willingly participate in a neonatal screening program for prenatal alcohol exposure, we offered meconium testing, with subsequent follow-up, interventions, and social supports, to women from a regional Ontario population delivering in a high-risk obstetric unit previously shown to have a high prevalence of alcohol-exposed neonates as determined by anonymous meconium testing. We assessed the rates of voluntary participation and positivity for alcohol exposure in this pilot screening program, and compared these with the rates observed with anonymous testing.

1.3 Methods

1.3.1 Subject recruitment

The target population to whom screening was offered were women residing in the region of Grey-Bruce, Ontario, who were delivering at St. Joseph’s Health Care
SJHC in London, Ontario. Grey-Bruce is located in Mid Western Ontario, Canada, and occupies an area of 8,586 km², comprising of a midsized city (Owen Sound, ~22,000 people), smaller towns, farming communities, first nation communities, and Mennonite settlements. There are ~1200-1400 births per year in the region, but high risk or complicated pregnancies are often transferred out of the region to tertiary and more specialized health-care centres, one of which is the high-risk obstetric unit of SJHC in London, Ontario (~50 births/year). We chose to launch the screening program at this high-risk obstetric unit since a previous study, using anonymous meconium testing, observed a 12-fold higher risk of FAEE-positive meconium in women referred to this site as compared to the general population of the region (30% vs. 2.5%) (Goh et al., 2010).

Written informed consent for meconium FAEE analysis and follow-up of those testing positive was sought from all Grey-Bruce women delivering at SJHC in London, Ontario from November 1st, 2008 to May 31st, 2010. Briefly, Grey-Bruce residents identified by nurses were informed of the study, offered screening, and given an Informed Consent document to review and sign if they chose to participate. Women were also informed of the study through pamphlets and posters in the maternity ward. It was stressed that a positive test or self-report of drinking in pregnancy would not necessitate involvement of child protection agencies, but would be used to initiate follow-up by the public health unit and to mobilize support services if needed.

1.3.2 Meconium collection, handling, and analysis

Meconium specimens from neonates born to consenting women were collected into 50-mL screw cap conical polypropylene tubes (Sarstedt AG and Co., Numbrecht, Germany) by nursing staff and labeled with the subject number to ensure confidentiality. Samples were stored onsite at -20°C and shipped on dry ice to the Motherisk Laboratory at Hospital for Sick Children in Toronto, Ontario on a biweekly basis, where they were stored at -80°C until analysis.

Meconium FAEEs were measured using headspace solid-phase microextraction and gas chromatography-mass spectrometry. The method involves the detection and
quantification of four FAEEs (ethyl palmitate, linoleate, oleate, and stearate) using corresponding $d_5$-ethyl esters as internal standards. It has been developed and validated in our laboratory, and published in detail elsewhere (Hutson et al., 2009, 2011). Heptane, ethyl esters (palmitate, linoleate, oleate, stearate), fatty acids (palmitic, linoleic, oleic, stearic), anhydrous ethanol-$d_6$, and thionyl chloride were obtained from Sigma Aldrich Co. (St. Louis, MO). Acetone was obtained from EMD Chemicals Inc. (Gibbstown, NJ). Chromatograms were analyzed using LabSolutions GCMSsolution software version 2.50SU1 (Shimadzu, Kyoto, Japan). A cumulative sum of $\geq 2.00$ nmol FAEE/gram meconium was considered positive for heavy alcohol exposure, as established in a population baseline study that measured meconium FAEEs in neonates born to abstainers, social drinkers, and confirmed heavy drinkers (Chan et al., 2003). The same cutoff was used in the previous anonymous prevalence study conducted at this site (Goh et al., 2010).

1.3.3 Maternal and neonatal characteristics

Subject characteristics along with pregnancy and delivery information were obtained from charts of consenting women. Additionally, information on alcohol use was obtained by the nursing staff using the Parkyn Screening Tool; a routine postpartum questionnaire administered to all women as part of Ontario’s Ministry of Health and Long Term Care “Healthy Babies Healthy Children” program. This questionnaire is meant to assess families with a new baby for risks of future health concerns and includes a question regarding alcohol use in pregnancy.

1.3.4 Follow-up and neurodevelopmental assessment

Children with positive meconium results were followed-up through Ontario’s Healthy Babies Healthy Children program, which involves postpartum home visits by public health nurses, who provide personalized support and education to families with newborns (Ontario Ministry of Health and Long-Term Care, 2003). In this study, the nurse assigned to a positive case was notified of the meconium test results, conducted an in-depth family assessment (including a screen for alcohol use disorders), and
enrolled the family in an ongoing home-visiting program with an individualized family service plan that included regular assessments of developmental milestones using the Ages and Stages Questionnaires® (ASQ). Children with FAEE-positive test results also received neurodevelopmental assessment by a certified clinical psychologist during two home visits; around 3 months and 1-1.5 years of age. The Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III®) was used, which measures infant cognitive, linguistic, and motor functioning.

If developmental delays were detected, additional service providers were engaged through referrals to intervention programs and specialized services, which were provided through Healthy Babies Healthy Children and Grey-Bruce Health Unit at no cost to the family. Need for referral to diagnostic services at the Hospital for Sick Children was assessed. Additional support services for the family were available if needed, including addiction treatment, counseling, parenting support, education on FASD, nutrition, employment, and recreation. If a sufficient number of positive cases were identified, the protocol included a secondary objective to determine whether meconium FAEE concentration correlated with neurodevelopmental outcomes.

1.3.5 Comparison with anonymous meconium testing

To determine whether women who consumed alcohol during pregnancy agreed to participate in the pilot open screening program, the rates of voluntary participation and positivity for prenatal alcohol exposure observed in our open program were compared to rates observed in the previous prevalence study conducted in the same obstetrical unit a year earlier, in which meconium was tested anonymously (Goh et al., 2010). Fisher’s Exact Test was used to compare the results of the two studies. Two-tailed P-value <0.05 was considered statistically significant.

1.3.6 Estimated costs of screening

To gauge the economic feasibility of such screening, we estimated the costs that would be associated with the proposed program if it was successfully implemented at
the high-risk obstetric unit at SJHC (a site with a particularly high prevalence of prenatal alcohol exposure), as well as, if screening were implemented more generally, at a large primary birthing site in the region where the prevalence of prenatal alcohol exposure is that of the general Grey-Bruce population (Owen Sound Hospital was used as an example). Costs associated with meconium testing and a 6-year developmental follow-up of those identified by the screen were considered in the estimates, which were summarized as costs incurred after one year of meconium screening at SJHC or Owen Sound Hospital and as costs per baby screened. All estimates were based on current prices in Canadian dollars and assumed perfect participation in the screening program. Additional costs that would be incurred by the public health unit in interventions and support services for those who develop disabilities were not included as these would vary greatly with each case and could not be accurately estimated.

For meconium testing, costs of FAEE analysis and shipping of meconium samples were considered. Sample collection containers, freezers, and nursing staff who would administer consent and collect samples are part of existing infrastructure and would likely not be associated with significant additional costs. The number of neonates expected to test positive for prenatal alcohol exposure, and thus be enrolled in follow-up, was based on the rates observed with anonymous testing at SJHC (Goh et al., 2010) and at primary Grey-Bruce birthing sites, such as Owen Sound Hospital (Gareri et al., 2008). The developmental follow-up of identified positive cases was to be conducted through Healthy Babies Healthy Children program by a public health nurse, as well as, by a clinical psychologist. We considered the number of visits required for the developmental assessments, length of visits, salary of the public health nurse, and cost of assessments by a clinical psychologist. It is important to note that the follow-up through Healthy Babies Healthy Children typically involves more frequent home visits by the public health nurse, as well as by a parent support worker, who work with the family on various areas of needs. However, since these may vary in frequency depending on the family’s needs, here, we only estimated the number and cost of visits necessary for the developmental assessments.
1.3.7 Ethics
The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the research ethics boards of the Hospital for Sick Children and the University of Western Ontario.

1.4 Results

1.4.1 Willingness of mothers to participate in open screening program
Sixty mothers from Grey-Bruce were identified at SJHC and offered meconium screening, of which 47 women consented, totaling a 78% consent rate (Figure 4.1). The maternal and neonatal characteristics of consenting subjects are presented in Table 4.1. This consent rate was significantly lower than in the previous study where meconium was tested anonymously, in which 95% (54/57) of women agreed to testing (p = 0.014). Of interest, we documented one instance where a woman recalled participating in the anonymous prevalence study with her previous baby, but refused to participate in this open screening program when told there would be follow-up of positive cases. Additionally, we encountered a woman who, although agreed to participate, was uncooperative, repeatedly neglected to notify the nurses that meconium had passed, and discarded the samples so that none were collected.
Table 4.1 Characteristics of participating women and neonates.

<table>
<thead>
<tr>
<th>Maternal Medical History*</th>
<th>Number of Cases</th>
<th>Percent of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chronic Conditions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression</td>
<td>6</td>
<td>13.64</td>
</tr>
<tr>
<td>Asthma</td>
<td>5</td>
<td>11.36</td>
</tr>
<tr>
<td>Genetic/hereditary disorder</td>
<td>4</td>
<td>9.09</td>
</tr>
<tr>
<td>Endocrine disorders</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>Sexually transmitted disease</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>Congenital anomalies</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>OTHERa</td>
<td>12</td>
<td>27.27</td>
</tr>
<tr>
<td><strong>Previous pregnancy-related history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous caesarian section</td>
<td>8</td>
<td>18.18</td>
</tr>
<tr>
<td>Anatomical uterine anomalies</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>Previous postpartum depression</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>Previous fetal demise</td>
<td>2</td>
<td>4.55</td>
</tr>
<tr>
<td>Previous chromosomal and other congenital anomalies</td>
<td>2</td>
<td>4.55</td>
</tr>
<tr>
<td>Previous neonatal death</td>
<td>2</td>
<td>4.55</td>
</tr>
<tr>
<td>OTHERb</td>
<td>4</td>
<td>9.09</td>
</tr>
<tr>
<td>*<em>Current Pregnancy Complications</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premature pregnancies (&lt; 37 weeks) (n=43)</td>
<td>18</td>
<td>41.86</td>
</tr>
<tr>
<td>Twin pregnancies (n=47)</td>
<td>10</td>
<td>21.28</td>
</tr>
<tr>
<td>Gestational hypertension</td>
<td>4</td>
<td>9.09</td>
</tr>
<tr>
<td>Placental abruption</td>
<td>4</td>
<td>9.09</td>
</tr>
<tr>
<td>Preterm premature rupture of membranes</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>Postdated labour</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>Breech present</td>
<td>3</td>
<td>6.82</td>
</tr>
<tr>
<td>OTHERc</td>
<td>12</td>
<td>27.27</td>
</tr>
<tr>
<td>*<em>Delivery information</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cesarean section</td>
<td>16</td>
<td>36.36</td>
</tr>
<tr>
<td>Vaginal</td>
<td>28</td>
<td>63.64</td>
</tr>
<tr>
<td>**Fetal/Neonatal Characteristics †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premature babies(&lt; 37 weeks) (n=52)</td>
<td>26</td>
<td>50.00</td>
</tr>
<tr>
<td>Intrauterine growth restriction</td>
<td>4</td>
<td>7.55</td>
</tr>
<tr>
<td>Chromosomal and or congenital malformation</td>
<td>3</td>
<td>5.66</td>
</tr>
<tr>
<td>Fetal arrhythmia</td>
<td>2</td>
<td>3.77</td>
</tr>
<tr>
<td>Large for gestational age</td>
<td>1</td>
<td>1.89</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>1</td>
<td>1.89</td>
</tr>
<tr>
<td>Admitted to NICU</td>
<td>27</td>
<td>50.94</td>
</tr>
<tr>
<td><strong>APGAR score (Mean ± SD):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1min (n=51)</td>
<td>7.27 ± 2.26 (median = 8)</td>
<td></td>
</tr>
<tr>
<td>5min (n=52)</td>
<td>8.25 ± 1.54 (median= 9)</td>
<td></td>
</tr>
</tbody>
</table>
*Data were obtained for 44 of 47 consenting women (unless otherwise stated) whose charts contained completed antenatal forms with the information of interest. The remainder did not have completed antenatal forms in the chart when data was collected.

† Data were obtained for 53 of 57 neonates (unless otherwise stated) whose charts contained the information of interest. The remainder did not have the information in the chart at the time of data collection.

a Other histories of chronic maternal conditions included epilepsy, hepatitis, hypertension, diabetes, group B Strep-positive, bipolar disorder, unspecified psychiatric condition, and idiopathic thrombocytopenic purpura.

b Other previous pregnancy-related histories included postpartum hemorrhage, oligohydramnios, gestational diabetes, and gestational hypertension.

c Other pregnancy complications included advanced maternal age (≥40 years old), gestational diabetes, placenta previa, oligohydramnios, chorioamnionitis, preeclampsia, HELLP syndrome, postpartum hemorrhage, and Rh sensitization
1.4.2 Positivity for prenatal alcohol exposure in pilot screening program

Samples were collected from 50 neonates, of which 39 were successfully analyzed (Figure 4.1). Only one of the 39 samples tested above the positive cutoff (≥2 nmol/g) for FAEE (52 nmol/g), which translated to a 3% positivity rate for prenatal alcohol exposure. Eleven samples were non-analyzable because of presence of contaminants and postnatal stool that impeded chromatography. The observed 3% positivity rate for prenatal alcohol exposure was ten-fold lower than the 30% (15/50) positivity rate observed under anonymous conditions in the previous study by Goh and colleagues (p < 0.001).
1.4.3 Maternal self-report of alcohol use in pregnancy

Chart reviews did not reveal that substance abuse (alcohol or drugs) was the primary reason for referral to SJHC in any of the cases. Three women admitted to consuming any amount of alcohol in pregnancy on their prenatal intake forms, including the woman whose baby’s meconium tested positive for FAEEs. However, none reported alcohol use that was concerning to health-care providers, with one woman stating that she drank 2 drinks per week, another reporting that she consumed an “occasional drink”, and the third stating she drank only prior to her knowledge of pregnancy.

1.4.4 Estimated costs of screening

The estimated costs of the proposed screening program at SJHC and at Owen Sound Hospital (primary regional birthing site) are presented in Table 4.2. As expected, the annual costs of meconium testing alone would be much higher at a primary birthing site ($51,571 - 107,042/year) compared to SJHC ($8,371 - 12,271/year), owing to the much larger number of births. However, despite the much greater number of babies that would be screened at Owen Sound Hospital, roughly the same number of babies (15 - 16/year) would be flagged for follow-up after one year of meconium testing because of the much lower prevalence of FAEE-positive meconium (2.5% vs. 30%) (Gareri et al., 2008). Thus, the costs of a 6-year follow-up of all positive cases identified after a year of meconium screening would be comparable between the two sites, falling in the range of $50,000 - 94,000. The cumulative cost of all program components; that is, meconium testing for one year and 6-year follow-up of all identified positive cases, would be almost twice as much at Owen Sound Hospital ($106,171-200,642) as at SJHC ($58,771 - 98,671) while identifying roughly the same number of cases.

Costs associated with the proposed screening program might be lowered in several ways. Firstly, batch processing of samples in established designated laboratories and weekly batch shipping of samples would halve the total costs associated with meconium testing in a large birthing site like Owen Sound Hospital (as compared to twice-weekly shipping and present cost of FAEE analysis). Furthermore, a
large proportion of follow-up costs were associated with neurodevelopmental assessments by a clinical psychologist, which currently range in price from $700 to $1500 per assessment depending on the qualifications of the assessor. Limiting it to only two assessments (e.g., at 1 year and 6 years of age) would reduce the total cost of follow-up to $2,660 – 4,260/baby. Alternatively, the clinical psychologist may be called upon only if the public health nurse detects developmental delays during brief assessments with tools such as the ASQ. This would significantly reduce the costs, but might increase the risk of not finding more subtle cognitive and behavioral issues that might lead to secondary disabilities later on.

Table 4.2. Estimated costs of the proposed meconium screening program if implemented at the high-risk obstetric unit in St. Joseph’s Health Care or at a primary regional birthing site (Owen Sound Hospital).

<table>
<thead>
<tr>
<th>Input costs and details</th>
<th>St. Joseph’s Health Care*</th>
<th>Owen Sound Hospital†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meconium Testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAEE analysis(^a)</td>
<td>$72-150/sample</td>
<td>$3,600 - 7,500/year</td>
</tr>
<tr>
<td>Shipping supplies(^b)</td>
<td>Containers: $18/box</td>
<td>$2,691/year</td>
</tr>
<tr>
<td></td>
<td>Dry ice:$2.25/lb</td>
<td></td>
</tr>
<tr>
<td>Shipping(^c)</td>
<td>To Motherisk Laboratory,</td>
<td>$2,080/year</td>
</tr>
<tr>
<td></td>
<td>Toronto, ~$40/box</td>
<td></td>
</tr>
<tr>
<td>Total cost of meconium testing</td>
<td>Including shipping</td>
<td>$8,371 - 12,271/year</td>
</tr>
<tr>
<td></td>
<td>$167.42 - 245.42/baby</td>
<td>$79.34 - 164.68/baby</td>
</tr>
<tr>
<td></td>
<td>Excluding shipping</td>
<td>$6,291 - 10,191/year</td>
</tr>
<tr>
<td></td>
<td>$125.82 - 203.82/baby</td>
<td>$76.14 - 158.28/baby</td>
</tr>
</tbody>
</table>

Developmental Follow-Up of Positive Cases (6-year)\(^d\)

| Assessments by public health nurse\(^e\) | $1,260/baby | $18,900 | $20,475 |
| Assessments by clinical psychologist\(^f\) | $2,100 - 4,500/baby | $31,500 - 67,500 | $34,125 - 73,125 |
| Total cost of follow-up               | $3,360 - 5,760/baby | $50,400 - 86,400 | $54,600 - 93,600 |

Total Cost of Screening Program

| Meconium testing over one year and 6-year follow up of all identified positive cases | $58,771 - $98,671 | $106,171 - $200,642 |
1.5 Discussion

To our knowledge, this is the first study to utilize biomarkers of fetal alcohol exposure in an open screening program designed to facilitate diagnosis and treatment of alcohol-affected children. The participation and positivity rates in this pilot program were significantly lower than those observed when testing was offered anonymously in the same high-risk unit, suggesting that many women who consumed alcohol in pregnancy refused to participate, not wishing to be identified by the screen. Of interest, if we assume that all refusals were in fact positive samples, the positivity rate in our population would total 27%, which is similar to the positivity rate observed by Goh and colleagues with anonymous meconium testing (Goh et al., 2010). These results suggest that, despite the offered benefits and the probable value of such screening programs to child health, women’s unwillingness to consent may diminish the value of open
population-based screening requiring parental consent. Since this was likely due to embarrassment, guilt, and fears of stigmatization and child apprehension (despite assurance otherwise); our results further shed light on important social factors surrounding alcohol use in pregnancy that are impeding not only the recognition of this problem, but also the efforts to address it.

Strategies to improve participation would need to be investigated if meconium screening with parental consent were implemented in clinical practice. For example, the “opt-out” method to gain consent was shown to yield higher testing rates in neonatal HIV screening (Public Health Agency of Canada, 2006). Participation rates may also increase with social marketing, public education, and as the test becomes established in society. Alternatively, jurisdictions may argue that testing should be conducted without maternal consent since it is in the best interest of the child. If this occurs, the screening program piloted here may serve as a model for a program that can be implemented in a clinical setting since it utilized currently existing services in the community. The developmental follow-up of children identified by the screen was integrated into Ontario’s Healthy Babies Healthy Children program, and assistance to children displaying delays was provided through existing community health programs and services, such as speech and language, infant and child development, and parenting support; which may all be adapted to incorporate interventions and strategies shown to be effective in assisting children with FASD and their families (Bertrand, 2009; Peadon et al., 2009).

Potential benefits of such programs were exemplified by the single positive case identified by the screening. Follow-up of the infant’s development proved of immense value as delays in motor development became apparent starting at 6 months of age. At 14 months, an assessment conducted by a clinical psychologist revealed delays in gross motor and expressive language functioning. These early assessments prompted referrals to appropriate infant and child development programs in the area. Although it is unclear whether the observed delays were alcohol-related or perhaps due to other
factors, this particular infant has benefited from close monitoring. It is possible that positive examples such as this case may serve as an educational tool to increase maternal participation rates in future programs of this nature.

Low participation is not the only potential obstacle to implementing screening in clinical practice. If participation can indeed be improved, the costs and resources required for testing, follow-up, diagnosis, and interventions, as well as the system capacity to handle these cases, must be considered. Although screening targeted to high-risk populations, such as the high-risk obstetric unit at SJHC, is an opportunity for cost-savings (as demonstrated in our cost estimates), such high-risk sites would first need to be identified, and many alcohol-exposed babies not delivered at these sites will be missed. Importantly, our estimates did not consider the costs of interventions, diagnosis, and support programs, which at present, may not even be equipped to handle the large number of cases expected if screening is implemented, and would thus need to be prepared in advance (Goh et al., 2008).

To date, two health economics studies examining similar hypothetical screening programs showed that reduction in secondary disabilities and primary prevention of FASD by intervention and education of mothers may lead to societal savings even if screening is implemented universally and not just targeted to high-risk populations (Gifford et al., 2010; Hopkins et al., 2008). However, many input variables in these studies were theoretical and incomplete, and we believe that the present study can aid future cost-effectiveness and cost-benefit studies since it provides a realistic model for program implementation along with estimated costs of program components. Furthermore, both of the above mentioned health economics studies assumed perfect maternal participation and did not consider how low participation in screening and interventions might impact the cost-effectiveness and cost-benefit ratios of such programs. Since we demonstrated that this will be a major issue in screening for prenatal alcohol exposure, future health economics studies should account for this, at the very least, by including public education and marketing campaigns as additional
costs.

Several ethical considerations could also hinder screening implementation. Although screening can improve quality of life through early diagnosis, detecting maternal drinking during pregnancy and labeling children as “at-risk” may carry serious psycho-social implications for these children and their families, and affect relationships both within the family and between the family and society, including their service providers (Marcellus, 2007). The test results may potentially be misused by courts, social services, insurance companies, and even within the health-care system through stigmatization of patients resulting in their under-treatment. To ensure that families experience maximum benefits and minimum risks, issues surrounding confidentiality, access to results, and their appropriate use, must be carefully considered.

Using meconium analysis as a means of screening for alcohol use in pregnancy has many advantages but also some important limitations. Meconium screening is advantageous over self-reporting since it is an objective method for assessing in utero alcohol exposure, and is valued because its collection is easy and non-invasive because it is discarded material. However, the window for meconium collection is limited to the first few days of life, which may be problematic. In our study a large number of samples were non-analyzable because of delayed sample collection that led to contamination with postnatally produced stool. These samples were excluded from analysis since contamination interferes with the chromatography (high background noise impedes quantification) and since these samples were no longer representative of in utero exposure. Second, since FAEEs in meconium are heat and light sensitive, storing and shipping conditions must be carefully monitored, which may be problematic in remote communities. Specimens that are not immediately frozen or shipped on ice are at risk for FAEE degradation. Moore and colleagues reported that samples stored at room temperature in the light lost 86% of the total FAEE concentration within 24 h, refrigerated samples lost only 10% within 24 h and up to 18% within 3 days, and that the most stable specimens were those stored in the freezer, which lost only 11% of the
total FAEEs over 6 days and did not lose ≥50% for at least 43 days (Moore et al., 2003). Overall, improper sample collection and handling may be an important issue affecting the usefulness of this test.

An important advantage of meconium is that, in contrast to urine or blood, it is a cumulative matrix that may provide a record of exposure over a prolonged period of time (last two trimesters). It is important to keep in mind, however, that meconium begins to form in the fetal intestine in the second trimester and accumulates mostly in the latter part of pregnancy (Burd and Hofer, 2008). Thus, prenatal alcohol exposure may not be detectable by meconium analysis if it had occurred earlier in pregnancy but the mother had largely abstained later on. Moreover, more research is needed on the timing of meconium accumulation in order to determine the gestational age at which a sufficient volume of meconium has accumulated to allow for FAEE analysis, and the gestational time window during which prenatal alcohol exposure will be detectable via meconium analysis (Burd and Hofer, 2008). It must also be emphasized that although meconium FAEEs are well studied fetal biomarkers of prenatal alcohol exposure, additional research is needed on the ability of this test to identify different timing, patterns, and levels of drinking in pregnancy, as information is still lacking on how these variables can affect the sensitivity and specificity of the test.

At present, there is no consensus among studies on the positive cutoff that should be used because of heterogeneity of study methodologies and use of different FAEE species and analytical methods (Burd and Hofer, 2008). In this study (and in the anonymous study by Goh and colleagues), a 2 nmol/g cutoff (~600 ng/g) sum of four FAEEs was utilized as indicative of heavy prenatal alcohol exposure as was established in a large population-based FAEE baseline study (Chan et al., 2003). This cutoff was reported to have 100% sensitivity and 98.4% specificity for detecting heavy alcohol exposure, however, this was based on a very limited number of confirmed drinkers. For this reason, the sensitivity of this cutoff requires further validation across a broad spectrum of drinking behaviors; but given the large number of abstainers that were
assessed in this baseline study, it likely rules out the majority of non-drinkers. The 2 nmol/g cutoff is higher than cutoffs proposed by some groups (for example, Bearer et al. established a cutoff value of 32 ng/g for ethyl oleate alone) (Bearer et al., 2003), but is more comparable to the 500 ng/g cutoff utilized by others (Bakdash et al., 2010; Hicks, 2007). It cannot be excluded that this higher cutoff, while effectively ruling out non-drinkers, may preclude detection of lower levels of exposure.

Lastly, although associations have been reported between meconium FAEE levels and several alcohol-related outcomes (Brien et al., 2006; Derauf et al., 2003; Hutson et al., 2010; Jacobson, 2006; Noland et al., 2003; Peterson et al., 2008), the proportion of infants with FAEE-positive meconium who will go on to develop disabilities is unknown and we were unable to conduct a case-control study looking at developmental outcomes because of the low participation rate. Such information is important to obtain as it would further validate the use of these biomarkers in identifying children at risk for alcohol-related disabilities.

Our study has limitations. The pilot screening program was implemented at a birthing site outside of the Grey-Bruce region to which high-risk pregnancies are transferred, which was chosen due to the high prevalence of FAEE-positive meconium shown in a previous anonymous study. Because Grey-Bruce communities are fairly small in size, the results might have been different if screening had been implemented at a primary Grey-Bruce birthing site, where patients might be more trusting of their health-care providers, who likely provided them with prenatal care. Alternatively, because the community is small, women might be even less likely to consent because of fears of stigmatization and other social implications that might be more pronounced in a small community. Whatever the case may be, the results may not be applicable to a primary health-care setting in a small community, but possibly resemble what may be observed in a primary birthing site of large city, where women are less likely to be acquainted with the hospital personnel. Another limitation is that although we estimated the costs that would be associated with our proposed screening program, these only
give a rough idea of how much a program of this nature might cost, assuming problems like low participation can be worked out. These costs are based on current prices and will likely vary depending on the site of screening implementation, as well as, with time. It should be noted that we estimated only the cost of the screening program and did not include the additional costs that may be incurred but the public health unit in interventions and support services for those who develop disabilities. These could not be reliably quantified since the programs and services accessed by the family will depend on the overall family situation and the infant’s development.

To summarize, this is the first study to implement an open neonatal screening program for prenatal alcohol exposure aimed at facilitating detection and management of FASD. Follow-up, interventions, and support programs were individualized and offered within the framework of currently available services in the area, thereby modeling a program that could be implemented in clinical practice. We demonstrated that women’s unwillingness to partake in this screening, especially of those who consumed alcohol in pregnancy, may hinder the implementation of such screening programs in clinical practice. Future studies should explore strategies that may improve women’s willingness to consent, as well as, evaluate and address other potential barriers to screening by determining the cost-effectiveness, logistics, and best practices for program implementation.

1.6 Disclosure
GK was supported by a CIHR operating grant and by the Ivey Chair in Molecular Toxicology, University of Western Ontario. IZ was supported by OGS and the University of Toronto Open Fellowship. The authors are not aware of any financial or other relationship that might lead to a conflict of interest.

1.7 Acknowledgments
We would like to thank the nursing staff at St. Joseph’s Health Care for their help with patient screening and sample collection.
1.8 References


exposure among newborns in a high-risk obstetric unit. Alcohol 44: 629-634.


McNamara TK, Orav EJ, Wilkins-Haug L, and Chang G. (2005). Risk during pregnancy...


Pharmacology 16: e91-e102.


2 Clinical Use of Meconium Fatty Acid Ethyl Esters for Identifying Children at Risk for Alcohol-Related Disabilities - The First Reported Case.

2.1 Abstract

Fatty acid ethyl esters (FAEEs) in meconium are validated biomarkers of heavy fetal alcohol exposure that may potentially be used clinically for identifying children at risk for alcohol-related disabilities. However, until now, FAEEs have been largely used anonymously in epidemiological studies, and by child protection authorities in need for verification of heavy alcohol use in pregnancy. Here we describe the first case of a neonate identified as part of a research study on a pilot neonatal screening program for prenatal alcohol exposure. The neonate’s meconium tested high for FAEEs (52 nmol/g; positive cutoff ≥2 nmol/g), which prompted active follow-up of the infant’s development, identifying early neurocognitive problems and allowing initiation of a remedial program.

2.2 Introduction

Prenatal alcohol exposure (PAE) can result in a wide range of physical anomalies and cognitive and behavioural deficits known collectively as Fetal Alcohol Spectrum Disorders (FASD). In North America, FASD affects an estimated 1 percent of live births (Sampson et al., 1997), making it the leading preventable cause of mental retardation and a significant social and economic burden (Lupton et al., 2004; Stade et al., 2009). Although the ethanol-induced damage is irreversible, early diagnosis and management of FASD may decrease the risk of developing secondary disabilities and improve prognosis in affected children (Streissguth et al., 2004). However, identification of children affected by PAE is difficult, with diagnosis often hinging on maternal reports of alcohol use in pregnancy (Chudley et al., 2005); which are unreliable and difficult to obtain.

Fatty acid ethyl esters (FAEE) are products of non-oxidative ethanol metabolism
that are formed when ethanol is conjugated to endogenous free fatty acids or fatty acyl-CoA (Best and Laposata, 2003). During gestation, ethanol ingested by the mother crosses the placenta and undergoes metabolism in the fetal compartment to FAEEs, which then deposit and accumulate in meconium (Koren et al., 2008). As a result, elevated levels of FAEEs in neonatal meconium may serve as objective markers of maternal alcohol use in pregnancy, as has been established in numerous studies (Bearer et al., 2003, 2005; Chan et al., 2003; Ostrea Jr. et al., 2006).

Meconium analysis for FAEEs is currently utilized in the context of child protection, and has been used to anonymously obtain epidemiological data on the prevalence of PAE in select populations (Gareri et al., 2008; Goh et al., 2010; Hutson et al., 2007). As of yet, there has been little use of this test in the context of diagnosing FASD but it has been suggested that it can be used as a screening tool to identify children at risk for disabilities (Goh et al., 2008). Using meconium testing clinically as a screening tool would not only provide a history of PAE required to make a diagnosis but; if coupled to long-term developmental follow-up, interventions, and social supports; may aid early detection and management of alcohol-related disabilities.

We have conducted a study involving a pilot screening program of this nature in a high-risk obstetric unit in London, Ontario, the objective of which was to determine if women would willingly participate in screening that aimed to identify and follow-up ethanol-exposed newborns such that intervention efforts could be initiated in a timely manner if developmental delays emerged (Zelner et al., 2010). During this study, a highly FAEE-positive case was identified and the follow-up of this infant highlights the potential benefits of meconium screening for early identification of at-risk babies.

2.3 The Study

An open meconium screening program for prenatal alcohol exposure was piloted in a high-risk obstetric site previously shown to have a high prevalence of FAEE-positive meconium by anonymous testing. This study has been described in detail elsewhere (Zelner et al., 2010). Briefly, meconium screening with subsequent
developmental follow-up of FAEE-positive cases was offered from November 1, 2008 to May 31, 2010 to all women from a regional population in Ontario who delivered at St. Joseph’s Health Care in London, Ontario. With consent, meconium specimens were collected and shipped to the Motherisk Laboratory at Hospital for Sick Children in Toronto for analysis.

Meconium FAEEs were measured using headspace solid-phase microextraction followed by gas-chromatography with mass spectrometry (HS-SPME GC/MS) according to previously published methodology (Hutson et al., 2009). A positive cutoff of ≥2 nmol/g sum of four FAEEs (ethyl palmitate, linoleate, stearate, and oleate) was considered indicative of heavy PAE (Chan et al., 2003).

Study participants whose neonates tested positive for FAEEs were followed-up through the “Healthy Babies Healthy Children” (HBHC) program; an existing public health program for families with newborns in Ontario. This voluntary program offers free home-visits by a public health nurse who provides assistance, educates, assesses the family’s needs, and devises an appropriate family service plan if ongoing follow-up and support may be of benefit to the family (Ontario Ministry of Health and Long Term Care, 2003). The family service plan for those identified by the meconium screen included regular developmental assessments of the baby by the public health nurse using Ages and Stages Questionnaires® and additional neurodevelopmental testing by a certified clinical psychologist at 3 months and 1-1.5 years of age using the Bayley Scales of Infant and Toddler Development®, Third Edition (Bayley-III). The latter comprehensively assessed cognitive, linguistic, and motor functioning of the infant. Upon detection of developmental delays, the public health nurse made referrals to intervention programs and support services for the baby and, if needed, for the family; all of which were provided at no cost to the family.

The study was approved by the research ethics boards of the Hospital for Sick Children and the University of Western Ontario.
2.4 Identification and Follow-up of the First Positive Case

Meconium screening identified a neonate with high FAEE levels in meconium (52 nmol FAEE/g meconium), suggesting heavy in utero alcohol exposure (see Figure 4.2). Ethyl oleate and ethyl linoleate constituted the largest proportion of the total sum, with levels of 32.87 nmol/g and 17.58 nmol/g, respectively. This neonate was born full term (40 weeks gestation) to a young, primiparous, single mother after an uncomplicated pregnancy and delivery. No complications or concerns were noted in the chart with respect to poor neonatal outcomes such as growth restriction or low birth weight. The APGAR scores at 1 and 5 minutes were 9, and the infant passed the infant hearing test. On antenatal forms, it was reported that the mother had a history of depression a few years prior to pregnancy. She did not take preconceptual folate, denied use of street drugs, but reported smoking during pregnancy (5 cigarettes/day). She also admitted to daily alcohol consumption (3-4 drinks/day) prior to her knowledge of pregnancy (early first trimester). No other risk factors or concerns were noted.

![Figure 4.2 Gas chromatography-mass spectrometry chromatogram of the positive meconium sample from a neonate identified in a meconium screening program for prenatal alcohol exposure. Four fatty acid ethyl esters (FAEEs) were quantified using their corresponding d5-FAEEs as internal standards. The m/z values and abundance factors are displayed on the right.](image-url)
As a result of the positive meconium test, follow-up was arranged as per protocol through the HBHC program and a public health nurse was appointed to manage the case. A family service plan was devised to provide support and guidance in consideration with the mother’s young age and inexperience, low educational attainment and household income, potential challenges of single parenting, and the available resources/programs in the area of residence. The baby was of generally good health, and no concerns with regard to hearing or growth were reported by the public health nurse assigned to the case throughout the duration of the study.

Neurodevelopmental assessment conducted by a clinical psychologist at 3 months of age (using the BSID-III) did not suggest developmental delays. Specifically, the infant performed in the high average range for a 3-month old infant on the Mental and Motor scales of the Bayley (75th and 79th percentiles, respectively), and in the average range in expressive and receptive language abilities (50th percentile). Slight delays in motor development (initially in fine motor, and later in gross motor) became apparent in the 6-month and 8-month assessments conducted by the public health nurse using the Ages & Stages Questionnaire®. At 8 months, the infant would only sometimes perform activities such as rolling from back to the stomach, could not get into the crawling position, and when stood up against furniture, could not hold on without leaning against the furniture or crib wall.

At 14 months of age, the infant was again assessed by a clinical psychologist using the BSID-III, which confirmed the presence of developmental delays. While on the cognitive scale, the infant performed in the average range (63rd percentile), on the Motor Scale of the Bayley, the child performed in the low average range, scoring in the 50th percentile in fine-motor abilities, but in the 9th percentile in gross-motor abilities. Additionally, on the language scales, the infant performed in the low average range, scoring in the 50th percentile in receptive language abilities, but in the 5th percentile in expressive language abilities. The infant has been referred to an infant and child development program and will be enrolled in a language and speech development
program in the area.

2.5 Discussion

This case, to the best of our knowledge, is the first reported instance of using meconium FAEE in a clinical setting to identify and follow-up infants at risk for alcohol-related disabilities in order to facilitate early intervention. The child identified in our pilot screening program tested high for FAEEs, with ethyl oleate and ethyl linoleate levels comprising the largest proportion of the total sum. Both of these esters have been shown by other groups to be the strongest correlates of maternal alcohol consumption (Bearer et al., 1999, 2003; Ostrea Jr. et al., 2006). Developmental follow-up of this child revealed delays in motor and language abilities, particularly in gross motor and expressive language functioning, which were well below age expectations at 14 months (in the 9th and 5th percentile on the BSID-III, respectively). These delays prompted referral to available intervention programs in the area and special focus on developing the impaired skills.

It should be stressed that the child has not yet been referred for diagnostic assessment and that we cannot make conclusions with regard to the cause of the developmental delays as there may be numerous co-existing risk factor (which will be discussed below). It is of interest, however, that the observed delays in motor and language abilities have been described in children with FASD. Many studies on alcohol’s effects on the developing motor system have reported delayed motor development in infants and children with PAE, with both fine and gross-motor dysfunction, as well as, tremors, weak grasp, and poor hand-eye coordination (Kalberg et al., 2006; Mattson and Riley, 1998; O’Leary, 2004; Wacha and Obrzut, 2007). Animal studies have also provided evidence for motor dysfunction, and have consistently found impairments in balance, reflex development, and disturbances in gait following PAE (Meyer et al., 1990; Thomas et al., 1996). Studies have also described the detrimental effects of PAE on both receptive and expressive language abilities, noting articulation disorders and delays in language acquisition, comprehension, language and speech
development, and overall language competence (Coggins et al., 2007; Mattson and Riley, 1998; McGee et al., 2009; Wyper and Rasmussen, 2011).

In agreement with the developmental findings in this case report, two studies have found associations between meconium FAEEs and psychomotor development after controlling for other risk-factors. Peterson and colleagues (2008) have found that increasing levels of FAEEs were associated with poorer mental and psychomotor development during the first 2 years of age (Peterson et al., 2008), while Hicks and colleagues (2007) reported that children with elevated levels of FAEE in meconium were found to be delayed on the BSID-II Psychomotor Development Index at 2 years of age (Hicks, 2007).

In the present case, however, we cannot exclude the role that other risk factors; such as maternal psychopathy, IQ (which was not assessed), prenatal care, social history, and smoking in pregnancy; may have played in bringing about or, at least, contributing to the poor developmental outcomes. Furthermore, we cannot rule out prenatal exposure to other drugs and the effect that these may have had. Although the mother denied using illicit drugs, the reliability of such self-reported information on antenatal forms is questionable, especially considering that the claim of drinking cessation early in pregnancy is inconsistent with the positive meconium result as this matrix does not begin to form until the second trimester. Nonetheless, since many of these other risk factors may be associated with maternal drinking in pregnancy, it is reasonable to presume that the positive meconium test for PAE may identify newborns with several risk factors for poor developmental outcomes in addition to PAE.

In summary, the close follow-up of a baby identified as “at-risk” for alcohol-related disabilities by meconium testing, facilitated early detection of developmental delays and initiation of interventions. This reported case was identified as part of a larger study investigating the potential utility and logistics of offering meconium screening in a clinical setting, and was meant to exemplify how the piloted screening program functioned and the potential benefits that such programs may offer if
implemented in clinical practice. In our pilot program, follow-up and interventions were integrated into existing community health programs, which is likely the most logistically and economically feasible option. Some of these existing programs, particularly those focusing on infant and child development, may even be tailored by including intervention protocols and specific teaching methods that have been reported to be effective at improving skills like language and literacy, learning, math, communication, and behavior in children with FASD (Bertrand, 2009; Peadon et al., 2009). In the future, additional studies focusing on the effectiveness of such interventions will be needed to investigate the full benefits of such programs.

2.6 Acknowledgements and Funding
The study was supported by a CIHR operating grant (GK). GK is supported by the Ivey Chair in Molecular Toxicology, Department of Medicine, University of Western Ontario. IZ is supported by OGS and through the University of Toronto Open Fellowship. The authors have no conflicts of interest to disclose.

2.7 References


Chapter 5 Synthesis of Fatty Acid Ethyl Esters in Mammalian Tissues after Ethanol Exposure: A Systematic Review of the Literature

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[IZ determined selection criteria, performed paper selection, data extraction and summarization, and prepared the manuscript for submission]
1 Abstract

The ability to undergo non-oxidative metabolism from ethanol to fatty acid ethyl esters (FAEEs) varies greatly among tissues and organs. To gain a greater understanding of non-oxidative ethanol metabolism to FAEE, we aimed to collect all published data on FAEE synthesis in mammalian organs and tissues to identify all tissues, organs, and enzymes that are known to, or likely, possess FAEE-synthetic activity. A systematic search for relevant papers was performed and two independent reviewers examined potentially relevant abstracts (articles on FAEEs that pertain to ethanol exposure) to determine whether they met the inclusion criteria. Information on FAEE synthesis was retrieved from papers meeting the inclusion/exclusion criteria and summarized by organ/tissue/matrix examined. The systematic search through four databases yielded 78 articles that investigated FAEE synthesis by tissues, tissue fractions and cell lines, and 29 articles that attempted to purify and/or characterize the enzymes involved in FAEE synthesis. Two enzyme activities have been studied: FAEE synthase (FAEES, which conjugates ethanol and free fatty acid) and acyl-CoA: ethanol O-acyltransferase (AEAT, which conjugates ethanol and fatty acyl-CoA). Both activities are expressed by a variety of different enzymes. FAEES activity is the most widely studied and has been purified from several tissues and shown to be associated with several well-known enzymes, while the identity of enzymes possessing AEAT activity remains unknown. The organs and tissues that have been shown to synthesize FAEEs are discussed, with special emphasis on the studies that attempted to elucidate the enzymology of FAEE synthesis in those tissues.

2 Introduction

2.1 Alcohol metabolism

Both oxidative and non-oxidative pathways of ethanol metabolism exist in the human body. The oxidative pathway is the main route of ethanol metabolism, with 85% of ingested ethanol metabolized in the liver via enzymatic oxidation (Swift, 2003). In this
process, ethanol is converted to acetaldehyde by alcohol dehydrogenase (ADH), cytochrome P450 2E1 of the microsomal ethanol-oxidizing system, and/or catalase, which is then converted to acetate by aldehyde dehydrogenase (ALDH). Non-oxidative metabolism, which constitutes a minor pathway of overall ethanol metabolism, results in the enzymatic production of conjugation products of ethanol and endogenous substrates such as fatty acids, phosphatidylcholine, sulfate, or glucuronic acid. These derivatives of non-oxidative ethanol metabolism are termed fatty acid ethyl esters (FAEEs), phosphaditylethanol (PEth), ethyl sulfate (EtS), and ethyl glucuronide (EtG), respectively (Pawan, 1972; Zimatkin & Deitrich, 1997).

2.2 Synthesis of FAEEs

The esterification of ethanol with fatty acids is the most studied non-oxidative pathway to date and is the focus of this systematic review. Since the 1960s and 1970s when FAEEs were discovered in different cells and tissues, FAEE synthesis has been shown to occur to varying degrees in many mammalian organs. The observed wide variability in FAEE concentrations among different organs, tissues, and cells following ethanol exposure is a result of differential expression of enzymes with FAEE synthetic and hydrolytic activities, presence of competing pathways and substrates, differences in fatty acid substrate availability, tissue composition (which affects FAEE retention and accumulation), and presence of co-factors and FAEE carriers. Despite being the most studied non-oxidative pathway of ethanol metabolism, information on the enzymes that mediate FAEE synthesis in mammals and how these may differ among the different tissues is scattered and limited. Evidence suggests that the enzymology behind FAEE synthesis is complex and that the enzymes involved likely have other principle physiological functions (Best & Laposata, 2003). It is also likely that the importance of any single FAEE-synthetic enzyme to overall FAEE synthesis may be tissue-specific, judging from studies that utilized specific enzyme inhibitors in various tissues (Kaphalia et al., 1999, 2004b).
2.3 Rationale and objective

It is important to gain a greater understanding of the enzymology behind FAEE synthesis and the capacity of different tissues to synthesize these esters for several reasons. First, since they are used as biomarkers of ethanol exposure, the levels of these esters in a particular tissue or matrix following ethanol exposure will depend on the capacity of that tissue to synthesize, retain, and degrade these esters. As such, understanding the origins of FAEEs in a tissue and the enzymology behind FAEE synthesis may aid with the interpretation of tissue levels of FAEE and help with selection of appropriate matrices for analysis. Second, FAEEs have been shown to be toxic to different organs and tissues (Ansari et al., 1995; Szczepiorkowski et al., 1995; Werner et al., 2002) and, therefore, understanding what tissues and enzymes are important in FAEE synthesis may shed light on organ susceptibility to ethanol-induced damage. Third, understanding the enzymology may be important for predicting and understanding how ethanol can affect the metabolism of other xenobiotics and endogenous compounds that utilize the same metabolic pathways, and how their metabolism can be affected in turn. To address some of the knowledge gaps, we aimed to systematically collect and summarize all the available primary literature on this subject in order to identify all mammalian tissues, organs, and enzymes that are known to, or likely possess, FAEE-synthetic activity.

3 Methods

3.1 Literature search

A systematic search for relevant papers was performed by searching MEDLINE, EMBASE, Web of Science, and BIOSIS (MEDLINE and EMBASE were searched via ovidsp interface, while Web of Science and BIOSIS were searched via Web of Knowledge interface) from inception to 9 November 2011, using the search terms (as keywords or text words appearing in title or abstract): FATTY ACID ETHYL ESTERS, FATTY ACYL ETHYL ESTERS, FAEE, “Fatty Acids” AND “Ethyl Esters”, “FATTY
ACID/ACYL ESTERS” AND “ethanol”. This search was supplemented by iterative reviews of the reference lists of relevant published papers as well as a Google scholar search.

3.2 Identification of relevant articles

Two independent reviewers examined potentially relevant abstracts (articles on FAEEs that pertain to ethanol exposure) to determine whether they met the following inclusion criteria: (i) were published in English, (ii) investigated FAEE synthesis and/or accumulation in mammals, mammalian tissues, cells, or cell lines, secondary to ethanol exposure, and (iii) investigated/reported FAEE synthesis by mammalian enzyme(s). Papers were excluded if they: (i) were not published in English, (ii) investigated FAEE-synthetic activity in non-mammalian tissues or enzymes, (iii) investigated FAEE-synthetic activity of engineered enzymes, (iv) measured FAEE levels in tissues without knowledge of ethanol exposure (e.g. to ascertain ethanol consumption), or (v) were not complete original literature (abstracts, conference proceedings, editorials, reviews, etc). Whenever it was not possible to make this determination, the full text article was examined.

3.3 Extraction of data and reporting of results

The following data were extracted from the manuscripts by three reviewers: species or cell lines studied, tissues/organs studied, ethanol exposure conditions and/or conditions and substrates for FAEE synthesis, and enzyme purification/characterization results. Information on FAEE synthesis was grouped by organ/tissue/matrix and summarized in the appropriate sections and tables. Information on mammalian FAEE synthesis (pertaining to all tissues and organs) was summarized separately under the appropriate headings.
4 Results

4.1 Paper selection

The systematic search of four databases yielded a total of 7117 publications using the above mentioned search terms. After removing duplicates, one reviewer screened the remaining 3669 manuscripts based title/abstract to identify articles on FAEEs as it related to alcohol (ethanol) exposure. Two independent reviewers screened the identified 739 relevant titles and abstracts for inclusion/exclusion criteria. Full text versions were obtained whenever it was not possible to make a decision based on the abstract. In case of disagreement, a third reviewer was consulted. Reviewers identified 386 publications that may be pertinent to the subject matter of this review and full text versions were obtained for thorough review and data extraction after exclusion of meeting abstracts, short communications and other non-primary literature. A total of 142 publications were identified as meeting the inclusion/exclusion criteria. Of these, 78 investigated FAEE synthesis by organs, tissues, tissue fractions, and/or cell lines. A total of 29 attempted to purify and/or characterized the enzymes involved in FAEE synthesis. The publications pertaining to FAEE synthesis and enzymology became the main focus of this review. The process of inclusions and exclusions is depicted in Figure 5.1.
4.2 Mammalian FAEE synthesis: General considerations

Since the early report of FAEE detection by Goodman & Deykin (1963) in the total body lipid extracts of homogenized Sprague Dawley rats given $^{14}$C-ethanol intravenously, the presence of FAEE in various mammalian organs after exposure to ethanol has been confirmed and investigated in many studies. Almost all tissues and
organs tested to date have been shown to possess the ability to synthesize FAEEs upon ethanol exposure, the complete list of which can be found in Table 5.1. FAEE synthesis has been studied primarily in rats, rabbits, and humans, but a few studies also utilized other mammalian species such as mice, goats, and pigs. Few negative studies exist, suggesting that the capacity to metabolize ethanol to FAEE is a widely expressed enzymatic activity. The liver, pancreas, and heart are the best-studied organs with respect to non-oxidative ethanol metabolism to FAEE, with the highest number of publications. In 1996, Treloar et al. proposed a generally accepted terminology of FAEE-synthetic enzymes, delineating two enzyme activities: FAEE synthase (FAEES) and acyl-CoA: ethanol O-acyltransferase (AEAT). The term FAEES is used to designate the FAEE-synthetic activity that uses ethanol and free fatty acids as substrates, while AEAT describes the FAEE-synthetic activity that uses ethanol and fatty acyl-CoA (FA-CoA) as substrates. FAEES activity is much better studied than AEAT activity and enzymes with FAEES activity have been purified from several tissues, as will be discussed in more detail below. Although AEAT activity is not as well studied as FAEES activity, and AEAT has not been purified, it has been demonstrated in microsomes and tissue homogenates in virtually all organs tested (Diczfalusy et al., 2001a; Polokoff & Bell, 1978).
Table 5.1 Mammalian tissues and organs in which FAEE-synthetic activity has been measured.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species assayed</th>
<th>Samples tested</th>
<th>Cells and cell lines (intact and fractions)</th>
<th>Activity assayed</th>
<th># of pub's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Rat, rabbit, human,</td>
<td>Tissue homogenate (whole, postnuclear fraction, microsomal fraction, cytosolic</td>
<td>HepG2, FuSAH</td>
<td>FAEE (fatty acid +</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>fraction), tissue lysate, acetonitrile powder extract⁷</td>
<td></td>
<td>ethanol)</td>
<td>X</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Pig, rat, human,</td>
<td>Pancreatic extract⁶, pancreatic slices, tissue homogenate (whole, postnuclear</td>
<td>AR42J, acinar cells</td>
<td>AET (FA-CoA +</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>rabbit, mouse, pig</td>
<td>fraction, microsomal fraction, cytosolic/soluble fraction, supernatant as protein</td>
<td></td>
<td>ethanol)</td>
<td>X</td>
</tr>
<tr>
<td>Heart</td>
<td>Rat, rabbit, human,</td>
<td>Isolated perfused heart, perfused heart in vivo, heart slices, tissue homogenate</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>mouse, pig</td>
<td>(whole, microsomal fraction, supernatant as protein+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>enzyme fraction and soluble/cytosolic fraction).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood and</td>
<td>Human, rat, rabbit</td>
<td>Plasma (post-heparin, normal), whole blood, serum, platelet-rich/poor blood,</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>vasculature</td>
<td></td>
<td>perfusate from isolated perfused heart, aorta homogenate.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Rat, human, rabbit,</td>
<td>Gray and white matter tissue homogenates (whole, supernatant as enzyme fraction,</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>microsomal protein).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose</td>
<td>Rabbit, human, rat</td>
<td>Tissue homogenate (whole, inflammatory, protein), acetonitrile powder</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extract⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Rat, rabbit, human</td>
<td>Tissue homogenate (whole, supernatant as protein fraction, microsomal protein,</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetonitrile powder extract⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI tract</td>
<td>Rat, human</td>
<td>Intestinal slices (jejunal rings), microsomal protein from intestinal mucosa,</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tissue homogenate of stomach, intestine, gastric circular muscle, duodenal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mucosa (whole, supernatant as protein source), acetonitrile powder extract⁶,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small intestine in vivo (intraduodenal infusion).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Rat, rabbit, human</td>
<td>Tissue homogenate (whole and supernatant as enzyme fraction, microsomal protein)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Fetal tissue</td>
<td>Human, mouse, rat</td>
<td>Tissue homogenate (supernatant) of human and mouse placenta, mouse fetux/embray,</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>human fetal brain.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hair</td>
<td>Human</td>
<td>Hair on scalp and collected</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Testis</td>
<td>Rat</td>
<td>Tissue homogenate (supernatant as enzyme fraction), acetonitrile powder</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Spleen</td>
<td>Rat, human</td>
<td>Tissue homogenate, microsomal protein.</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Kidney</td>
<td>Rat</td>
<td>Microsomal protein, acetonitrile powder extract⁶</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>Human</td>
<td>Tissue homogenate.</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Eye</td>
<td>Rat</td>
<td>Homogenates of choroid, cornea, iris, ciliary body, lens (ND), retina</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Milk</td>
<td>Goat</td>
<td>Freshly secreted milk</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

*Studies that measured FAEE-synthetic activity of purified or commercial enzymes are not listed in this table. These can be found in Table 4. FAEE synthesis by the bone marrow was mentioned in one abstract but excluded as it did not meet the inclusion/exclusion criteria. Negative studies: Large (1982) did not observe FAEE synthesis from FA-CoA and ethanol in rabbit heart homogenates. Hamamoto et al. (1993) did not observe FAEE synthesis from ethanol and free fatty acid in skeletal muscle and brain of rats. Born et al. (2006) did not observe FAEE synthesis from free fatty acid and ethanol in the eye lens of rats. AET: acyl-coenzyme A: ethanol O-acetylmtransferase; FA-CoA, fatty acid coenzyme A; FAEE, fatty acid ethyl ester; FAEEs, fatty acid ethyl ester syntheses; ND, not detected; RBC, red blood cell; WBC, white blood cell.

⁷For extraction protocol, see Newcomer & Ratnay (1983).

⁸For extraction protocol, see Tsujita & Calza (1992).
4.3 Localization of FAEES and AEAT activity

It is apparent that FAEES and AEAT activities are expressed by different enzymes, with AEAT activity localized to the microsomal fraction and FAEES activity in both cytosolic and microsomal fractions (as well as mitochondrial, lysosomal, and likely others; Kabakibi et al., 1998; Treloar et al., 1996). AEAT activity has been shown to have a luminal enzyme activity in that the active site is located on the cytoplasmic surface of microsomal vesicles (Diczfalusy et al., 1999, 2001a; Polokoff & Bell, 1978). It uses fatty acyl-coenzyme A (FA-CoA) as its substrate rather than the free fatty acid released by hydrolysis of FA-CoA. This is shown by the increased rate of FAEE formation from oleic acid in human liver microsomes with the addition of coenzyme A, adenosine triphosphate, and Mg\(^{2+}\), and the highest rates with oleoyl-CoA as substrate (Treloar et al., 1996). Although there is some disagreement between several early studies over the presence of FAEES activity, particularly in microsomes (Grigor & Bell, 1973; Newsome & Rattray, 1966), later studies have shown that significant FAEES activity is found in both microsomal and cytosolic fractions of various organs (higher in cytosolic fractions). This suggests either weak binding of the enzyme responsible to the microsomal membrane, or the existence of more than one enzyme catalyzing the reaction (Treloar et al., 1996).

4.4 Differential inhibition of FAEES and AEAT activity

Studies utilizing enzyme inhibitors indicate that the two activities are mediated by distinct enzymes (Table 5.2). One group showed that the serine esterase inhibitor bis-para-nitrophenylphosphate (BNPP) increased AEAT activity but inhibited FAEES activity in rat and human liver microsomes (Diczfalusy et al., 1999, 2001a, 2001b). Another inhibitor, p-hydroxymercuribenzoic acid (P-HMB, a cysteine-reacting agent) inhibited AEAT activity in human liver microsomes but had no effect on FAEES activity (Diczfalusy et al., 1999, 2001b). In rat liver microsomes, the acyl-CoA: cholesterol acyltransferase (ACAT, EC 2.3.1.38) inhibitor DL-melinamide decreased AEAT activity,
but had no effect on FAEES activity (Diczfalusy et al., 1999). Overall, inhibitor studies suggest that the enzyme(s) responsible for FAEES activity in liver microsomes contains an active site serine involved in the catalytic actions of the protein and is likely associated with serine-dependent esterases, which could be a microsomal carboxylesterase (CE, EC 3.1.1.1) as suggested by others. In contrast, the situation with microsomal enzyme(s) with AEAT activity is less clear, with some conflicting results with regard to the effect of serine esterase inhibitors (Diczfalusy et al., 1999, 2001a, 2001b; Polokoff & Bell, 1978; Treloar et al., 1996), some of which may be a result of species differences (see below). In general, it appears that, at least in humans, AEAT activity is unlikely to be mediated by serine-dependent CEs. The increased AEAT activity observed in the presence of BNPP is likely due to inhibition of microsomal CEs that compete with AEAT for the substrate (FA-CoA) and can degrade the synthesized FAEEs.
Table 5.2 Effect of specific enzyme inhibitors on FAEE synthetic activity in organs/tissues/cells assayed.

<table>
<thead>
<tr>
<th>Inhibitors tested (target)</th>
<th>Effect on FAEE synthesis, AEAT or FAEES activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>THL (lipases)</td>
<td>FAEE synthesis in isolated rat acini&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>DL-melanimide (ACAT)</td>
<td>FAEE synthesis in meconium&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sandoz S8-025 (ACAT)</td>
<td>AEAT activity in rat liver microsomes but ↔ AEAT activity in human, ↔FAEES activity in both&lt;sup&gt;3,6&lt;/sup&gt;</td>
</tr>
<tr>
<td>pCMBS (acyl-CoA hydrolase)</td>
<td>↔AEAT activity in human liver microsomes&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>DFP (CE, specifically serine esterase)</td>
<td>AEAT activity in rat liver microsomes&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>pMSP (CE, specifically serine esterase)</td>
<td>AEAT activity in human liver microsomes&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>FNMP (CE)</td>
<td>AEAT activity in rat liver microsomes&lt;sup&gt;3&lt;/sup&gt;, ↑AEAT activity in human and rat liver microsomes but ↓FAEES&lt;sup&gt;3,6&lt;/sup&gt;</td>
</tr>
<tr>
<td>phMB (a cysteine-reacting compound)</td>
<td>↑AEAT in rat liver microsomes, ↓AEAT in human, ↔FAEES in both&lt;sup&gt;3,6&lt;/sup&gt;</td>
</tr>
<tr>
<td>TOTP and TOTP metabolites (3/serine esterase)</td>
<td>FAEES in hepatic FN fraction of rats dosed with TOTP and HepG2 cells (intact, PN fraction) ↓FAEES activity in rat plasma and whole blood ↔FAEES activity in rat brown fat homogenate/PN fraction ↔FAEES activity in tissue homogenates and PN fractions of pancreatic tissue in rats dosed with TOTP and AR42' cells&lt;sup&gt;8,9,10&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-BCP (cholesterol esterase)</td>
<td>↔FAEES in HepG2 and hepatic FN fraction in rats dosed with 3-BCP ↔FAEES activity in PN fractions of AR42' cells and pancreatic tissue in rats dosed with 3-BCP&lt;sup&gt;8,9,10&lt;/sup&gt;, FAEE synthesis in intact AR42' cells&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Haber et al., 2004; <sup>2</sup>Zelner et al., 2012; <sup>3</sup>Pokoloff & Bell, 1978; <sup>4</sup>Treolar et al., 1996; <sup>5</sup>Dziakul et al., 1999, 2001a; <sup>6</sup>Kaphalia et al., 1999, 2004b; <sup>7</sup>Meier et al., 2002, 2004; <sup>8</sup>Wa et al., 2008. 3-BCP, 3-benzyl-5-chloro-2-pyrene; ACAT, acetyl-coenzyme A: cholesterol acyltransferase; AEAT, acetyl-coenzyme A: ethanol O-acyltransferase; BNPP, bis-(4-nitrophenyl) phosphate; CoA, coenzyme A; DFP, diisopropyl fluorophosphates; FAEE, fatty acid ethyl ester; FAEES, fatty acid ethyl ester synthase; pCMBS, p-chloromercuribenzenesulfonate; phMB, p-hydroxymercurobenzoic acid; PMSF, phenylmethylsulfonyl fluoride; PN, postnuclear; THL, tetrahydrolipstatin; TOTP, tri-o-tolyl phosphate.
4.5 Species differences in AEAT activity

It appears that there are species-specific differences with regard to enzymes expressing AEAT activity (Table 5.2). As mentioned above, P-HMB (a cysteine-reacting agent which inhibited AEAT activity in human liver microsomes) increased AEAT in rat liver microsomes, while DL-melinamide (a potent ACAT inhibitor that inhibited AEAT activity in rat liver microsomes) had no effect on AEAT activity in human liver microsomes, suggesting that it may not be associated with ACAT in humans (Diczfalusy et al., 1999, 2001b). In agreement with this, another ACAT inhibitor, Sandoz 58-035, at a concentration that completely inhibits ACAT activity, resulted in only small decreases in AEAT activity in human liver microsomes (Treloar et al., 1996). Moreover, serine and carboxylesterase inhibitors such as diisopropyl fluorophosphates (DFP) decreased AEAT activity in rat liver microsomes (Polokoff & Bell, 1978), while others, such as phenylmethylsulfonyl fluoride, increased AEAT activity in human liver microsomes, likely due to inhibition of substrate (FA-CoAs) and/or product degradation (FAEES) in these assays (Diczfalusy et al., 1999, 2001a). Although this observation may be due to differences in inhibitor structures, it may also be a species-specific difference. The differential effect of inhibitors on AEAT activity in rat and human liver microsomes suggests that AEAT activity in rat microsomes is potentially associated with ACAT, while the enzyme with AEAT activity in humans may be mediated by other enzymes, for example acyl-CoA hydrolase, since p-chloromercuribenzenesulfonate (an acyl-CoA hydrolase inhibitor) was shown to decrease AEAT activity in human liver microsomes (Treloar et al., 1996).

4.6 Tissue differences in FAEES activity

As an additional source of complication, FAEES activity has been shown to be differentially affected by inhibitors depending on the tissue assayed. Tri-o-tolylyphosphate (TOTP, a serine esterase inhibitor) and TOTP metabolites decreased FAEES activity in hepatic post-nuclear fractions of tissue homogenates, in plasma and
whole blood from rats dosed with TOTP, and in HepG2 cells incubated with these inhibitors (Kaphalia et al., 1999, 2004b; Mericle et al., 2002). These findings suggest that the enzyme(s) with FAEES activity in these tissues are most probably related to a CE (Table 5.2). However, TOTP had no effect on FAEES activity in the rat pancreatic post-nuclear fraction of tissue homogenate, brown fat, or in AR42J cell lines. Furthermore, the protein expressing FAEES activity in the pancreas did not cross-react with antibodies to rat adipose tissue FAEES (shown to be a CE), which recognizes the 60 kDa and 84 kDa proteins in liver postnuclear fractions and plasma (Kaphalia et al., 1999; Mericle et al., 2002). The lack of inhibition of FAEES or esterase activity by TOTP or its metabolites in these tissues suggests that the enzyme(s) with FAEES activity are structurally and functionally different than those in liver and plasma. This was an unexpected observation since pancreatic cholesterol esterase (ChE, EC 3.1.1.13), which was hypothesized to be responsible for FAEES activity in the pancreas, is known to be a serine esterase (Kissel et al., 1989) and should have been inhibited by TOTP. Of interest, 3-benzyl-6-Chloro-2-pyrone (3-BCP), an inhibitor of ChE, also did not inhibit FAEES/esterase activity in postnuclear fractions of pancreatic homogenate or in AR42J cells, an observation that could not be explained (Kaphalia et al., 2004b). In contrast, a later study by the same group, in which intact AR42J cells were exposed to ethanol in the presence and absence of 3-BCP, found that 3-BCP inhibited FAEE synthesis by these cells (Wu et al., 2008). Therefore, further studies are needed to understand why TOTP metabolites and 3-BCP differentially affect FAEES activity and FAEE synthesis in different tissues, as it may be related to the differences in enzymes expressing FAEES activity, presence of other FAEE-synthetic enzymes, or possible detoxification pathways for these inhibitors in the organs examined.

4.7 Tissue capacity and relative importance of FAEES and AEAT activity

With regard to relative tissue capacity to synthesize FAEE after ethanol exposure, 16 studies have measured FAEE-synthetic activity in more than one organ under the same assay conditions, thereby allowing for comparison between organs
(Table 5.3). These studies consistently found that FAEES activity is the highest in the pancreas (all studies that assayed this tissue, n = 12), followed by the liver (in all studies that evaluated both organs). The lowest FAEES activity appears to be in blood/serum, skeletal muscle, and testis. As for the rest of the tissues, the ranking varies from study to study, which may be due to species-specific differences, sample chosen for testing (e.g. homogenate versus microsomal fraction), fatty acid substrate used, and assay conditions. In both multi-organ studies that evaluated AEAT activity, the liver and intestinal mucosa had the highest AEAT activity among all organs analyzed, as measured in tissue homogenates obtained from patients undergoing elective surgery (Diczfalusy et al., 2001a), and in microsomes from the same organs in rats (Polokoff & Bell, 1978). While FAEES and AEAT activities per gram of liver in humans were shown to be comparable in vitro to rat liver microsomes in one study (on a units/g liver basis; Treloar et al., 1996), another group showed that under optimal conditions, the capacity of AEAT was considerably higher in rat liver microsomes (Diczfalusy et al., 1999). Also, the same group demonstrated that AEAT activity is considerably higher than FAEES activity in most human tissues (with the exception of the pancreas where the two activities were comparable), suggesting that AEAT activity may be more important quantitatively.
Table 5.3 Relative tissue capacities for FAEE production.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sample/enzyme source tested</th>
<th>Substrates/additives</th>
<th>Incubation conditions (pH, temp, time)</th>
<th>Rank of organs tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapoint &amp; Lange, 1986</td>
<td>Human</td>
<td>Tissue homogenate (10% w/v in 50 mM Tri-HCl pH 8) (postmortem)</td>
<td>[14C] oleate (0.4 mM) and ethanol (200 mM)</td>
<td>60 minutes at 37°C (pH 8)</td>
<td>Pancreas &gt; liver &gt; adipose &gt; heart = brain (cerebral cortex) &gt; skeletal muscle = aorta (nmol/g/hr)</td>
</tr>
<tr>
<td>Makinoue et al., 1991</td>
<td>Rat</td>
<td>Tissue homogenate (10% w/v in 50 mM Tri-HCl pH 8.4)</td>
<td>[14C] oleate (0.4 mM) and ethanol (0.2 M)</td>
<td>45 minutes at 37°C in 50 mM sodium phosphate buffer (pH 7.2)</td>
<td>Pancreas &gt; liver &gt; lung &gt; heart &gt; brain (nmol/g tissue/hr)</td>
</tr>
<tr>
<td>Makinoue et al., 1992</td>
<td>Rabbit</td>
<td>Tissue homogenate (10% w/v in 50 mM Tri-HCl pH 8.4)</td>
<td>[14C] oleate (0.4 mM) and ethanol (0.2 M)</td>
<td>45 minutes at 37°C in 50 mM sodium phosphate buffer (pH 7.2)</td>
<td>Pancreas &gt; liver &gt; lung &gt; heart &gt; brain (nmol/g tissue/hr)</td>
</tr>
<tr>
<td>Carlson et al., 1995a</td>
<td>Rat</td>
<td>Tissue homogenate (10% w/v in 50 mM Tri-HCl buffer pH 8.0)</td>
<td>[1-14C] oleate (0.4 mM) and ethanol (0.2 M)</td>
<td>45 minutes at 37°C in 50 mM sodium phosphate buffer (pH 7.2)</td>
<td>Pancreas &gt; liver &gt; lung (liver higher than pancreas in one experiment) (nmol/g tissue/hr)</td>
</tr>
<tr>
<td>Hayakawa et al., 1990</td>
<td>Rat</td>
<td>Supernatant of tissue homogenate (enzyme fraction, homogenized in 0.01 M Tri-HCl with 1 mM 2-mercaptoethanol, centrifuged at 15,000 g for 10 min, supernatant used in the enzyme assays)</td>
<td>[14C] oleate (0.4 mM) and ethanol (200 mM)</td>
<td>1 hour at 37°C in 50 mM sodium phosphate buffer (pH 7.2)</td>
<td>Pancreas &gt; liver &gt; heart &gt; brain and skeletal muscle ND (nmol/l/hr mg protein)</td>
</tr>
<tr>
<td>Suzuki et al., 1990</td>
<td>Rat</td>
<td>Supernatant of tissue homogenate (prepared in 20 mM sodium phosphate buffer, pH 7.4, centrifuged at 2000 g for 10 min, supernatant used in the enzyme assays)</td>
<td>[14C] oleate (0.4 mM) and ethanol (200 mM)</td>
<td>Time not stated (up to 90 minutes, probably 30) at 37°C in 50 mM sodium phosphate buffer, pH 7.2</td>
<td>Pancreas &gt; liver &gt; intestine &gt; liver &gt; stomach &gt; heart &gt; testes (nmol/l/hr mg protein)</td>
</tr>
<tr>
<td>Shurna et al., 1991</td>
<td>Human</td>
<td>Supernatant of tissue homogenate (28 000 g)</td>
<td>[14C] oleate (0.4 mM) and ethanol (200 mM)</td>
<td>45 minutes at 37°C in 60 mM sodium phosphate buffer, pH 7.2</td>
<td>Pancreas &gt; liver = brain = muscle &gt; heart &gt; lung (nmol/hf per mg)</td>
</tr>
<tr>
<td>Heilh et al., 1995a</td>
<td>Human</td>
<td>Tissue homogenate supernatant (as enzyme source, centrifuged at 150 g for 5 min, supernatant at 15 850 g for 3 min, supernatant removed) (surgical specimen)</td>
<td>[14C] oleate (0.6 mmol/100 µL) and ethanol (0.1 M) + essentially fatty acid free BSA</td>
<td>3 hours at 37°C pH 7.5 (homogenate supernatant in PBS)</td>
<td>Pancreas &gt; liver = brain &gt; muscle &gt; heart &gt; lung (nmol/l/hr mg protein)</td>
</tr>
<tr>
<td>Werner et al., 2001</td>
<td>Rat</td>
<td>Tissue homogenate (1/2 w/v in PBS solution with 1 mmol/L benzamidine, 20 mg/L phenyl(methyl)sulfonyl fluoride, 100 mg/L soybean trypsin inhibitor, 10 mg/L aprotinin)</td>
<td>Ethanol (1 mmol/L) and (1-[14C] oleate (300 mmol/100 µL) + essentially fatty acid free albumin (2 mg/mL) + essentially fatty acid free albumin (2 mg/mL)</td>
<td>3 hours at 37°C</td>
<td>Pancreas &gt; liver (nmol/l/hr mg protein)</td>
</tr>
<tr>
<td>Baurer et al., 1992</td>
<td>Mouse</td>
<td>Tissue homogenate supernatant (centrifuged at 200 g for 5 min)</td>
<td>[14C] oleate (0.4 mM) and ethanol (200 mM)</td>
<td>1 hour at 37°C in 60 mM Tris (pH 7.5)</td>
<td>Liver &gt; placenta &gt; heart (nmol/hf/hr)</td>
</tr>
<tr>
<td>Tsujita &amp; Okuda, 1992a</td>
<td>Rat</td>
<td>Acetone powder extract†</td>
<td>[14C] oleate (0.4 mM) and ethanol (600 mM)</td>
<td>Time not stated at 37°C in 60 mM sodium phosphate buffer, pH 7.0</td>
<td>Liver &lt; adipose &lt; small intestine &lt; kidney &lt; lung &lt; testes (nmol/l/hr mg protein)</td>
</tr>
<tr>
<td>Gukovskaya et al., 2002</td>
<td>Rat</td>
<td>Acinar cell and tissue lysates (tissue or acini in 10 mmol/L Tris-HCl buffer (pH 8.2) containing 1 mmol/L phenyl(methyl)sulfonyl fluoride, 1 mmol/L β-mercaptoethanol, and a protease-inhibitor cocktail. Cell lysates were centrifuged for 20 minutes at 15,000 g and the supernatants assayed)</td>
<td>[14C] oleate (0.1 mmol/L) and ethanol (200 mM and 1 M)</td>
<td>1 hour at 37°C in 33 mmol/L sodium phosphate buffer (pH 7.2)</td>
<td>Pancreatic acini &gt; pancreatic tissue &gt; liver (nmol/l/hr mg protein)</td>
</tr>
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(continued)
Table 5.3 (Continued).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Sample/enzyme source tested</th>
<th>Substrates/additives</th>
<th>Incubation conditions (pH, temp, time)</th>
<th>Rank of organs tested</th>
</tr>
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<tbody>
<tr>
<td>Mericle et al., 2002</td>
<td>Rat</td>
<td>Postnuclear fraction obtained from 10% tissue homogenate In 0.05 M phosphate buffer, pH 7.2, or plasma</td>
<td>[1-14C] oleate (2 μmol/2 mL) and ethanol (50 μmol/2 mL)</td>
<td>2 hours at 37 °C in 50 mM sodium phosphate buffer (pH 7.4)</td>
<td>Pancreas &gt; plasma &gt; liver (nmol/mg protein/h)</td>
</tr>
<tr>
<td>Mericle et al., 2004</td>
<td>Rat</td>
<td>Postnuclear fraction obtained from 10% tissue homogenate In 0.05 M phosphate buffer, pH 7.2, or whole blood.</td>
<td>[1-14C] oleate (2 μmol/2 mL) and ethanol (50 μmol/2 mL)</td>
<td>2 hours at 37 °C in 50 mM sodium phosphate buffer (pH 7.4)</td>
<td>Pancreas &gt; liver &gt; brown fat &gt; whole blood (nmol/h/mg protein)</td>
</tr>
<tr>
<td>Diczfalussy et al., Human 2001a</td>
<td>Human</td>
<td>Tissue homogenate (in 50 mM potassium phosphate buffer with 0.5 mM EDTA, pH 7.4) (from biopsy)</td>
<td>AEAT: ethanol (0.86 M) and [1-14C] palmitoyl-CoA (100 μM) + 2 mg/mL HSA FAEES: ethanol (0.86 M) and [1-14C] palmitate (400 μM)</td>
<td>15-20 minutes at 37 °C in 100 mM potassium phosphate buffer with 1 mM EDTA (pH 7.4)</td>
<td>AEAT: Liver &gt; duodenum &gt; pancreas ≈ gastric ventricular mucosa ≈ heart ≈ lung ≈ gallbladder &gt; adipose FAEES: Liver ≈ pancreas &gt; gallbladder ≈ lung &gt; heart ≈ duodenum &gt; gastric ventricular mucosa ≈ adipose Also assessed serum: AEAT low and FAEES ND (nmol/min/g tissue) Intestinal mucosa ≈ liver &gt; isolated fat cell &gt; kidney &gt; brain &gt; lung ≈ spleen ≈ heart ≈ skeletal muscle (nmol/min/mg)</td>
</tr>
<tr>
<td>Polokoff &amp; Bell, 1978</td>
<td>Rat</td>
<td>Microsomal protein</td>
<td>Ethanol (10 μl in 0.2 mL) and [3H] palmitoyl-CoA (40 μM) + 8 mM MgCl₂ and 0.25 mg/ml BSA</td>
<td>2 minutes at room temperature, in 175 mM Tris-HCl (pH 7.0)</td>
<td></td>
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</table>

Comparison of FAEE-synthetic activity among mammalian tissues/organisms in multi-organ studies. ‘>>>’ indicates a difference of at least 100-fold in synthesis rate.
Publications by Lange & Sobel (1983b) and Kinunen & Lange (1984) were excluded since they measured organ-specific accumulation (reported as nmol/g) rather than synthetic activity (nmol/mg or nmol/min/mg).
AEAT, acyl-coenzyme A: ethanol O-acyltransferase; BSA, bovine serum albumin; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; FAEE, fatty acid ethyl ester; FAEES, fatty acid ethyl ester synthase; HSA, human serum albumin; ND, not detected; PBS, phosphate buffered saline; w/v, weight/volume.

*aNo control mentioned.*

*bProtocol from Mogelson & Lange (1984).*

*cProtocol from Tsujita & Okuda (1992).*

*dProtocol from Kaphalia et al. (1997).*
4.8 FAEE synthesis and enzymology in mammalian organs and tissues

Pancreas.

Elevated pancreatic FAEE concentrations have been observed in rats and rabbits acutely and chronically exposed to ethanol (Estival et al., 1981; Hamamoto et al., 1990; Manautou & Carlson, 1991), as well as in postmortem pancreatic samples from subjects acutely intoxicated at the time of death, where FAEE concentrations were the highest of all other parenchymal organs (Laposata & Lange, 1986). The likely origin of these esters is the pancreatic tissue itself as it possesses one of the highest capacities to synthesize FAEEs (Table 5.3), with acinar cells likely being the main source of non-oxidative ethanol metabolism in the pancreas (Gukovskaya et al., 2002). FAEE-synthetic activity has been demonstrated in pancreatic extract, pancreatic slices, pancreatic homogenates (and various fractions), isolated acinar cells, pancreatic microsomes, as well as pancreatic cell lines (AR42J; Table 5.1). The pancreas possesses both FAEES and AEAT activities, being the only organ where the AEAT activity and FAEES activity are comparably high (Diczfalusy et al., 2001b).

In one of the first reports, Newsome & Rattray (1965) showed that enzymatic esterification of ethanol with free fatty acids in porcine pancreatic extract (“porcine pancreatin”) was increased by bile salt addition, suggesting involvement of ChE. The authors noted that more than one enzyme activity may be involved: an esterase-type process mediating the esterification of ethanol with fatty acid in the “soluble” state, and a lipase-type reaction mediating the lesser esterification observed with acid in the “emulsified” state. Involvement of more than one enzyme was also suggested by Gukovskaya et al. (2002), who reported that the ethanol dose dependence of FAEE synthesis in rat pancreatic acinar cells was approximated by a sigmoid curve (not a hyperbola), which may imply the presence of two or more isoforms of FAEES. Indeed, several pancreatic enzymes have since been shown to possess FAEES activity,
including ChE and triglyceride lipase (TGL, EC 3.1.1.3; Table 5.4). Commercial bovine and porcine pancreatic ChE have been shown to catalyze the synthesis of FAEE from ethanol and free fatty acids (Bhat & Ansari, 1990; Lange, 1982; Tsujita & Okuda, 1994b). ChE purified from pig pancreas was associated with FAEE-synthetic activity based on co-elution and co-precipitations by antibody against purified ChE and identical inhibition of FAEE synthetic activity (from oleate), Bt-NH-Ph hydrolyzing activity, and trioleylglycerol-hydrolyzing activities of the purified enzyme by DFP (Tsujita & Okuda, 1994b). In support of this, FAEES activity purified from the microsomal fraction of pancreatic tissue in rats was also shown to be a ChE based on co-elution of synthetic and hydrolytic activities, and its functional and structural properties (Kaphalia & Ansari, 2003; Table 5.5).

Pancreatic TGL is another enzyme shown to catalyze FAEE formation from ethanol and free fatty acid as demonstrated using a porcine commercial enzyme and characterization of the enzyme with FAEES activity purified from the cytosol (soluble fraction) of human pancreatic tissue (Riley et al., 1990). This purified FAEES was suggested to be a TGL based on its activities, binding properties, and N-terminal amino acid sequence (Riley et al., 1990). In support of lipase involvement, Haber et al. (2004) showed that FAEE production by isolated rat pancreatic acini after incubation with clinically relevant concentrations of ethanol (50 mmol/L and 100 mmol/L) is significantly decreased (91–93% inhibition) in the presence of the lipase inhibitor tetrahydrolipstatin (THL). None of the glutathione-S-transferase (GST, EC 2.5.1.18) isozymes purified from rat and human pancreas showed FAEES activity (Sharma et al., 1991; Singhal et al., 1991), and purified FAEES from human pancreas, which eluted in 3 peaks (suggesting several isoenzymes), did not display GST activity (Sharma et al., 1991).

Liver.

As with the pancreas, human (acutely intoxicated subjects and alcoholics) and animal studies (mice, rats, and rabbits) have demonstrated the accumulation of FAEEs in the liver after ethanol exposure (Clugston et al., 2011; Estival et al., 1981; Hungund
et al., 1988; Kinnunen & Lange, 1984; Laposata & Lange, 1986; Loftus et al., 2011; Manautou & Carlson, 1991). In fact, FAEE levels in this organ can be used as post-mortem markers of pre-mortem ethanol intake (Refaai et al., 2002; Salem et al., 2001). The likely origin of these esters is the liver itself as it has been demonstrated to have one of the highest capacities for FAEE synthesis, with both FAEES and AEAT activity among the highest of all organs (Diczfalusy et al., 2001b; Hamamoto et al., 1990; Laposata & Lange, 1986). Hepatic FAEE-synthetic activity has been demonstrated in liver homogenates, microsomes, tissue extracts, and hepatic cell lines (HepG2 and Fu5AH; Table 5.1). In 1990, Laposata et al. showed that FAEE synthesis by cultured rat hepatoma cells was linearly related to the ethanol concentration in the culture medium, and that the enzymes responsible for FAEE were primarily membrane bound and concentrated in the mitochondrial-lysosomal and microsomal fraction of the rat hepatocytes, rather than in the cytoplasm. Studies using human HepG2 cell line, which is inherently deficient in oxidative pathways, showed that the fatty acid used for FAEE synthesis is derived from a designated intracellular pool of fatty acids (Dan et al., 1998), that the addition of lipoproteins or albumin to the culture medium of HepG2 cells stimulates FAEE secretion when the cells are exposed to ethanol (Hasaba & Laposata, 2001), and that the FAEE species secreted are different from the FAEEs which are present within the organs exposed to ethanol, possibly indicating some selectivity among FAEE molecules for release from the cell (Dan & Laposata, 1997).

FAEES activity in liver microsomes is most probably expressed by various CE isozymes. Commercial porcine hepatic CE was demonstrated to have FAEES activity (Table 5.4; Kaphalia et al., 2004b), and CE and FAEES activities have been shown to co-purify from rat hepatic microsomes and resolve into two peaks: 180 kDa (major), and 60 kDa (minor; Kaphalia et al., 1997; Table 5.5). In the latter study, both FAEES and CE activities associated with the 180 kDa protein (a trimer) were inhibited with β-esterase inhibitor (DFP), and the N-terminal sequence of the first 27 amino acids of the purified enzyme was identical to that of rat hepatic microsomal CE (Kaphalia et al., 1997). Furthermore, this enzyme could also be immunoprecipitated with antibodies against rat
adipose tissue FAEES (shown to be a CE), but not with those against rat pancreatic 
ChE (Kaphalia et al., 1997). The microsomal minor fraction (60 kDa, low molecular 
weight) of FAEES activity was shown to be associated with five distinct isozymes that 
expressed both FAEES and CE (p-nitrophenol acetate- or pNPA-hydrolyzing) activity 
(Kaphalia & Ansari, 2001). These purified low molecular weight proteins were all 
monomers and also cross-reacted with antibodies to rat adipose tissue FAEES, but not 
with antibodies to rat pancreatic ChE, suggesting that they too are related to various 
hepatic microsomal CEs (Kaphalia & Ansari, 2001). However, some inconsistency 
exists as rat hepatic CEs ES-4 and ES-10 were shown to be devoid of FAEES activity in 
another study, which may be a result of isozyme or assay differences (Diczfalusy et al., 
1999).

Heart

FAEEs have been shown to be present in the myocardium after exposure to 
ethanol in animals (Lange et al., 1981; Lange & Sobel, 1983a; Mogelson & Lange, 
1984; Yoerger et al., 2006) and in humans, most notably, in autopsy studies that 
demonstrated elevated FAEE levels in the myocardium of subjects acutely and 
chronically exposed to alcohol pre-mortem (Kinnunen & Lange, 1984; Lange & Sobel, 
1983b; Laposata & Lange, 1986; Yamazaki et al., 1997). As with the liver and pancreas, 
the origin of these FAEEs is likely the myocardial tissue itself as FAEE synthesis has 
been demonstrated in whole/perfused hearts, heart slices, heart homogenates, and 
tissue fractions (Table 5.1). Early experiments performed by Lange laboratory identified 
FAEEs as products of rabbit myocardial ethanol metabolism in isolated perfused rabbit 
hearts, heart slices, and heart homogenates (Kinnunen & Lange, 1984; Lange et al., 
1981; Lange & Sobel, 1983a). The fatty acid source for FAEE synthesis in rabbit heart 
homogenates incubated with ethanol was shown to be non-esterified fatty acids 
themselves, and not diacylglycerides, triacylglycerides, cholesterol esters, or FA-CoA 
(Lange, 1982). This contrasts the findings in other tissues, particularly in the liver, where 
FA-CoA was an excellent substrate for FAEE synthesis (Polokoff & Bell, 1978; Treloar
et al., 1996), and the fact that AEAT activity has been demonstrated in heart homogenates and microsomes of the heart by other groups (Diczfalusy et al., 2001a; Polokoff & Bell, 1978). The reasons for the discrepancy in findings with respect to AEAT activity in the heart are unclear, but may be due to methodological differences between the studies such as homogenate preparation, incubation conditions, as well as possible species-specific differences (rabbit vs. human). In vivo FAEE synthesis by the heart has been shown to be very rapid, as the pig heart produced substantial levels of FAEEs within five minutes of ethanol infusion to the coronary arteries, with greater FAEE concentration at the site where the ethanol directly interacts with the myocardium (Yoerger et al., 2006).

Two FAEES activity peaks, which were separable by diethylaminoethyl (DEAE)-cellulose chromatography, were identified in the soluble portion of rabbit myocardium (Mogelson et al., 1984) and four in the human (Bora et al., 1989a, 1989b, 1989c, 1992a, 1996), all of which catalyze FAEE formation from free fatty acids and ethanol in the absence of ATP and coenzyme A (Table 5.1). In rabbit myocardium, the major peak of enzyme activity was purified to homogeneity and determined to be a 50 kDa dimer (26 kDa identical or nearly identical subunits), that is distinct from ChEs (Mogelson et al., 1984). Four FAEES enzyme activities were demonstrated in human myocardium by Lange’s group, which were purified to homogeneity and eventually designated as synthase-I, synthase-II, synthase-III (in order of their elution from DEAE-cellulose at pH 8.2) and finally, synthase/carboxylesterase (Bora et al., 1989a, 1989b, 1989c, 1992a, 1996). Human myocardial FAEES-III, like the major soluble FAEES in rabbit myocardium, was shown to be a soluble dimeric enzyme comprised of two identical or nearly identical subunits of 26 kDa each (active enzyme is 54 kDa; Bora et al., 1989b, 1989c). Human myocardial FAEES-I was also indicated to be a dimer of the same molecular weight, cross-reacted with antibodies to FAEES-III, and had a highly homologous amino acid composition to FAEES-III (Bora et al., 1989a).
Both synthase-I and III were suggested to belong to the GST family of enzymes based on activity (both had glutathione transferase activity), structural similarity, and amino acid sequence homology to GSTπ-1 (Bora et al., 1989a, 1989b, 1989c, 1991). However, site-directed mutagenesis of FAEES-III by this group showed a differential effect of amino acid substitutions on FAEES and GST activities (Bora et al., 1992b), which raises some doubt that one enzyme protein has both activities. Indeed, the suggestion that FAEES-III and I belong to the GST family of enzymes was seriously challenged by other groups who did not find associations between GST and FAEES enzyme activities (Board et al., 1993; Sharma et al., 1991; Singhal et al., 1991; Suzuki et al., 1990). These groups showed that in human and rat tissues FAEES and GST activity were distinctly separated during chromatographic fractionation and that purified GSTs (various classes) were devoid of FAEES activity (Sharma et al., 1991; Singhal et al., 1991; Suzuki et al., 1990). It was suggested that the two FAEES isozymes isolated by Bora et al. may have been co-purified with GST isoenzymes because of incomplete separation of π and μ class GSTs and FAEES due to similarities in their molecular weight and charge (Sharma et al., 1991). It should be noted that in contrast to Bora’s purification scheme, Sharma et al. (1991) added an extra step during the purification procedure after DEAE-cellulose (glutathione Sepharose 6B affinity chromatography), which bound that majority of GST activity and not FAEES activity. Furthermore, a clone that encodes FAEES-III described by Bora et al. (1991) constructed by another group using site-directed mutagenesis of GSTP1 cDNA was devoid of both FAEES and GST activity, suggesting that the cDNA reported by Bora et al. may have resulted from a cloning artifact (Board et al., 1993).

Less controversy surrounds FAEES-II and synthase/carboxylesterase. FAEES-II was isolated, purified, and characterized in the human myocardium, and, unlike synthases I and III, shown to be a monomer with a molecular mass of 65 kDa (Bora et al., 1992a). Although it dimerized easily, dimerization was not required for its catalytic activity. Since FAEES-II did not cross-react with antibodies to FAEES-III or ChE, lacked ChE and GST activities (although this latter point may be moot), it was deemed not
related to FAEES-I, FAEES-III, or ChE. Furthermore, the N-terminal 17 amino acid sequence of FAEES-II did not correspond to any known N-terminal sequence, indicating that this may be a novel protein (Bora et al., 1992a). Of interest, a common feature of the three synthases is the specificity for unsaturated octadecanoic acids, as rates of synthesis are higher than with saturated fatty acids. Since the binding affinities for the different fatty acids are essentially the same, this may be attributed to the differential thermodynamic properties of the enzyme-substrate complex (Bora et al., 1992a).

The fourth FAEES enzyme (the last to be purified) was shown to be a 62 kDa protein that had both synthase and CE activities and no GST activity (Bora et al., 1996). The N-terminal sequence of the first 17 amino acids was 88% homologous to CE from rat liver and adipose (Bora et al., 1996), and its cDNA was found to be identical to CEs isolated from human eye and liver (Kroetz et al., 1993). Antibodies to this enzyme cross-reacted with human cytosolic, microsomal, as well as mitochondrial fractions, while the microsomal fraction had more than one band suggesting that there may be more than one synthase/CE in the microsomes (Bora et al., 1996). In 2003, Bora et al. conducted molecular characterization of this enzyme by site directed mutagenesis and found that both ser204 and his451 mutations in the active site caused an almost total loss of CE enzyme activity, and significantly lowered FAEES enzyme activity.

In addition to these purified myocardial enzymes, in 1997, Chang et al. demonstrated that isolated rat hearts perfused with chylomicrons labeled with 14C-triglyceride fatty acids in the presence of ethanol synthesize FAEEs as a result of lipoprotein lipase (LPL, EC 3.1.1.34) activity, while addition of apoprotein CII (a LPL cofactor) increases both triglyceride hydrolysis and FAEE synthesis. Thus, LPL, which is present on the lumenal surface of the capillary endothelium of cardiac and skeletal muscle, adipose, and other extra-hepatic tissues (Chang et al., 1997), may be yet another enzyme that mediates FAEE synthesis in the myocardium.
FAEE synthesis in adipose tissue is of potential interest for at least two reasons. First, adipose tissue has the highest content of free fatty acids compared with any other tissue, which means that it is abundant in substrates for FAEE synthesis; second, hydrophobic compounds like FAEEs may accumulate in adipocyte triacylglycerols (Tsujita & Okuda, 1992), thus having a prolonged half-life in this tissue. In post-mortem studies, concentrations of FAEEs were shown to be significantly higher in adipose tissue of acutely intoxicated subjects at the time of death than in controls, as well as in chronic alcoholics with undetectable blood alcohol concentrations (BACs) at the time of death, suggesting that the FAEEs are trapped within a pool of triacylglycerols and stored in this tissue (Laposata et al., 1989; Laposata & Lange, 1986). The half-life of FAEEs in adipose tissue of animals (rabbits and rats) chronically fed ethanol is approximately 16–24 h (Depergola et al., 1991; Laposata et al., 1989), which is much greater than that of ethanol. On this basis, a series of human and animal studies showed that FAEEs in adipose tissue can serve as post-mortem markers of pre-mortem ethanol intake when no blood sample is available (Bjorntorp et al., 1990; Depergola et al., 1991; Refaai et al., 2002; Salem et al., 2001). Adipose tissue has been shown to synthesize FAEEs from free fatty acids and ethanol (FAEES activity) in proportion with the ethanol concentration (Laposata et al., 1989; Laposata & Lange, 1986), and FAEES activity in adipose tissue of humans was found to be elevated after prolonged ethanol exposure, and to remain elevated for some time, making it another potential marker of chronic ethanol exposure (Bjorntorp et al., 1990; Depergola et al., 1991). With regard to AEAT activity, FAEE formation from ethanol and FA-CoA has been demonstrated in rat microsomal protein from isolated fat cells (Polokoff & Bell, 1978) and in human adipose tissue homogenates, where it was higher than its FAEES activity (Diczfalusy et al., 2001a).

FAEES activity was suggested to be a CE by Tsujita & Okuda in 1992, who obtained the enzyme associated with FAEES activity by acetone precipitation and
successive chromatographies from rat adipose tissue (Table 5.5). They reported that FAEE-synthesizing activity eluted in two peaks from DEAE-cellulose, the first (major) coinciding with p-nitrophenol butyrate (pNPB)-hydrolyzing (i.e. CE) activity. Further purification of this major peak showed that the two activities were associated, as judged by their co-elution, co-purification co-precipitation by antibody against FAEEs, and identical inhibition by DFP. The purified 60 kDa enzyme had little activity on long-chain triacylglycerols, but acted on more hydrophilic substrates (butyrate and tributyrin), suggesting that the enzyme is a typical CE. Furthermore, the N-terminal amino acid sequence of the first 27 residues of the purified enzyme was identical to that of CEs in rat liver, while the first 19 residues were identical to that of CE from rat lung. Of interest, the purified adipose tissue FAEEs was detected immunologically in liver, lung, and testis, but not in kidney or small intestine, while the anti-IgG to the purified enzyme removed most of the FAEEs activities in adipose tissue, testis, and lung. Minimal enzymatic activity was removed in the liver and intestine, and none in the kidney. Surprisingly, TOTP (a serine esterase inhibitor), which inhibited FAEEs activity in the liver and blood of rats treated with the inhibitor, did not inhibit FAEEs activity in rat brown fat (Mericle et al., 2004). As suggested by the authors, a possible reason for this may be that the active metabolites of TOTP do not translocate to the brown fat or that there is more than one enzyme responsible for the FAEEs activity in adipose tissue, at least one of which is not inhibited by TOTP (possibly the second peak identified by Tsujita & Okuda, 1992).

**Brain.**

The presence of FAEEs in the brain after ethanol ingestion was documented in several studies in both humans and animals, indicating not only that the non-oxidative pathway of ethanol metabolism exists in the brain, but that it is functional in vivo (Laposata et al., 1987; Laposata & Lange, 1986; Hungund et al., 1988; Refaai et al., 2003). Like adipose, the brain, having a relatively high lipid content, may be a good depot for lipophilic FAEEs. Studies have shown that FAEEs were present in both
cerebellum and cerebral cortex in individuals acutely intoxicated at time of death (Laposata et al., 1987; Laposata & Lange, 1986; Refaai et al., 2003; Yamazaki et al., 1997), and higher BACs were associated with higher FAEE concentrations in the cerebral cortex (Laposata et al., 1987). FAEEs activity has been demonstrated in ten different locations in homogenates of postmortem human brain, with gray matter sites (cerebral cortex, cerebellum, thalamus, basal ganglia, hippocampus) containing more activity than sites with gray and white matter (midbrain, pons, medulla), with the lowest activity observed in white matter sites (centrum semiovale, corpus callosum; Laposata et al., 1987). The localization of FAEEs activity in neurons was confirmed by Isenberg et al. (1992) who, using human and rodent neuroblastoma and glioma cell lines, not only demonstrated the presence of FAEEs-III in cultured neural cells but also demonstrated that cells with neuronal properties have higher activity than glioma cell lines (synthesis from oleate and ethanol). AEAT activity has also been described in microsomes from rat brain (Bishop & Hajra, 1984; Polokoff & Bell, 1978). In the latter study, it was found that both free fatty acid and FA-CoA could serve as acyl donors for esterification (AEAT and FAEEs activity), and that there were two acylation systems for ethanol – one active at neutral pH and one at low pH – suggesting the existence of several enzymes or isozymes with the ability to esterify ethanol (Bishop & Hajra, 1984).

Two forms of FAEEs were isolated by ion-exchange chromatography from the soluble (cytosol) fraction and tissue pellet (membrane bound) of both white matter (centrum semiovale) and gray matter (cerebral cortex) homogenates (Laposata et al., 1987) (Table 5.5). In the cytosolic fraction of gray matter, two peaks emerged from the DEAE column, each containing about 50% of the enzyme activity, while in white matter, the first peak activity contained 75% and the second peak 25% of enzyme activity (Laposata et al., 1987). In the pellet fraction, the two peaks were resolved in relative proportion identical to those of the corresponding gray and white matter soluble fractions described above. It was concluded that FAEEs is present in cytosol or loosely bound to membrane fractions.
Blood.

Numerous studies have shown FAEEs to be present in human blood following ethanol ingestion with the levels of these esters correlating with BACs (Doyle et al., 1994; Laposata et al., 1995). The utility of FAEEs in blood as markers of acute and chronic ethanol intake in humans has been well established as FAEEs persist in blood for a prolonged period of time compared to ethanol (Best et al., 2003; Bisaga et al., 2005; Borucki et al., 2004, 2005, 2007; Doyle et al., 1996; Kaphalia et al., 2004a; Soderberg et al., 1999). Blood FAEE levels are higher in chronic alcohol abusers compared to acute (presumably because of inhibition of hepatic ADH activity; Kaphalia et al., 2004a) and may remain elevated for a prolonged period of time (up to 99 h), likely due to a slow reflux of FAEEs from storage compartments (e.g. liver, pancreas, adipose; Borucki et al., 2004, 2005, 2007). Since nearly 70% of serum FAEEs are bound to lipoproteins and albumin, the association between FAEE levels and BAC is enhanced by consideration of serum albumin (Morfin et al., 2007) and triglyceride concentrations (Soderberg et al., 1999). Of interest, there appears to be a gender difference in FAEE concentrations following ethanol ingestion, with FAEE peak concentrations being approximately 2-fold greater in men than in women given equal weight-based amounts of ethanol, which is surprising since women have higher BAC (Best et al., 2006; Soderberg et al., 1999).

FAEEs in blood may originate from different organs and be excreted into blood and/or be synthesized by blood cells or enzymes. Normal and post-heparin rat plasma have been shown to esterify ethanol with free fatty acids, as well as to transesterify it with cholesterol oleate and triolein, which could also serve as substrates (FA sources; Newsome & Rattray, 1966). FAEES activity has been demonstrated in human whole blood and plasma incubated with oleate and ethanol, with FAEES activity predominantly found within white blood cells/leukocytes (Gorski et al., 1996; Wright et al., 1987). FAEE synthesis by the mononuclear fraction of human white blood cells incubated with ethanol is very rapid, occurring within seconds of ethanol exposure and selectively
producing ethyl oleate (Alhomsi et al., 2006). Human platelets and red blood cells also possess FAEE-synthetic activity (Gorski et al., 1996; Salem et al., 2005). Even though red blood cells produce less FAEE than white blood cells on a per cell basis, they may account for a significant portion of the total FAEEs activity in whole blood because they are more numerous than white blood cells (by three orders of magnitude; Best et al., 2003; Gorski et al., 1996).

One study partially purified FAEEs activity from the cytosolic fraction of human leukocytes by DEAE-cellulose chromatography and found that it separated into two peaks, similarly to the minor and major FAEEs in myocardium (10:1 minor to major ratio), but no attempt was made to characterize these enzymes (Wright et al., 1987; Table 5.5). Inhibitor studies indicate that enzyme(s) associated with FAEEs activity in blood/plasma may be CEs. Plasma FAEEs in rats was shown to be inhibited after intraperitoneal injection of TOTP (a serine esterase inhibitor), much like in the liver, and this parallel dose-dependent inhibition of hepatic and plasma FAEEs, as well as pNPA-hydrolyzing activity, indicates that FAEEs and esterase activities may be expressed by CEs in these tissues (Mericle et al., 2002). This is further supported by the fact that the protein expressing FAEEs activity in plasma cross-reacts with antibodies to rat adipose tissue FAEEs, also shown to be a CE (Tsujita & Okuda, 1992).

Important blood cell-independent sources of FAEE synthetic activity in blood are hepatic and pancreatic enzymes that can be released during liver and pancreatic damage, thereby greatly enhancing FAEE synthesis in blood (Aleryani et al., 1996). Another source of FAEEs in the blood may be the endothelium. Human aorta homogenates incubated with ethanol and oleate have been shown to possess FAEEs activity (Laposata & Lange, 1986), and FAEEs have been determined to be synthesized in the vascular space by LPL either bound to the endothelial surface or released into circulation bound to chylomicrons (Chang et al., 1997). Tsujita & Okuda (1994a) showed that during the purification of LPL from rat post-heparin plasma, the elution pattern of the FAEEs activity coincided with that of the triolein-hydrolyzing activity and
DFP inhibition profiles of the two enzyme activities were identical (Table 5.4). Although albumin bound fatty acids did not act as substrates for FAEE synthesis by LPL (and are thus an unlikely substrate for FAEE synthesis in blood \textit{in vivo}), LPL catalyzed FAEE formation in a concentration dependent manner from chylomicrons in an ethanol/water mixture in the presence of high albumin. In support of this, an early study showed that FAEE synthesis occurred in human post-heparin plasma incubated with phosphatidylethanolamine and ethanol, which was presumed to be mediated by phospholipase but could also have been LPL as it can also hydrolyze PE (Jackson, 1983; Vogel et al., 1965).

\textit{Gastrointestinal tract, gallbladder, and spleen.}

Because both fatty acids and ethanol are absorbed by the intestinal mucosa, the intestine may be a major site of FAEE synthesis. FAEE-synthetic activity has been demonstrated in rat intestinal slices (jejunal rings from rats fasted overnight) in the presence of taurocholate (Baraona et al., 1975), during intraduodenal infusion of ethanol \textit{in vivo} (Chen et al., 1994), in the enzyme fraction of rat stomach and intestine homogenates (Suzuki et al., 1990), and in the acetone extract of rat small intestine incubated with ethanol and oleate (Tsujita & Okuda, 1992). Overall, the intestine has relatively high FAEES activity compared to other organs tested, typically third after pancreas and liver, but no study to date has purified this activity from this tissue. FAEES from rat adipose tissue (a CE) was not detected immunologically in acetone powder extracts of the rat small intestine, but the anti-IgG to the purified adipose FAEES did remove 29% of FAEE-synthesizing activity in the small intestine, suggesting the possible existence of a similar enzyme but also involvement of other enzymes in FAEE synthesis in the small intestine (Tsujita & Okuda, 1992). Interestingly, there was no correlation between FAEES and cocaethylene synthetic activity in human spleen, duodenum, and colon homogenates; the duodenum expressed only FAEES activity and different colon specimens showed minimal FAEES and a range of cocaethylene synthase activities (Heith et al., 1995). This was unexpected since both activities were
mediated by CEs in other tissues (Heith et al., 1995), and suggests that there may be several enzymes displaying FAEES and/or cocaethylene synthetic activity in the gastrointestinal tract.

With respect to AEAT activity, the presence of ethanol acyltransferase in microsomal protein from rat tissues, including intestinal mucosa and spleen was demonstrated (Polokoff & Bell, 1978), and a relatively high AEAT activity, as compared to other organs tested, was also found in homogenate protein of human duodenal mucosa, with intermediate levels in gastric ventricular mucosa and gallbladder (Diczfalusy et al., 2001a). Interestingly, duodenal mucosa was found to contain high AEAT activity and very low FAEES activity, suggesting that the intestinal AEAT activity may contribute significantly to total FAEE synthesis. This is because fatty acids from ingested fat are taken up in the intestinal mucosal cells, where they are converted to CoA esters prior to esterification into triacylglycerols (fatty acids are rapidly esterified to CoA when entering cells), phospholipids, and cholesteryl esters. Since ingested alcohol is also taken up by the intestinal mucosa, a significant amount may be esterified to fatty acids in the mucosal cells (Diczfalusy et al., 2001a). An important consideration in the gastrointestinal tract with regard to FAEE-synthetic enzymes is the presence of digestive enzymes such as ChE, and pancreatic and gastrointestinal lipases that are secreted into the lumen, which have been shown to have FAEE synthetic activity.

**Lung.**

FAEE accumulation and synthesis have been demonstrated in rat and rabbit lungs after exposure to ethanol (Manautou & Carlson, 1991; Manautou et al., 1992). It was found that FAEES activity in lung homogenates is intermediate among the organs tested, indicating that this non-oxidative pathway of ethanol metabolism is present in the lung. FAEES activity in rat lung was also demonstrated in acetone tissue extracts incubated with ethanol and oleate (Tsujita & Okuda, 1992). Although no specific enzymes have been implicated or purified from the lungs, the anti-IgG to the purified FAEES from rat adipose tissue (shown to be a CE) (Tsujita & Okuda, 1992) showed the
presence of this protein in the lungs, and removed most of the FAEES activity (62%), suggesting that the FAEE-synthetic enzyme(s) in this tissue is either identical or very similar to the enzyme in adipose tissue (a CE). Furthermore, the N-terminal amino acid sequence of the first 19 residues of the purified adipose tissue enzyme was identical to that of CE from rat lung. In support of CE involvement, FAEES activity has also been reported in human lung homogenates, where it correlated with cocaethylene synthetic activity (also mediated by CEs), suggesting they may be expressed by the same enzyme (Heith et al., 1995). AEAT activity has also been demonstrated in microsomal protein from rat lungs (Polokoff & Bell, 1978), and in human lung homogenates where it is considerably higher than FAEES activity (Diczfalusy et al., 2001b).

**Fetal tissues (placenta, fetal organs, meconium).**

Non-oxidative metabolism of ethanol and FAEE accumulation have been demonstrated in maternal and fetal tissue in mice (Bearer et al., 1992) and rats (Hungund & Gokhale, 1994). In rodents, placentae, maternal tissue, and fetuses accumulate significant amounts of FAEEs 1 h after maternal ethanol administration, and FAEEs were shown to persist for at least 7 days in the placenta of mice (estimated half-life in the placenta of at least 50–80 h), and at least 14 days in fetal rat organs (Bearer et al., 1992; Hungund & Gokhale, 1994). The kinetic patterns for tissue FAEEs approximated that of maternal BAC in ethanol-dosed pregnant rats (Vallaro et al., 1997). With regard to FAEE origin in fetal tissues, placental perfusion studies have shown that FAEEs detected in fetal matrices are likely produced by the fetus from ethanol that has been transferred and metabolized by the fetus itself since maternal FAEEs are not transferred to the fetus (Chan et al., 2004b). Indeed, FAEES activity has been demonstrated in supernatant from tissue homogenates of human term placenta and mouse placenta (at gestational day 14), mouse embryo, primary cultures of rat fetal brain cells, fetal and postnatal rat brain, and in human fetal brain (2nd trimester) incubated with ethanol and oleic acid, demonstrating that the enzyme activity necessary for FAEE synthesis is present in fetuses and fetal brain tissue relatively early in
gestation (Bearer et al., 1992, 1995). Although the FAEE-synthetic enzymes in fetal tissues are unknown, it was shown that during the purification of the major cytosolic GST expressed in human placenta (GST3), FAEEs activity and GST activity clearly separate during chromatography on DEAE-cellulose, suggesting that in this tissue, these activities result from different enzymes (Suzuki et al., 1990).

FAEEs have also been detected in the meconium, which is a matrix unique to the fetus. Xenobiotics to which the neonate is exposed to in utero may be excreted in the fetal bile or urine into the amniotic fluid (and then subsequently swallowed), thus accumulating directly in the meconium. FAEEs in this matrix have been shown to be biomarkers of fetal ethanol exposure that may permit newborn testing to identify children at risk for alcohol-related disabilities (Bearer et al., 1999, 2003, 2005; Brien et al., 2006; Chan et al., 2004a; Klein et al., 1999; Littner et al., 2008; Ostrea Jr. et al., 2006). As mentioned above, since FAEEs do not cross the placenta (Chan et al., 2004b), FAEEs found in meconium may be synthesized by various fetal tissues (as discussed above) but it has also been shown that meconium itself possesses FAEE-synthetic activity as FAEEs are formed in meconium samples incubated with ethanol (Klein et al., 1999; Zelner et al., 2012). It is possible that lipases in the neonatal gastrointestinal tract (pancreatic and gastrointestinal) are responsible for this formation since the lipase inhibitor THL significantly decreased FAEE formation from added ethanol in meconium samples in vitro (Zelner et al., 2012).

**Eye.**

In 2006, Bora et al. found significantly higher levels of FAEEs in the choroid of 10-week alcohol-fed rats than in those fed alcohol for 1 week. They also demonstrated FAEEs activity, which was increased four-fold in the choroid of alcohol-treated rats compared with controls, with no change in cornea, iris, ciliary body, lens or retina. FAEEs isolated from choroid of brown Norway rats was a 60 kDa single band, the N-terminal sequence of which was identical with rat adipose tissue FAEEs (a CE) isolated by Tsujita & Okuda (1992) (Table 5.5).
**Milk.**

In a 1966 report in Nature, Patton and McCarthy showed the presence of FAEEs in freshly secreted goat milk incubated with labeled fatty acid dissolved in ethanol (to facilitate its dispersion in milk) and in milk lipids from two lactating goats after oral ethanol administration. However, when ethanol was infused directly into the mammary gland by way of the teat canal, no formation of FAEEs in milk drawn was observed, supposedly due to rapid ethanol oxidation in the mammary glands (Patton & McCarthy, 1966). These results suggested that humans who consume alcohol might secrete traces of FAEEs in milk and that enzymes with FAEE-synthetic capabilities are secreted in milk. One such enzyme may be the hormone-dependent lipase, which is an equivalent of ChE and is secreted into the mammary gland to aid newborns with digestion (Small et al., 1991).

**Skeletal muscle.**

Low FAEEs activity has been demonstrated in homogenates of skeletal muscle from humans (Laposata & Lange, 1986), and was undetected in skeletal muscle homogenates from male Wistar rats (Hamamoto et al., 1990). The presence of AEAT activity in microsomal protein from skeletal muscle (ethyl palmitate formation from ethanol and palmitoyl-CoA) was also demonstrated to be in low range of all organs tested (Polokoff & Bell, 1978). Surprisingly, Salem et al. (2006) found that the concentration of FAEEs in skeletal muscle from Wistar rats after ethanol exposure was similar to that of the liver, and that, on a total FAEE mass basis within the body, the skeletal muscle FAEE levels accounted for much more of the total body FAEE load than that of the liver and heart. These findings are in contrast with a study by Laposata & Lange (1986) that reported low FAEE concentration in skeletal muscle of autopsy subjects. This discrepancy may be due to rapid degradation of FAEEs in skeletal muscle since by the time the autopsy was performed in the 1986 study, most of the FAEEs in skeletal muscle may have been hydrolyzed (Salem et al., 2006).
Hair and skin.

The vast majority of studies investigated FAEE levels in hair of individuals with known histories of ethanol exposure or of ethanol-exposed animals to assess their utility as biomarkers of chronic ethanol exposure (Auwarter et al., 2001; Caprara et al., 2005; de Giovanni et al., 2007; Kulaga et al., 2006, 2009; Pragst et al., 2000, 2001; Wurst et al., 2004). Human studies found that FAEEs were present at greater concentrations in hair of individuals with a history of heavy alcohol consumption (fatalities and alcoholics in treatment) as compared to social drinkers and teetotalers. Since in almost all cases the segmental concentrations increased from proximal to distal end of the hair, FAEEs are likely deposited in hair mainly from sebum (Auwarter et al., 2001, 2004). Thus, it is likely that FAEEs are synthesized from ethanol in the sebaceous cells; however, it cannot be excluded that smaller amounts of FAEEs can also be incorporated into the hair from systemic circulation into the hair root, or can be formed from ethanol and fatty acids in basal cells of the hair root (Auwarter et al., 2004). Further studies by the Pragst group also showed that it is possible to use hair samples other than scalp hair, such as pubic, axillary, beard and body hair, for analysis of FAEEs as markers for alcohol misuse (Hartwig et al., 2003b; Susse et al., 2010).

FAEE formation in the holocrinic sebum gland may be mediated by lipases, as these are found in the sebaceous glands and in the external root sheath of the hair follicle. Additionally, other enzymes participating in lipid metabolism as well as bacterial lipases may also play a role. Of interest, false positive test results for hair FAEEs may occur due to external contamination of the hair with ethanol; however, whether this process is enzymatic remains to be determined (de Giovanni et al., 2008; Gareri et al., 2011; Hartwig et al., 2003a). Much like the hair, skin surface lipids, which consist mainly of sebum, may be another matrix for measurement of FAEEs as alcohol abuse markers (Gonzalez-Illan et al., 2011; Pragst et al., 2004).
No studies to date have focused specifically on non-oxidative metabolism to FAEEs or FAEE accumulation in the testis or kidney. However, some multi-organ studies have measured FAEE-synthetic activity in these organs in addition to others. Both FAEES and AEAT activity have been investigated and demonstrated in rat kidney tissue (Polokoff & Bell, 1978; Tsujita & Okuda, 1992), while only FAEES activity has been investigated in rat testes, which was found to be low in tissue homogenates (Hamamoto et al., 1990; Suzuki et al., 1990). With regard to enzymology, the only report on FAEES activity in testis and kidney was conducted in rats and investigated the effect of anti-IgG to rat adipose tissue FAEES (a CE) on FAEES activity in these tissues (Tsujita & Okuda, 1992). It was found that the anti-IgG removed most of the FAEES activity in acetone powder extracts of testis (82%), but did not affect the activity in kidney. Furthermore, adipose tissue FAEES was detected immunologically in the testis, but not in the kidney, suggesting that the FAEE-synthetic enzyme(s) in the testis is either identical or very similar to the enzyme in adipose tissue (a CE), while FAEES activity in the kidney is mediated by another enzyme(s) (Tsujita & Okuda, 1992).
Table 5.4 Studies that measured FAEES/AEAT activity of known commercial or purified enzymes.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Enzyme</th>
<th>FAEES activity</th>
<th>Other enzymatic activity(ies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lange, 1982</td>
<td>Bovine commercial <em>pancreatic</em> cholesterol esterase (EC 3.1.1.13)</td>
<td>✓ ethanol and $[^{14}C]$ linoleic acid in 30 mM phosphate (pH 7.35, 30 minutes at 37°C)</td>
<td>✓ Cholesterol ester synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Ethanol and $[^{14}C]$ fatty acid in 50mM phosphate buffer (pH 7.35, 2 hours at 37°C)</td>
<td>✓ Conjugation of fatty acids to haloethanols</td>
</tr>
<tr>
<td>Bhat &amp; Ansari, 1990</td>
<td>Bovine commercial <em>pancreatic</em> cholesterol ester hydrolase (EC 3.1.1.13)</td>
<td>✓ FAEES synthesis from ethanol and trioleylglycerol: $[^{14}C]$ trioleylglycerol and ethanol in 100mM potassium phosphate with sodium cholate (required; pH 7, 1 hour at 37°C)</td>
<td>✓ Ethyl oleate hydrolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
<td>✓ Trioleylglycerol-hydrolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>✓ Cholesterol ester synthesis</td>
<td>✓ Trioleylglycerol-hydrolysis</td>
</tr>
<tr>
<td>Tsujita &amp; Okuda, 1994b</td>
<td>Porcine <em>pancreatic</em> carboxylester lipase (EC 3.1.1.13) - purified, not commercial</td>
<td>✓ $[^{14}C]$ oleate and ethanol in 100mM potassium phosphate (pH 7, 1 hour at 37°C)</td>
<td>✓ Cholesterol ester synthesis</td>
</tr>
<tr>
<td>Rixey et al., 1990</td>
<td>Porcine <em>pancreatic</em> commercial triglyceride lipase (EC 3.1.1.3)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in 60mM sodium phosphate buffer (pH 7.2 at 37°C)</td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
</tr>
<tr>
<td>Tsujita &amp; Okuda, 1994a</td>
<td>Rat lipoprotein lipase purified from postheparin plasma (EC 3.1.1.34)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in 60mM sodium phosphate buffer (pH 7.6 at 37°C)</td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
</tr>
<tr>
<td>Bora et al., 1998b</td>
<td>Bovine commercial liver GST-P (π class; EC 2.5.1.18)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in 60mM sodium phosphate buffer (pH 7.2, 45 minutes at 37°C)</td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
</tr>
<tr>
<td>Suzuki et al., 1990</td>
<td>Human purified GSTs from liver (GST1 and 2) and placenta (GST3) representing three evolutionary classes (μ, α, and π). (EC 2.5.1.18)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in 60mM sodium phosphate buffer (pH 7.2 at 37°C)</td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
</tr>
<tr>
<td>Sharma et al., 1991</td>
<td>Human GSTs purified from pancreas, liver, heart, muscle, brain, lung (EC 2.5.1.18)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in 60mM sodium phosphate buffer (pH 7.2, 45 minutes at 37°C)</td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
</tr>
<tr>
<td>Singhal et al., 1991</td>
<td>Rat purified <em>pancreatic</em> GSTs - 7 isozymes belonging to 3 evolutionary classes (μ, α and π). (EC 2.5.1.18)</td>
<td>✓ $[^{14}C]$ oleate and ethanol (method by Mogelson &amp; Lange, 1984)</td>
<td>✓ FAEES synthesis from ethanol and chylomicrons: $[^{14}C]$ ethanol/water mixture and chylomicrons in 50mM Tris-HCl buffer (pH 8.4, 37°C)</td>
</tr>
<tr>
<td>Kaphalia et al., 2004b</td>
<td>Porcine commercial hepatic CE (EC 3.1.1.1) and <em>pancreatic</em> Che (EC 3.1.1.13)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in phosphate buffer (pH 7.4, 2 hours at 37°C)</td>
<td>✓ AEAT activity; $[^{14}C]$ palmitoyl-CoA and ethanol in potassium phosphate buffer (pH 7.4, ~15 minutes at 37°C)</td>
</tr>
<tr>
<td>Diez-Falussy et al., 1999</td>
<td>Rat hepatic CEs ES-4, ES-10 (EC 3.1.1.1)</td>
<td>✓ $[^{14}C]$ oleate and ethanol in phosphate buffer (pH 7.4, 20 minutes at 37°C)</td>
<td>✓ AEAT activity; $[^{14}C]$ palmitoyl-CoA and ethanol in potassium phosphate buffer (pH 7.4, ~15 minutes at 37°C)</td>
</tr>
</tbody>
</table>

AEAT, acyl-coenzyme A: ethanol O-acyltransferase; CE, carboxylesterase; CoA, coenzyme A; ES, esterase; FAEES, fatty acid ethyl ester synthase; GST, glutathione-S-transferase.

✓ Activity indicated was measurable.

✗ activity indicated was not measurable.
Table 5.5 Studies that purified/characterized FAEES activity in mammalian tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>References</th>
<th>Species (Fraction)</th>
<th>Peaks eluted (DEAE cellulose)</th>
<th>Molecular weight</th>
<th>Other enzymatic activity(ies)</th>
<th>Antibody cross-reactivity/precipitation</th>
<th>Amino acid sequence</th>
<th>Suggested enzyme(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>Riley et al., 1990</td>
<td>Human (cytosol)</td>
<td>3 peaks (human)</td>
<td>32kDa (monomer)</td>
<td>√ FAEES hydrolysis</td>
<td>Antibodies to α-GST did not immunoprecipitate FAEES activity of brain and heart homogenates (human).</td>
<td>N-terminal 20 a.a. identical to human trilglceride lipase.</td>
<td>Triglyceride lipase</td>
</tr>
<tr>
<td></td>
<td>Sharma et al., 1991; Singhal et al., 1991</td>
<td>Human, rat (soluble fraction)</td>
<td>2 peaks (human)</td>
<td>52kDa (monomer)</td>
<td>× GST activity</td>
<td></td>
<td>Not GST</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kapturin &amp; Anvari, 2003</td>
<td>Rat (microsomal fraction)</td>
<td>68kDa (possibly a dimer of 136 kDa)</td>
<td>√ Cholesterol ester hydrolysis</td>
<td>FAEES-I and III; √ GST activity</td>
<td>FAEES-I cross-reacts with antibodies to FAEES-I and III.</td>
<td>N-terminal 13 a.a. identical to rat pancreatic ChE.</td>
<td>ChE</td>
</tr>
<tr>
<td>Heart</td>
<td>Mogelson &amp; Lange 1984; Mogelson et al., 1988</td>
<td>Rabbit (soluble fraction)</td>
<td>2 peaks (human)</td>
<td>30kDa (dimer, ~36kDa identical subunits)</td>
<td>× Cholesterol ester hydrolysis</td>
<td>FAEES-I and III; √ GST activity</td>
<td>Residue composition distinct from bovine pancreatic cholesterol esterase</td>
<td>Not ChE</td>
</tr>
<tr>
<td></td>
<td>Bora et al., 1989a, 1989b, 1990c, 1992a, 1996</td>
<td>Human (soluble fraction)</td>
<td>4 peaks</td>
<td>42.1kDa (monomer)</td>
<td>FAEES-I and III; √ GST activity</td>
<td>FAEES-I cross-reacts with antibodies to FAEES-I and III.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose</td>
<td>Tsuji &amp; Okada, 1992</td>
<td>Rat (acetone powder extract)</td>
<td>60kDa single band</td>
<td>√ pNPA-hydrolysis</td>
<td>FAEES-I and III; √ GST activity</td>
<td>FAEES-I cross-reacts with antibodies to FAEES-I and III.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Kaphalia et al., 1997; Kaphalia &amp; Anvari, 2001</td>
<td>Rat (microsomal fraction)</td>
<td>2 peaks (human)</td>
<td>180kDa (trimer)</td>
<td>√ pNPA-hydrolysis</td>
<td>Antibodies to rat adipose tissue FAEES. Not rat pancreatic ChE.</td>
<td>Major: Fst 27 a.a. N-terminal identical to rat hepatic CE.</td>
<td>Hepatic ChE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60kDa (dimer; monomer)</td>
<td>× Cholesterol ester synthesis</td>
<td></td>
<td>Major: N-terminal sequence homology to various hepatic microsomal CEs and protease precursors.</td>
<td>Hepatic ChE</td>
</tr>
</tbody>
</table>
Table 5.5 (Continued).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>References</th>
<th>Species (Fraction)</th>
<th>Peaks eluted (DEAE cellulose)</th>
<th>Molecular weight</th>
<th>Other enzymatic activity(ies)</th>
<th>Antibody cross-reactivity/precipitation</th>
<th>Amino acid sequence</th>
<th>Suggested enzyme(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>Bora et al., 2006</td>
<td>Rat (choroid homogenate - soluble fraction)</td>
<td>69 kDa single band</td>
<td></td>
<td></td>
<td></td>
<td>N-terminal a.a. sequence identical to rat adipose FAEES</td>
<td>CE</td>
</tr>
<tr>
<td>Blood (WBC)</td>
<td>Wright et al., 1987</td>
<td>Human (cytosolic fraction of leukocytes) Lymph (soluble and pellet fractions of white and gray matter)</td>
<td>2 peaks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Laposata et al., 1987</td>
<td></td>
<td>2 peaks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For details on the specific purification steps, see cited reference. a.a., amino acid; CE, carboxylesterase; ChE, cholesterol esterase; FAA, fatty acid anilide; FAEE, fatty acid ethyl ester; FAEES, fatty acid ethyl ester syntheses; FAME, fatty acid methyl ester; GST, glutathione-S-transferase; kDa, kilo dalton; pNPA, p-nitrophenol acetate; pNPB, p-nitrophenol butyrate; WBC, white blood cell.

✓ Activity indicated was measurable.
× Activity indicated was not measurable.
4.9 Altering FAEE production

*Inhibiting oxidative metabolism.*

Formation of FAEEs is dose (and BAC) dependent as esterification of fatty acids with ethanol presumably becomes more prominent when BACs are high and oxidative pathways are saturated (Werner et al., 2001). Accordingly, tissues that have limited oxidative metabolism may have increased ethanol disposition through the non-oxidative pathway (Behonick et al., 1997). The linkage between the two metabolic pathways for ethanol has been demonstrated as increased tissue accumulation of FAEEs in the presence of inhibitors of oxidative ethanol metabolism (Manautou et al., 1992; Werner et al., 2001, 2002). Specifically, it was reported that inhibition of oxidative ethanol metabolism resulted in a two-to three-fold increase in non-oxidative ethanol metabolism to FAEEs in rat liver and pancreatic homogenates incubated with ethanol (Werner et al., 2001) and significantly increased FAEE concentrations in the pancreas, plasma, and liver *in vivo* in rats (Werner et al., 2002). These findings were corroborated by Best et al. (2006), who demonstrated increased plasma FAEE concentrations following inhibition of oxidative metabolism of ethanol by 4-methylpyrazole treatment in human subjects. This phenomenon was also confirmed in knock-out animals deficient in oxidative pathways, where higher FAEE levels in liver and pancreas were observed in ADH-negative mice fed ethanol as compared to controls (Bhopale et al., 2006; Kaphalia et al., 2010). Additionally, this was demonstrated in cell lines, as significantly higher levels of FAEEs were synthesized in HepG2 cells (which are inherently deficient in ADH) exposed to ethanol than in HepG2 cells transfected with ADH at all ethanol concentrations (Wu et al., 2006). Overall, it is apparent that oxidative and non-oxidative pathways are metabolically linked and the increased FAEE production is caused by a shift from
the oxidative toward the non-oxidative pathways of ethanol by substrate loading (Werner et al., 2001).

**Other mechanisms.**

A series of FAEE accumulation studies by Calabrese et al. investigated the effect of pyridoxine, pyrrolidone carboxylic acid, metadoxine, L-carnitine (involved in fatty acid transport during lipid breakdown, an obligate co-factor in mitochondrial fatty acid β-oxidation), and acetyl-L-carnitine on FAEE formation in rats. The authors showed that ethanol administration (2 g/kg daily for seven days) increased total FAEEs in brain, heart, kidney, liver, and plasma, while pre-treatment with pyridoxine, pyrrolidone carboxylic acid, or metadoxine (one hour before ethanol) decreased FAEE accumulation after ethanol administration (Calabrese et al., 1993, 1995, 2001; Calabrese & Rizza, 1999). The authors speculated that pre-treatment with either metadoxine or L-carnitine resulted in decreased FAEE accumulation by reducing free fatty acid levels needed for FAEE synthesis and enzyme activity at tissue levels while concomitantly decreasing BAC through increased clearance of ethanol from blood (Calabrese et al., 1993, 1995; Calabrese & Rizza, 1999). Pre-treatment with acetyl-L-carnitine, unlike L-carnitine, did not reduce BAC after the same ethanol dosage (in fact, there was a slight increase as may be expected from its ability to inhibit ADH; Calabrese et al., 2001). Thus, the authors stipulated, the decreased FAEE formation with acetyl-L-carnitine is consistent with a demonstrated decrease in FAEES activity and a possible reduction of free fatty acid levels rather than decreased BAC.

In an unrelated study, it was found that ganglioside GM1 treatment one and 24 h prior to ethanol exposure on both gestational day 7 and/or gestational day 14 reduced the accumulation of FAEEs in placentae, fetal tissue, and maternal organs (Hungund & Gokhale, 1994). The mechanism behind this
reduction in accumulation was not determined but the authors speculated that GM1 inhibits alcohol-induced phospholipase A2 activity and thereby minimizes the availability of free fatty acid substrates for FAEEs in the organs. Alternatively, GM1 may produce its protective effect by inhibiting FAEE synthesis, which may lead to reduced FAEE accumulation. Furthermore, [3H]-GM1 may cause membrane alterations such as increased rigidity, resulting in reduced penetration of ethanol into the membrane (Hungund & Gokhale, 1994).

*Induction of FAEEs activity.*

Several studies by a number of groups have investigated the induction of FAEEs activity in tissues and it appears that FAEEs activity is regulated, at least to some extent, by previous and chronic ethanol exposure. Laposata et al. (1987) reported elevated FAEEs activity in brains of alcoholics compared to non-alcoholics. In rats treated with ethanol daily (2 g/kg daily for seven days), a significant increase in FAEEs activity in liver tissue homogenate was demonstrated (Calabrese et al., 2001). FAEEs activity was shown to double in the infranatant of adipose tissue homogenate from rats ingesting ethanol after ten and seventeen weeks of ethanol exposure, and it decreased to normal after the ethanol ingestion was stopped with a half-life of approximately one week (Depergola et al., 1991). This was also shown in humans, where FAEEs activity in adipose tissue increased from teetotalers to social drinkers, and was two-fold higher in alcoholics, and five times higher in deceased alcoholics (Bjorntorp et al., 1990). This induction of enzyme activity appeared to have a half-life of several weeks, which is much longer than the half-life of elevation in FAEE concentration (after cessation of ethanol intake) and there appeared to be an inverse relationship between time since the most recent consumption of alcohol and FAEEs activity (Bjorntorp et al., 1990). Additionally, it was found that white blood cell FAEEs activity could be induced nearly two-fold upon ingestion of alcohol for six days but only for a limited time (returned to normal before ethanol
intake was stopped; Gorski et al., 1996). Of interest, alcoholic individuals were shown to have approximately half the white blood cell FAEES activity of normal individuals, which may be the result of years of ethanol abuse and/or down regulation of FAEES as an adaptive response to limit production of FAEEs since these esters are toxic (Gorski et al., 1996).

Some conflicting studies with respect to FAEES induction exist, however. In rats administered various inducers, it was found that β-naphthoflavone, phenobarbital, and ethanol did not induce FAEES activity in rat organs, and only Aroclor 1254 increased FAEES activity and only in the liver (Carlson et al., 1995). In another conflicting study, Hamamoto et al. (1990) showed a slight decrease in FAEES in the pancreas of rats administered ethanol for 7 weeks. The contrasting results with respect to induction by ethanol may be due to different induction phases and ethanol dosing regimens. Overall, the majority of studies support that FAEES activity is regulated to some extent by ethanol exposure. In further support of this, Pfutzer et al. (2002) showed that in rats, chronic ethanol consumption induced expression of FAEE-related genes (e.g. pancreatic ChE and ES-10 mRNA) in the pancreas and liver.

Genetics.

It has been shown that cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction leads to increased levels of FAEEs following alcohol administration to mice. Specifically, the mean FAEE concentration in the liver of CFTR mice following injection with 2 g/kg of ethanol was significantly greater than the amount detected in wild type, suggesting an association between CFTR dysfunction and changes in FAEEs upon ethanol exposure (Blanco et al., 2005). The mechanisms behind this observation are unknown.
4.10 Other conjugation products by FAEE-synthetic enzymes

Although beyond the scope of this review, it should be noted that enzymes with FAEE-synthetic activity have been shown to catalyze the conjugation of other exogenous substances and fatty acids. FAEES purified from rat liver microsomes was found to catalyze the formation of 2-butoxyethyl esters (Kaphalia et al., 1996), 2-chloroethyl esters (Kaphalia et al., 1997), and fatty acid anilides (Ahmad et al., 1993; Kaphalia et al., 1999). Methyl, propyl, and butyl esters of fatty acids are also formed by the actions of FAEES enzymes purified from human myocardium (Bora et al., 1992a), while FAEES purified from rat adipose tissue (a CE) was shown to promote the esterification of cocaine to ethanol to form cocaethylene (Heith et al., 1995). Bovine pancreatic ChE, which mediates FAEE formation, also mediated the conjugation of haloethanols (2-chloroethyl and 2-bromoethyl ester as well as other chloroethanols; Bhat & Ansari, 1990; Singhal et al., 1991). LPL, which was shown to catalyze FAEE formation, also catalyzed the formation of fatty acid alcohol esters with water-insoluble alcohols (monooleoylglycerol, dioleoylglycerol, trioleoylglycerol, oleic acid, palmitoyl oleate) as substrates in aqueous medium (Tsujita et al., 1999).

5 Discussion

Understanding ethanol metabolism to FAEEs in different tissues is important as the tissue capacity to synthesize FAEEs may predispose it to toxicity and affect the concentrations of these esters in tissues where they have been used as biomarkers of ethanol exposure. Over the years, two enzyme activities have been studied: FAEES, which uses ethanol and free fatty acid, and AEAT, which uses ethanol and fatty acyl-CoA. These two activities are expressed by different enzymes as evidenced by differences in their cellular localization and differential effect of enzyme inhibitors on these activities. The identities of all enzymes responsible for FAEE-synthetic activity as well as all
enzymes and substrates suspected to be involved in oxidative and non-oxidative ethanol metabolism are shown in Figure 5.2.

**Figure 5.2. Oxidative and non-oxidative ethanol metabolism in mammalian tissues.**

"FAEES" and "AEAT" refer to FAEE-synthetic activity that has not been purified and/or linked to a known enzyme in some tissues. Enzymes followed by a question mark are expected to utilize the substrate in question for FAEE synthesis but have not been tested. Similarly, lipid substrates followed by a question mark are expected to act as fatty acid sources during FAEE synthesis, but have not been explicitly tested. It should be noted that for complex lipid molecules (triglycerides, cholesteryl esters, and phospholipids) it is not clear whether the catalytic activity occurs through transesterification or conjugation of the liberated fatty acid. The relative importance of fatty acid liberation versus the conjugation of the fatty acid to ethanol requires further investigation. ADH, alcohol dehydrogenase; AEAT, acyl-CoA: ethanol O-acyltransferase; ALDH, aldehyde dehydrogenase; CE, carboxylesterase; ChE, cholesterol esterase or carboxylester lipase; CoA, coenzyme A; FAEES, fatty acid ethyl ester synthase; LPL, lipoprotein lipase; SULT, sulfotransferase; TGL, triglyceride lipase; UGT, UDP-glucuronosyltransferase.
Virtually every tissue tested has been shown to possess FAEE-synthetic activity, with pancreas and liver having the highest activities and being the most widely studied. The enzymes associated with FAEES activity have been purified or partially purified from the pancreas, liver, heart, brain, blood, and adipose tissue. These enzymes are active at low ethanol concentrations easily attained in vivo in humans and have high apparent Km values for ethanol (higher than physiologically achievable ethanol concentrations) (Mogelson & Lange, 1984; Mogelson et al., 1984), suggesting that these activities may be side reactions of enzymes with different roles. Indeed, FAEES activity has been shown to be associated with various CEs, ChE and lipases, depending on the tissue and cell fraction from which it was purified. Furthermore, several enzymes and isozymes with FAEES activity typically exist within a particular tissue, the relative contribution of which to total FAEE-synthetic activity varies by organ as evidenced by inhibitor studies. The involvement of GST in FAEES activity has also been suggested, but these findings were challenged by a number of groups and may have resulted from methodological limitations (Board et al., 1993; Sharma et al., 1991; Singhal et al., 1991; Suzuki et al., 1990). The identity of the enzyme with AEAT activity in humans remains unknown, but it is unlikely to be due to ACAT or diglyceride acyltransferase (EC 2.3.1.20) activities (the latter does not trans-esterify fatty acyl-CoA to ethanol), and is unlikely to be a serine esterase (unlike enzymes with FAEES activity) based on inhibitor studies (Diczfalusy et al., 1999, 2001a, 2001b). Given that LPL, ChE, and TGL have FAEES activity, additional substrates that can serve as a fatty acid source in FAEE synthesis also include lipoproteins, phospholipids, and cholesteryl esters, although some of these have not been explicitly tested. It should be noted that in some cases, it is not clear whether FAEE synthesis proceeds through direct trans-esterification or hydrolysis/liberation of the fatty acid and its subsequent conjugation to ethanol. The relative importance of the hydrolytic action, which makes the fatty acid substrate available for esterification with ethanol, versus
esterifying action that conjugates the fatty acid or fatty acyl-CoA with ethanol, is not known. It is also possible that in vivo these two steps are primarily mediated by different enzymes, a hydrolytic enzyme and an esterification enzyme, and that the hydrolytic enzyme is rate limiting (Best & Laposata, 2003). This is a difficult distinction to make in complex systems like tissue homogenates.

One particular organ system worth further discussion when it comes to enzymology of FAEE synthesis is the gastrointestinal tract. This organ system may be of particular importance when it comes to FAEE synthesis, since it is the site of intake of both fatty acids and ethanol and may be of particular enzymatic complexity. It is important to note that aside from FAEEs activity expressed by intestinal cells (which has not been purified), FAEE synthesis is likely also mediated by pancreatic digestive enzymes that participate in the metabolism of fats, which are released into the gastrointestinal tract during digestion. The lipase likely attacks triglyceride forming an acyl-enzyme intermediate and, during the deacylation process, ethanol binds to fatty acid as an acceptor (Tsujita & Okuda, 1994b). Thus, if ethanol is present during triglyceride degradation in the intestinal lumen, lipases (CE and triglycerides) may contribute to non-oxidative ethanol metabolism. On the endothelial surface, LPL can similarly catalyze FAEE formation during degradation of triglyceride-rich lipoproteins such as chylomicrons. Additionally, there is a potential contribution of bacterial enzymes to FAEE synthesis in the gastrointestinal tract, as the intestinal flora are extremely abundant and comprise hundreds of species of bacteria, each possessing their own enzymatic machinery including lipases and CEs that may also participate in FAEE formation (this is beyond the scope of this review). The proportion of FAEEs formed in the gastrointestinal tract that make it to systemic circulation requires further study since, although a fairly rapid hydrolysis of orally ingested FAEEs to free fatty acids and ethanol has been observed, whether these can be re-conjugated upon absorption in other tissues is not known (Saghir et al., 1997, 1999a, 1999b).
It is apparent that the presence or absence of oxidative pathways in a particular tissue also influences non-oxidative metabolism in that tissue since the two are metabolically linked (Werner et al., 2001). Tissues with minimal oxidative metabolism such as brain, lung, and heart, are expected to metabolize ethanol predominantly through non-oxidative pathways, while factors that affect the oxidative pathways will also affect FAEE production by a shift in substrate loading. Factors that will lead to increased FAEE production include exposure to inhibitors of oxidative metabolism, high ethanol concentrations that may saturate oxidative enzymes, and decreased ADH activity as a result of chronic alcohol abuse (Werner et al., 2001). The latter factor may be of importance since ADH deficiency in chronic alcoholics may lead to increased body burden of ethanol and its disposition via non-oxidative metabolism (Kaphalia et al., 2010). Furthermore, there is evidence that enzymes with FAEE-synthetic activity may be regulated by ethanol exposure, at least to a certain extent, which will further influence FAEE production and levels in tissues depending on the individual’s history of alcohol use (Björntorp et al., 1990; Gorski et al., 1996).

Since it is clear that numerous enzymes have the ability to synthesize FAEEs, genetic variability in one particular enzyme is unlikely to affect overall FAEE production in an individual. However, in some tissues where FAEE synthesis is primarily mediated by one particular enzyme or isoenzyme, such genetic variability may influence FAEE concentrations in the tissue or matrix following ethanol exposure. This may be of importance if it is a matrix in which FAEE concentrations are used as biomarkers of ethanol exposure. Additionally, in tissues where a particular enzyme mediates the bulk of FAEE synthesis, exposure to enzyme inhibitors or competing substrates may significantly influence FAEE production, especially since FAEE synthesis is likely a side-activity of enzymes with other physiological functions. There are few publications on the effects of different agents on FAEE production and accumulation.
(Calabrese et al., 1993, 1995, 2001; Hungund & Gokhale, 1994), none of which is mechanistic, so this particular area requires more study.

The vast majority of studies included in this systematic review utilized proper controls, such as boiled homogenate and microsomal samples, to account for non-enzymatic FAEE formation, which has been shown to be low (Kinnunen & Lange, 1984). Studies in matrices like hair and skin did not investigate whether FAEE synthesis is enzymatic, but it is likely given the number of lipases present in sebum and sebaceous glands. In studies that measured FAEES and AEAT activities in homogenates, microsomal preparations, and cell lines, it is important to note that the reported activities likely reflect the difference between the rates of synthesis and hydrolysis of the ethyl esters and that the reported specific activities may be in lower limits since they can be compromised by the presence of substantial amounts of unlabeled free fatty acids in cytosolic and microsomal samples assayed. Caution should also be exercised when interpreting results of studies measuring and comparing FAEES and AEAT activity in intact and crude systems where inter-conversion of substrates likely occurs, in assays utilizing homogenates or cell fractions where cell architecture is disrupted, and in purified enzymes. In vivo, there are many factors that might influence the relative importance of these two activities, including the concentration of the relevant substrates, access to the active sites of the enzymes, and presence of lipid-binding proteins (which may be important in the presentation of the enzyme and/or removal of synthesized FAEEs) at the site of FAEE synthesis. These factors further complicate the interpretation of in vitro “activity” results.

Additional difficulty in interpreting the results of the studies discussed in this review stems for the fact that FAEE formation in various tissues is a result of a complex interplay of several enzyme activities. For this reason, there is need for caution when interpreting results from studies that utilized crude systems (e.g. tissue homogenates) as more than one enzyme may be contributing to the
synthetic activity, competing for substrates, or utilizing the product. Since FAEES activity is likely due to side activities of various lipases, ChE, and CEs, the individual contribution to overall FAEE synthesis, and thus the effect of inhibitors, is expected to vary by tissue, meaning that results from one tissue should not be generalized to another. Furthermore, trying to tease out the enzymes involved in FAEE synthesis by using inhibitors in crude systems is problematic since inhibition of one enzyme with FAEES activity may simply shift FAEE synthesis to another enzyme if more than one enzyme is contributing to the synthetic activity. Additionally, since some enzymes involved in lipid metabolism require co-factors for optimal activity (e.g. apoprotein CII, bile salts), the results of some in vitro studies on FAEE synthesis may not be accurate if these were left out in the assays. One last point that should be emphasized is that species differences with regard to enzymes with AEAT activity are apparent, and it cannot be excluded that this may also be the case with some enzymes possessing FAEES activity, although this has not been investigated.

Overall, it can be concluded from the review of literature that FAEE synthesis is complex and involves numerous enzymes and isoenzymes, the differential expression of which in tissues influences their synthetic capacity and thus the levels of these esters in matrices. This is complicated by differential expression of FAEE-hydrolytic enzymes, presence/availability of substrates (fatty acid sources), tissue composition (which influences FAEE retention/accumulation), FAEE-binding proteins, and competing pathways and substrates. It is possible that FAEE levels in some tissues will be greatly influenced by presence of competing substrates or genetic variability in a particular enzyme responsible for the bulk of FAEE synthesis in that tissue, but will have no effect on another tissue where another family of enzymes is involved. Likewise, it is possible the ethanol can affect the metabolism of endogenous compounds and xenobiotics normally metabolized by these enzymes through competition. Matrices where concentrations of FAEEs are used
as biomarkers of ethanol exposure should be further investigated to determine the enzyme(s) involved, as well as the effect of previous ethanol exposure on the activity and expression of these enzymes in order to ensure accurate interpretation of FAEE levels.

6 Acknowledgements

The authors would like to express their sincerest gratitude to Cheri Nickel at The Hospital for Sick Children for her immense help conducting the literature searches.

7 Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

8 References


Borucki K, Kunstmann S, Dierkes J, et al. (2004). In heavy drinkers fatty acid ethyl esters in the serum are increased for 44 hr after ethanol consumption. Alcoholism Clin Exp Res 28:1102–1106

Brien JF, Chan D, Green C, et al. (2006). Chronic prenatal ethanol exposure and increased concentration of fatty acid ethyl esters in meconium of term fetal guinea pig. Ther Drug Monit 28:345–350


Dan L, Laposata M. (1997). Ethyl palmitate and ethyl oleate are the predominant fatty acid ethyl esters in the blood after ethanol ingestion and their synthesis is differentially influenced by the extracellular concentrations of their corresponding fatty acids. Alcohol Clin Exp Res 21:286–292


Hartwig S, Auwaerter V, Pragst F. (2003a). Effect of hair care and hair cosmetics on the concentrations of fatty acid ethyl esters in hair as markers of chronically elevated alcohol consumption. For Sci Int 131:90–97


Kulaga V, Caprara D, Iqbal U, et al. (2006). Fatty acid ethyl esters (FAEE); comparative accumulation in human and guinea pig hair as a biomarker for prenatal alcohol exposure. Alcohol Alcohol 41:534–539


Pfutzer RH, Tadic SD, Li HS, et al. (2002). Pancreatic cholesterol esterase, ES-10, and fatty acid ethyl ester synthase III gene expression are increased in the pancreas
and liver but not in the brain or heart with long-term ethanol feeding in rats. Pancreas 25:101–106


Salem RO, Laposata M, Rajendram R, et al. (2006). The total body mass of fatty acid ethyl esters in skeletal muscles following ethanol exposure greatly exceeds that found in the liver and the heart. Alcohol Alcohol 41:598–603


Chapter 6 Summary of Research Findings and Discussion

Screening programs for prenatal alcohol exposure using objective methods like biological markers may be an important strategy for the management of FASD in our society. The use of meconium testing for FAEEs is presently limited to research studies and child protection cases, but the clinical value of such screening lies in the fact that by providing an accurate history of fetal alcohol exposure, they can facilitate early recognition and diagnosis of alcohol-affected individuals. This would, in turn, allow for early intervention in these individuals and specialized support. Early diagnosis has been shown to be associated with a decreased risk of secondary disabilities such as disrupted school experience, unemployment, institutionalization, and trouble with the law; likely because it permits such early intervention and support (Streissguth et al., 2004). Furthermore, multiple studies have shown that specific interventions and teaching techniques can improve skills like mathematics, behavioural regulation, peer relations and social communication, executive function, compliance, learning readiness, and challenging behavior of clinical concern in individuals affected by FASD (Bertrand, 2009; Peadon et al., 2009). Such improvements would be expected to increase productivity, reduce the incidence of adverse life outcomes, improve the quality of life, and reduce societal burden of FASD.

Before meconium FAEE testing can be used clinically for the identification of newborns at risk for FASD, several key knowledge gaps have to be addressed. Specifically, it is necessary to conduct further validation of the test and explore its ability to identify ethanol-related adverse effects in newborns, to identify factors that can potentially influence the validity and accuracy of the test, and to assess the clinical utility of meconium testing and feasibility of implementing meconium screening programs. The studies conducted as part of this doctoral thesis aimed to address (or at least begin to address) some of the knowledge gaps outlined above.
The present chapter deals with the significance of the specific research findings outlined in the preceding chapters, and summarizes these findings in terms of what it means for meconium testing for FAEE and for potential implementation of such testing in neonatal screening programs for prenatal alcohol exposure.

1 Summary and Significance of Research Findings

I. Assessing the relationship between ethanol-induced organ-system injury and FAEE concentrations in meconium of fetal sheep (Chapter 2).

Previous studies have shown that FAEE concentrations in neonatal meconium constitute a sensitive and specific biomarkers of heavy maternal drinking in the latter half of pregnancy (Bearer et al., 2003; Koren et al., 2008). However, the sensitivity and specificity of meconium FAEE concentrations for detecting lower levels of fetal ethanol exposure, and their relationship with the resultant manifestations of ethanol-induced organ or system injury (effect) were unknown. This was important to determine in order to evaluate the ability of meconium testing to identify individuals adversely affected by prenatal alcohol exposure. The results of this study showed that:

- FAEE concentrations were significantly elevated in meconium of fetal sheep exposed to daily, relatively moderate-dose ethanol in late gestation (0.75 g/kg over 1 hour), as compared with controls.

- Positive cutoff values could be established that differentiated between ethanol exposed and non-exposed fetuses with high sensitivity and specificity (meconium concentrations of ≥0.0285 nmol total FAEE/g had 93.3% sensitivity and specificity in detecting the fetal ethanol exposure regimen).

- When the studied animals (regardless of exposure status) were classified according to the positive cutoff, those testing above the cutoff, as a group, frequently differed from those testing below the cutoff with respect to various
pathological endpoints, including nephron endowment, lung collagen deposition, cardiomyocyte maturation, and tropoelastin gene expression in cerebral vessels.

- In all studied animals as a group (ethanol-exposed and controls combined), meconium FAEE concentration was correlated with many of the pathological endpoints in fetal organs (nephron endowment, lung collagen deposition and surfactant gene expression, relative heart weight and cardiomyocyte maturation, tropoelastin and collagen gene expression in cerebral vessels, and TNF-α gene expression in the placenta).

These results demonstrate that, in fetal sheep, meconium FAEE could serve as a highly sensitive and specific biomarker of late gestation ethanol exposure at doses that do not result in overt fetal dysmorphology, but rather in subtle pathological changes in a number of organs. Furthermore, the relationship between meconium FAEE concentrations (biomarkers of exposure) and the observed ethanol-induced pathology (effect) provides evidence in support of the potential use of meconium FAEE in the identification of neonates at risk for adverse effects as a result of in utero ethanol exposure. This is in agreement with previous studies that have demonstrated the association between meconium FAEE concentration and lower APGAR scores (Derauf et al., 2003), growth restriction (Derauf et al., 2003; Noland et al., 2003), lower score on executive functioning tasks at age 4 (Noland et al., 2003), and decreased psychomotor performance in children at age 2 (Peterson et al., 2008).

This is the first study to date that examined the association between meconium FAEE concentration and multi-organ injury following relatively moderate doses of ethanol exposure. Due to the absence of apparent physical dysmorphology, the pathological manifestations of ethanol injury observed in this study are unlikely to be identified at birth, which highlights the potential value of meconium testing for FAEE as a screen for identifying those at risk for dysfunction. The negative effects on fetal organs described here may be important to identify as they can lead to sub-optimal organ function and
susceptibility to dysfunction, especially if challenged by other risk factors commonly seen in ethanol-exposed neonates (e.g., prematurity, low birth weight, poor nutritional status). Furthermore, the effect that these ethanol-induced system-wide changes can have on the brain may be of developmental and functional significance, potentially placing the individual at risk for poor developmental outcomes. Future research is required to definitively determine the physiological and developmental significance of organ injury resulting from lower levels of fetal ethanol exposure, and into the sensitivity and specificity of meconium FAEE concentrations in detecting the wide range of “real-world” patterns of alcohol use in humans. Nonetheless, the results of this study are encouraging and provide additional evidence validating the use of meconium FAEE concentrations as biomarkers of prenatal ethanol exposure and highlighting their potential utility in identifying neonates at risk for ethanol-related adverse effects.

II. Determining the affect of delayed meconium collection and contamination with postnatal stool on meconium analysis for FAEEs (Chapter 3).

Sample contamination with postnatally produced stool, which can occur as a result of delayed sample collection, is a potential confounder that can influence the accuracy and validity of meconium FAEE testing. This study investigated whether delayed sample collection (leading to collection of samples potentially contaminated with postnatal stool) can result in false positive test results for FAEEs by prospectively collecting serial excretions from neonates born to nondrinking mothers to capture the transition from meconium to postnatal stool. FAEE testing of 136 samples collected from 30 neonates during their first few days of life revealed that:

- Although the first-collected meconium sample testing negative for FAEEs in all babies, later samples tested above the 2 nmol/g positive cutoff in 63% of babies.
• The median time to appearance of FAEE-positive samples was 59.2 hours postpartum.
• Four of the 30 babies (~13%) excreted FAEE-positive meconium less than 24 hours postpartum, with the earliest positive sample collected 18.9 hours postpartum.
• In vitro experiments demonstrated that ethanol and FAEE concentrations can be increased in late samples (likely containing postnatal stool) after sample incubation with glucose (but not in the presence of antibiotics), and that FAEEs are readily formed in blank meconium in the presence of ethanol.
• Lipase activity is present in meconium and postnatal stool, and both lipase activity and FAEE formation from ethanol in blank meconium is significantly reduced in the presence of a lipase inhibitor.

This study demonstrated that collection of samples excreted later in the postpartum period can lead to false positive test results for FAEEs, which could be because of contamination with dietary components of postnatally produced stool and ethanol-producing microorganisms that are acquired in the first few days of life. We propose that ethanol fermented from ingested carbohydrates by the developing bacterial flora in the neonatal gut is conjugated to fatty acids by lipases in meconium and postnatal stool, thereby giving rise to positive test results that are not due to prenatal ethanol exposure.

These findings uncovered an important limitation to meconium analysis for FAEEs and have important implications with regard to proper sample collection protocols. This is the first study to demonstrate that false positive results can occur with meconium testing for FAEE. This means that strict sample collection protocols and sample acceptability guidelines must be devised and adhered to in order to ensure that the FAEE content is representative of in utero ethanol exposure because false positive test results could have serious legal, social, and clinical implications for the child and family in question. All efforts must be made to collect the earliest neonatal excretion to ensure the accuracy of the results,
and the possibility of contamination must be considered when interpreting FAEE concentrations in samples collected after the first day of life and/or if several bowel movements have already occurred. Furthermore, caution should be exercised when assessing samples with apparent signs of contamination with postnatally produced stool.

This study also shed light on the origin and synthesis of FAEEs in meconium. Several digestive enzymes have been previously shown to synthesize FAEEs in the presence of ethanol, including several lipases (carboxylester lipase and pancreatic triglyceride lipase) (Best and Laposata, 2003), and thus their involvement in meconium FAEE synthesis was suspected. The results suggest that meconium possesses the enzymatic machinery necessary to synthesize FAEE, and that this activity may be related to lipases. It seems likely that FAEEs found in meconium as a result of maternally ingested ethanol during pregnancy are, at least in part, also synthesized by lipases in this matrix. The contribution of other enzymes with FAEE synthetic activity cannot be excluded since, as evident from the systematic review (Chapter 5), FAEE synthesis is mediated by numerous enzymes and isozymes.

III. Assessing the potential clinical utility of voluntary meconium screening for prenatal alcohol exposure in a high-risk obstetric population (Chapter 4).

Using meconium FAEE testing for neonatal screening could not only identify neonates at-risk for FASD and provide accurate exposure history required for diagnosis, but, if implemented along with a comprehensive follow-up program and interventions, could also facilitate early recognition and treatment of FASD (Gifford et al., 2010; Goh et al., 2008; Hopkins et al., 2008). However, since informed consent from a competent patient or appointed guardian prior to treatment or testing is an ethical and legal component of medical practice
(Etchells et al., 1996; Flagler et al., 1997), a screening program of this nature would require consent of the child’s legal guardian (typically the parent). This study assessed whether women would willingly partake in a meconium screening program for prenatal alcohol exposure coupled to long-term developmental follow-up and interventions. A pilot screening program of this nature was launched in a high-risk obstetric unit previously shown to have a high prevalence of FAEE-positive meconium via anonymous meconium testing. The results demonstrate that:

- The participation rate in the screening program was significantly lower than when testing was conducted anonymously (78% vs. 95%, respectively; p < 0.05).
- The positivity rate was 3% in contrast to 30% observed under anonymous conditions (p < 0.001).
- Follow-up of the infant’s development proved of immense value as delays in motor development became apparent starting at 6 months of age. At 14 months, an assessment conducted by a clinical psychologist revealed delays in gross motor and expressive language functioning.

The results suggest that the majority of mothers who consumed alcohol in pregnancy refused to participate in the pilot screening program despite its potential benefit to child health. This indicates that maternal unwillingness to consent may limit the effectiveness of meconium testing for population-based open screening, and sheds light on the extent to which factors like stigmatization, embarrassment, guilt, and fears of child apprehension can be detrimental to screening efforts.

Although no specific policies currently exist for meconium testing, informed consent from the patient, guardian, or court with the authority to give it, is an ethical and legal necessity in medical practice, without which, medical testing or treatment cannot proceed (Etchells et al., 1996). Thus, the issue of participation must be considered and addressed if such screening programs are implemented in clinical practice. Public education, social marketing, and various
strategies that could improve participation may need to be implemented for such programs to be of benefit. For example, the “opt-out” method to gain consent was shown to yield higher testing rates in neonatal HIV screening (Public Health Agency of Canada, 2006) and may thus prove to be of benefit in neonatal alcohol screening. In the "opt-out" approach, women are informed and told that testing will be conducted with other routine neonatal tests, but are given an option to decline testing if they wish. In other words, women have to explicitly decline rather than consent as is done with the standard “opt-in” method. In both cases, written informed consent is required; it is merely the way in which the patients are initially informed of the testing that is different. The “opt-out” approach is currently employed in some provinces (e.g., Alberta, Manitoba, Quebec) for neonatal HIV screening and has shown to yield considerably higher testing rates than the “opt-in” approaches employed in other provinces (e.g., Ontario, New Brunswick, Nova Scotia) (Public Health Agency of Canada, 2006).

Participation rates may also increase as the test becomes established in society. It is possible that positive examples such as the positive case presented in this thesis (Chapter 4) may serve as educational tools to build trust in the system and increase maternal participation rates by making it more acceptable to the population. Alternatively, jurisdictions may argue that testing should be conducted without maternal consent since it is in the best interest of the child. The legality and ethics of doing so are discussed in more detail below. If this does occur, the screening program piloted here may serve as a model for a program that can be implemented in a clinical setting since it utilized currently existing services in the community, which may the most cost-effective and feasible strategy for implementing such programs.
IV. Examining the capacity for FAEE synthesis and the enzymology of this non-oxidative metabolic pathway of ethanol in mammalian organs and tissues (Chapter 5).

The capacity for non-oxidative ethanol metabolism to FAEEs varies greatly among tissues and organs, which is likely related to differential expression of enzymes with FAEE synthetic activity and presence or absence of competing metabolic pathways (e.g., oxidative), among other factors. In this study, published literature on the subject was systematically reviewed to provide a more complete insight into the enzymology behind FAEE-synthesis and the capacity of different tissues to synthesize these esters. The results of this study revealed that:

• Almost all tissues and organs (tested to date) have been shown to possess the ability to synthesize FAEEs upon ethanol exposure, with pancreas and liver being the most widely studied.
• Two distinct enzyme activities have been discerned: FAEE synthase (FAEES, which conjugates ethanol and free fatty acid) and acyl-CoA: ethanol O-acyltransferase (AEAT, which conjugates ethanol and fatty acyl-CoA).
• Based on inhibitor and localization studies, it is apparent that FAEES and AEAT activities are expressed by different enzymes, with AEAT activity localized to the microsomal fraction and FAEES activity found in both cytosolic and microsomal fractions (as well as mitochondrial, lysosomal, and likely others).
• FAEES activity is the highest in the pancreas followed by the liver, AEAT activity is the highest in the liver and intestinal mucosa.
• The enzymes associated with FAEES activity have been purified or partially purified from the pancreas, liver, heart, brain, blood, and adipose tissue and have been shown to be associated with various CEs, ChE and lipases, depending on the tissue and cell fraction from which they were purified. Furthermore, several enzymes and isozymes with FAEES activity typically exist within a particular tissue. The identity of enzymes possessing AEAT activity remains unknown.
Factors that can lead to increased FAEE production include exposure to inhibitors of oxidative metabolism, high ethanol concentrations that may saturate oxidative enzymes, and decreased ADH activity as a result of chronic alcohol abuse.

From this work, it is apparent that the capacity to metabolize ethanol to FAEE is a widely expressed enzymatic activity. The concentration of FAEEs in a particular tissue or matrix will depend on the capacity of that tissue to synthesize, retain, and degrade these esters. It is clear that FAEE synthesis involves numerous enzymes and isoenzymes, the differential expression of which in tissues influences their synthetic capacity and thus the levels of these esters in the matrix. This is complicated by differential expression of FAEE-hydrolytic enzymes, presence/availability of substrates (fatty acid sources), tissue composition (which influences FAEE retention/accumulation), FAEE-binding proteins, and competing pathways and substrates. FAEEs found in meconium are likely synthesized by the enzymes (likely lipases) in meconium itself, by enzymes in the GI tract (secreted and intracellular), and perhaps also by enzymes in other tissues and later deposited in meconium. The ontogeny of these various enzymes and differential expression in fetal tissues likely dictates their overall contribution to non-oxidative ethanol metabolism and to meconium FAEE-content.

It is possible that FAEE levels in some tissues will be greatly influenced by presence of competing substrates or genetic variability in a particular enzyme responsible for the bulk of FAEE synthesis in that tissue, but will have no effect on another tissue where another family of enzymes is involved. Likewise, it is possible the ethanol can affect the metabolism of endogenous compounds and xenobiotics normally metabolized by these enzymes through competition. Matrices where concentrations of FAEEs are used as biomarkers of ethanol exposure, such as meconium, should be further investigated to determine the
enzyme(s) involved in the synthesis and degradation of FAEEs, the effect of competing pathways and substrates on FAEE synthesis, and the potential effect of genetic variability and chronic alcohol exposure on the activity and expression of these enzymes. This will facilitate a better understanding and interpretation of FAEE concentrations in these matrices and help with the identification of confounding variables that can affect the test results.

2 Challenges and considerations in implementing neonatal meconium screening programs for prenatal alcohol exposure

The purpose of any screening program is to identify individuals who are likely to have a specific condition so that a comprehensive diagnostic assessment can follow. Thus, a meconium screening program will only be beneficial if it is integrated in a consistent long-term funding program that makes adequate resources available for follow-up, diagnosis and interventions (Chudley et al., 2005; Goh et al., 2008). As such, even if the consent rate is improved, it is extremely important to determine whether it will be cost-effective or even economically feasible to follow-up all the positive cases identified by meconium testing without overwhelming the health care system. Additionally, increasing the diagnostic capacity will be essential as the current diagnostic clinics are simply unprepared to deal with the large influx of cases that would be expected if a screening program were launched (Goh et al., 2008). Similarly, existing support and intervention programs may also be unprepared, unequipped, and require additional training of staff to handle the influx of ethanol-exposed children who develop disabilities and require specialized support services. To more accurately estimate the costs that may be associated with such an influx, it will be important to determine what proportion of infants testing positive for meconium FAEEs
actually go on to develop disabilities and will require access to diagnostic clinics and intervention programs.

The reduction in secondary disabilities associated with FASD may lead to societal savings that could offset screening costs. Furthermore, by identifying two potential patients – the neonate and the drinking mother, screening can present an opportunity for intervention in both (Koren et al., 2008). Treatment, management, and education of mothers can potentially prevent future alcohol-exposed pregnancies thereby decreasing the incidence of FASD (primary prevention of FASD) and have a significant impact beyond FASD in addressing issues of mental health, substance abuse, and child protection (Leonardson and Loudenburg, 2003). Two health economics studies examining hypothetical screening programs reported that reduction in secondary disabilities and primary prevention of FASD by intervention and education of mothers may lead to societal savings if screening is implemented universally or targeted to high-risk populations (Gifford et al., 2010; Hopkins et al., 2008). Hopkins et al. (2008), reported significant cost savings with a program that was aimed solely at improving literacy (an incremental cost-effectiveness ratio for mandating a universal screen of all newborns in Ontario of $65,874 per quality-adjusted life years), whereas Gifford et al. (2010) calculated that the savings may range from $6 to $97 for every $1 spent on screening as a result of treatment of the identified mothers alone. However, many input variables in these studies were theoretical, incomplete, or simply not taken into account, and it is hoped the study presented in this thesis (Chapter 4) can aid future cost-effectiveness and cost-benefit studies by providing a realistic model for program implementation along with estimated costs of program components.

A number of social and legal-ethical issues complicate the use of biomarkers for detecting alcohol use among pregnant women and could thus hinder screening implementation. Several potential concerns relating to
constitutional law have been raised, centering on informed consent and the right to self-determination and autonomy, reproductive rights, the right to privacy, and the right to equal treatment (Zadunayski et al., 2006). From the bioethical perspective, potential meconium screening programs have been evaluated based on the principles of autonomy, beneficence, non-maleficence, and justice (Marcellus, 2007). These are discussed in the following paragraphs.

With regard to informed consent and autonomy, the overriding general principle is that medical treatment cannot be given without the consent of the individual or a guardian with the authority to give it (Etchells et al., 1996). The person consenting must be informed and be able to understand the nature and effect of the proposed treatment. With regard to discarded samples such as meconium or placenta, the situation is less clear and the requirement of informed consent for alcohol and drugs testing often depends on hospital policies where such specimens are collected (Bearer, 2001). It has been argued that when children are born in hospitals, recovered meconium that becomes available during routine care may be legally tested for medical purposes without consent, although, ethically, mothers should be informed (Dickens, 2011). Furthermore, an argument has been made that meconium actually belongs to the newborn, and that mothers, as trustees, must manage it in the child’s best interests (Dickens, 2011). Thus, it can be argued that because screening is of potential benefit to the child, the mother may not legally be free to refuse services meant to aid her child’s health assessment and care (Dickens, 2011). On the other hand, others have argued that the ethical necessity of informed consent, in conjunction with section 7 of the Canadian Charter of Right and Freedoms, would prevent the implementation of mandatory screening without room for an informed refusal or deferral as this would infringe upon the section 7 Charter rights of women (Zadunayski et al., 2006). An additional concern raised against mandatory screening is that such policy may push some women to stay outside of the health care system and decline prenatal and antenatal care out of fear of social and
legal repercussions, which would be particularly detrimental for these women and their newborns (Marcellus, 2007; Zadunayski et al., 2006).

Another ethical concern is raised with regard to the benefits of screening and its potential negative implications. As mentioned above, a meconium screening program will only be beneficial if it is integrated in a consistent long-term follow-up program with adequate resources for diagnosis and interventions. If the health care system simply does not have the resources to provide improved care or access to services, assessing exposure will not be of benefit to the individuals or society. Furthermore, although screening can improve quality of life through early diagnosis (Streissguth et al., 2004), labeling children as “at-risk” for disabilities may carry serious psycho-social implications for these children and their families (Marcellus, 2007). The test results may potentially be misused by courts, social services, insurance companies, and even within the health-care system due to stigmatization of patients. Additionally, in some jurisdiction, physicians may be obligated to report maternal drug or alcohol use to local health services departments, including child protection services, which could result in punitive action and/or child apprehension (Zadunayski et al., 2006). From this, it is clear that if a screening program is implemented in clinical practice, it will be imperative to first determine who will have access to the test results, how the confidentiality of the patients will be protected, and how this information should or should not be used to ensure that the families benefit from screening with minimum risks.

Lastly, there is considerable concern regarding the ethics of targeted screening as compared to universal screening, which relates back to the principle of justice. It is argued that targeted screening can lead to over-identification of already marginalized populations (Marcellus, 2007). Furthermore, since no reliable risk factors for prenatal alcohol use exist, there is no valid justification for screening the newborns of some women but not others (Zadunayski et al., 2006).
Targeting any specific racial and socio-economic groups would essentially equate to stereotyping and profiling. Screening must be conducted with fairness and equality such that the potential benefits and harm of such programs is equally distributed among society and not restricted to one particular group (Zadunayski et al., 2006). To avoid any stigmatizing selection of target populations, universal testing of neonatal meconium is preferred, however, it raises economical and ethical concerns of cost-effectiveness with regard to allocation of already scarce resources (Dickens, 2011).

Overall, if neonatal screening for prenatal alcohol exposure moves into the clinical arena and becomes the standard of care, policies that will aim to maximize the benefits and minimize the harms for both the child and the mothers will need to be drafted. Furthermore, these policies and guidelines will have to be subject to ongoing amendments as the legal and ethical considerations related to such screening are reviewed, deliberated on, and refined over time.

3 Limitations and Future Directions

There are some important challenges and limitations to using meconium testing for FAEE as a biomarker of fetal ethanol exposure and a screen for the identification of infants at risk for FASD. Although meconium, in contrast to urine or blood, is a cumulative matrix that can provide a long record of exposure (last two trimesters), it begins to form in the fetal intestine in the second trimester and accumulates mostly in the latter part of pregnancy (Burd and Hofer, 2008). This means that prenatal alcohol exposure may not be detectable by meconium analysis if it had occurred earlier in pregnancy but the mother later abstained from drinking. More research is needed on the timing of meconium accumulation and the gestational time window during which prenatal alcohol exposure will be detectable via meconium analysis (Burd and Hofer, 2008).
Additionally, the time window for meconium collection after birth is approximately 72 hours, but, as determined from the research presented in this thesis (Chapter 3), samples should ideally be collected within the first 24 hours of birth to minimize the risk of obtaining false positive results and contamination with postnatal stool. This may be particularly problematic in premature babies that often have delayed excretion times (Kumar and Dhanireddy, 1995). Additional research must be conducted on whether this delayed passage of meconium could lead to false positive results even in the very first meconium sample. Overall, improper sample collection and handling may be an important issue affecting the usefulness of this test.

Further validation of meconium FAEE as biomarkers of prenatal ethanol exposure is required. Specifically, research is needed on the ability of this test to identify different timing, patterns, and levels of drinking in pregnancy, as information is still lacking on how these variables can affect the sensitivity and specificity of the test. Additionally, it is unknown what proportion of infants testing positive for meconium FAEEs actually go on to develop disabilities and thus the predictive value of this test with respect to child development and FASD diagnoses must be determined in the future.

Lastly, before FAEE screening can move into the mainstream, and perhaps become part of the standard of care, comprehensive cost-effectiveness and cost-benefit studies will be needed to determine whether it is an economically viable and ethically sound option. Such studies should strive to utilize accurate input variables. It will also be important for such health economics studies take into account how low participation in screening and interventions may impact the cost-effectiveness and cost-benefit ratios of such programs since this will be an important issue based on the results of the studies presented in this thesis (Chapter 4).
4 Conclusions

Several new pieces of evidence are presented in this thesis regarding the validity, usefulness, limitations, and potential clinical utility of meconium FAEE analysis as a screening tool for prenatal alcohol exposure. First, this research showed that meconium FAEE concentrations are predictive of certain fetal organ-system injury, thereby providing additional evidence in support of the use of this test as a screening tool for the identification of infants at-risk for disabilities. Second, this research revealed that an important limitation of the test is a risk of false positive results due to delayed sample collection. This has important implications with respect to sample collection protocols and interpretation of FAEE concentrations in samples collected later in the postpartum period. Third, this research demonstrated that women’s unwillingness to consent diminishes the utility of meconium FAEE testing for population-based open screening and that increasing participation rates will be an important challenge if neonatal screening programs for prenatal alcohol exposure are implemented in clinical practice. Lastly, this research revealed the complexities of FAEE synthesis in mammalian tissues, the involvement of numerous enzymes and isozymes, and that the differential expression of these enzymes and presence of competing metabolic pathways may influence FAEE concentrations in a tissue or matrix following ethanol exposure. More in-depth knowledge of FAEE synthesis is important for the appropriate selection of matrices for FAEE analysis and accurate interpretation of FAEE concentrations in various tissues following ethanol exposure. In summary, the results presented in this thesis fill several knowledge gaps that existed with respect to the potential benefits and limitations of meconium analysis for FAEEs, and contribute new knowledge that will help bring meconium FAEE testing into the clinical arena.
5 References


Appendices

Appendix A. Documentation of Ethical Approval

Research Ethics Board (REB)

The Research Ethics Board for The Hospital for Sick Children is organized and operates according to the principles and practices outlined in the Tri-Council Policy Statement, the ICH Harmonized Tripartite Guidelines: Good Clinical Practice, and Division 3 and the Medical Devices Regulations of the Food and Drug Act as well as the Natural Health Products Regulations of Health Canada. This signed document is in lieu of the Health Canada Research Ethics Board Attestation Form.

Approval & Terms of Agreement

Investigators:

Dr. Gideon Koren, S. Shor, J. Gareri, I. Nulman

Study Title:
Pilot Study on Neonatal Screening for Prenatal Ethanol Exposure in the Region of Grey Bruce, ON

REB File number: 1000010902  Level of Continuing Review: II D

Protocol Version Date: July 16, 2007
Consent & Assent Form Version Date(s): July 16, 2007
Investigator’s Brochure Version Date: n/a

Other Approved Document Dates: Recruitment Tools (Short Summary Given to Mothers) – July 16, 2007

I agree to carry out the proposed research involving human subjects in accordance with the above-noted guidelines and regulations (as applicable) and using only the REB-approved study protocol and consent/assent form(s). I shall notify the division/department head and the REB prior to implementing any amendments in the protocol and consent/assent forms and of any deviations or any changes in study activity. I shall also notify the REB of any unexpected adverse events as per REB guidelines. As applicable, I certify that the research contract and corresponding protocol are consistent and will inform the contract manager of any protocol amendments as required.

I agree that, in accordance with the Personal Health Information Protection Act of Ontario, I am responsible for adhering to all conditions and restrictions imposed by the REB governing the use, security, disclosure, return and disposal of the research subjects’ personal health information. I am also responsible for reporting immediately any privacy breaches to the REB Chair and to Janice Campbell, the Sick Kids privacy officer. I will ensure that the personal health information is used, only as necessary, to fulfill the specific research objectives and related research questions described in this application and approved by the REB.

Signature of Primary Investigator

DATE Nov 2007

I approve of this research protocol, agree to share responsibility for its proper conduct, and will ensure that the REB is notified of concerns, as appropriate.

Signature of Division/Department Head

DATE Nov 22/04

The REB of the Hospital for Sick Children has reviewed and approved the above-named research study.
Dr. Melvin Freedman, REB Chair
555 University Avenue, Toronto, Ontario, MSG 1X8
Tel: 416-813-8152 Fax: 416-813-5085 Email: melvin.freedman@sickkids.ca

DATE OF APPROVAL  NOV 2 3 2007  EXPIRY DATE  November 2008

Office of Research Ethics
The University of Western Ontario
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Website: www.uwo.ca/researchethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. G. Koren
Review Number: 13582E
Review Date: September 12, 2007
Protocol Title: Pilot study on Neonatal Screening for Prenatal Ethanol Exposure in the Region of Grey Bruce, Ontario.
Department and Institution: Paediatrics, University of Western Ontario
Sponsor:
Ethics Approval Date: November 28, 2007
Expiry Date: September 30, 2009
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 3 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expeditied review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change in the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation and/or advertisement must be submitted to the office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB, Dr. John W. McDonald

Ethics Officers to Contact for Further Information

Janica Sutherland (sutherlandj@uwo.ca)
Jennifer McEwen (mcwenej@uwo.ca)
Grace Kelly (kelleyg@uwo.ca)
Denise Grafton (graftond@uwo.ca)

This is an official document. Please retain the original in your files.
Notification of REB Initial Approval (Expedited)

Date: February 19, 2008

To: Dr. Denise Feig
Leadership Sinai Centre for Diabetes
Mount Sinai Hospital
60 Murray Street
5th Floor, Diabetes Centre
Toronto, Ontario M5G 1X5

Re: 07-0330-E
Fatty Acid Ethyl Esters (FAMEs) as Biological Marker in Meconium for Suspected Cases of In Utero Alcohol Exposure: A Pilot Study in Newborns Born to Diabetes-Affected Mothers.

Sponsor: Canadian Institutes of Health Research (CIHR)
REB Review Type: Expedited
REB Initial Approval Date: 19 February, 2008
REB Expiry Date: 19 February, 2009
Documents Approved:
- Protocol (dated 16-Jan-2008)
- Consent Form (dated 16-Jan-2008)
- Pamphlet (dated 16-Jan-2008)
- Questionnaire/Survey (rec. 12-Dec-2007)
Documents Acknowledged: Yes
Health Records Access:

The above named study has been reviewed and approved by the Mount Sinai Hospital Research Ethics Board. If, during the course of the research, there are any serious adverse events, confidentiality concerns, changes in the approved project, or any new information that must be considered with respect to the project, these should be brought to the immediate attention of the REB. In the event of a privacy breach, you are responsible for reporting the breach to the MSH REB and the MSH Corporate Privacy Office (in accordance with Ontario health privacy legislation – Personal Health Information Protection Act, 2004). Additionally, the MSH REB requires reports of inappropriate/unauthorized use of the information.

If the study is expected to continue beyond the expiry date, you are responsible for ensuring the study receives re-approval. The REB must be notified of the completion or termination of this study and a final report provided. As the Principal Investigator, you are responsible for the ethical conduct of this study.


Sincerely,

Ronald Heslegrave, Ph.D.
Chair, Mount Sinai Hospital Research Ethics Board
Appendix B. Consent forms and data collection documents for Grey Bruce/ St. Joseph Health Care (Chapter 4).

INFORMED CONSENT

PILOT STUDY ON NEONATAL SCREENING AND FOLLOW-UP FOR PRENATAL ETHANOL EXPOSURE IN THE REGION OF GREY BRUCE, ONTARIO

Primary Investigator:
Dr. Gideon Koren
Director, Motherisk Program
The Hospital for Sick Children
416-813-5781

Co-Investigator
Irene Zelner
Graduate student, University of Toronto
416-813-7709; irene.zelner@sickkids.ca

Purpose of the Study:

This study is a region-wide research study that is carried out in conjunction with the Grey Bruce Health Unit in order to determine the degree of benefit families will experience if screening for heavy prenatal alcohol exposure using meconium (first bowel movement) becomes a standard tool in clinical practice. Furthermore, we would like to determine the relationship between meconium test results and the neurodevelopment of the child.

Description of the Research:

Information regarding the study is provided in a pamphlet, after which time a trained research coordinator, nurse, or midwife, will administer the informed consent.
This informed consent asks for your permission to obtain results of the standardized TWEAK questionnaire, Antenatal 1 and 2 Forms, delivery information, infants hearing tests results, and information regarding the demographic characteristics of the family. The TWEAK questionnaire assesses alcohol use and consists of five questions. Antenatal 1 and 2 forms consist of health and pregnancy information obtained by the obstetrician during prenatal visits and will provide information on possible complications in pregnancy. The delivery information is filled out by the delivering physician and provides the history of the birthing process, including delivery method and child’s health at the point of delivery. Furthermore, for research purposes, we ask for your permission to collect meconium samples from your child(ren), and to conduct follow-up calls and visits to assess the neurodevelopment of your child.

If you agree to participate, a registered nurse or the study coordinator will collect the meconium samples and label them with an assigned number in order to protect your confidentiality. The samples will then be shipped to the Hospital for Sick Children where they will be tested for the presence of fatty acid ethyl esters (FAEE); which are alcohol-alcohol related substance that are indicative of significant alcohol exposure in pregnancy if present in high amounts.

If the meconium test is positive for the presence of FAEEs, your child will be matched with an equal number of children with negative test results and whose mothers tested negative on the TWEAK questionnaire. The matching will be done according to the gestational age and sex of the infant at the time of delivery, as well as demographic characteristics of the family (marital status, household income, education of mother). The children in these groups will be followed-up for 2 years with regular assessment of developmental milestones via phone conversation with parents and/or communication with the public health nurse assigned to the family (the follow-up procedure is described below in more detail). Around three months and one and a half years of age the child will undergo neurodevelopmental testing by a certified clinical psychologist. This assessment will occur during a scheduled home visit (approximately 2 hours in length) and will
involve the testing of your child’s development using Bayley Scales of Infant and Toddler Development, a validated tool for detecting developmental delays in the very young. The test consists of five scales, three of which are administered with child interaction to assess cognition, motor skills, and language (involve playful and engaging toys and activities); and two scales involve parent questionnaires to assess social-emotional development and adaptive behavior. At one of these visits, the clinical psychologist will also assess your (the mother’s) cognitive function in order to allow for accurate interpretation of the child’s results from neurodevelopmental testing.

If the child exhibits developmental delays, early identification will enable referral to any or all child development services, speech and language support and, or occupational therapy at no cost to the family. Furthermore, support services for the mothers such as addiction and mental health services will be available if needed through the Grey Bruce Health Unit, and the mothers may be followed-up in order to observe for changes in their quality of life.

More Detailed Description of the Follow-Up:

The standard procedures that are implemented by the Grey Bruce Public Health Unit will be followed. These procedures include a phone call within 48 hours of hospital discharge to arrange for a postpartum home visit by a Public Health nurse. During the home visit the Public Health nurse provides information and education to new parents regarding child care and other relevant topics, conducts an in-depth assessment, and, if deemed needed, devises a family service plan that may benefit the family (with the family’s permission). The Public Health nurse and parent support worker continue to work with the family in accordance with the devised family service plan to support healthy child development.

As part of this study, the Public Health nurse will be notified of the meconium test results and will act as a case manager and coordinate other service providers as needed. The Public Health nurse will also communicate to the researchers the results of the in-
depth assessment, testing of developmental milestones, and details of the family service plan. In addition, you may be contacted for follow-ups via phone, as part of the study. During the quick phone interviews, you will be asked for various developmental milestones in order to detect possible developmental delays and provide early interventions. The calls will be conducted on a monthly basis by Irene Zelner or the local Health Unit.

Two follow-up home visits will also take place; one at around three months of age and the second at one and a half years of age, during which the child will undergo neurodevelopmental testing by a certified clinical psychologist. At one of these follow-up home visits, the clinical psychologist will also assess your (the mother’s) cognitive function in order to allow us to accurately interpret your child’s test results from neurodevelopmental testing.

If at any stage of the study developmental delay is detected, either via milestones, neurodevelopmental testing or parental concerns, appropriate interventions will be tailored for the child’s needs through the Healthy Babies/Healthy Children Program. As mentioned above, support services for the mothers will also be available if needed, and the mothers may be followed-up in order to observe for changes in their quality of life.

**Summary of Study Interventions:**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample collection</td>
<td>A meconium sample will be collected from your baby’s diaper by the nurses and shipped to the Hospital for Sick Children for FAEE analysis.</td>
</tr>
<tr>
<td>Follow-up</td>
<td>If the meconium test is positive, or if you are chosen to be in the control group, your baby will be followed up via regular and timely assessments of developmental milestones (time commitment will depend on your family’s needs).</td>
</tr>
<tr>
<td>Developmental assessment at 3 and 18 months.</td>
<td>If the meconium test is positive or if you are chosen to be in the control group, a more in-depth assessment of your child’s development will be conducted by a clinical psychologist during two 2-hr home visits.</td>
</tr>
</tbody>
</table>
If the meconium test is positive or if you are chosen to be in the control group, at one of the follow-up home visits, the clinical psychologist will also assess your (the mother’s) cognitive function in order to allow us to accurately interpret your child’s test results from neurodevelopmental testing. This will take approximately 45 minutes.

**Potential Harms:**

We know of no harm that taking part in this study could cause you.

**Potential Discomforts or Inconvenience:**

One may experience anxiety while waiting for meconium and TWEAK test results. In case you experience any anxiety or discomfort please do not hesitate to contact Irene Zelner, the study coordinator, via phone at 416-813-7709 or via email irene.zelner@sickkids.ca.

**Potential Benefits:**

*To individual participants:*

Early diagnosis and time-sensitive intervention for children at risk of FASD. This in turn, can reduce the risk of the child developing secondary disabilities that can lead to an improvement of the child’s quality of life.

*To society:*

Successful establishment of meconium testing for alcohol exposure in pregnancy will have a significant impact on the detection, early clinical diagnosis, and intervention for children at risk of FASD, leading to reduced development of secondary disabilities associated with FASD, improved quality of life and decreased costs experienced by the justice system and the social services that are associated with secondary disabilities of FASD.
Confidentiality:

We will respect your privacy. No information about who you are and who your child is will be given to anyone or be published without your permission, unless the law requires us to do this. For example, the law requires us to give information about you or your child if a child has been abused, if you or your child has an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.

Hospital for Sick Children Clinical Research Monitors and regulator of the study may see your or your child’s health record to check on the study.

By signing this consent form you agree to let these people look at your or your child’s records and obtain the above mentioned information. We will provide you with a copy of this research consent and leave a copy of this consent in your or your child’s health records.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members, such as Canadian Institutes of Health Research monitors and the Hospital for Sick Children Research Ethics Board monitors. Following completion and publication of the research study, the data will be kept for five years and then destroyed as required by the Hospital for Sick Children policy. If the results of this study are published, your name will not be used and no information that discloses your identity will be released or published without your explicit consent to the disclosure.

The results of the tests we describe in this form will be used only for this study. If another health care professional caring for you or your child needs to see these study results, we will not allow disclosure of the results without your permission. We recommend that you obtain the results of these tests from health care professionals who
were trained at reading and understanding the results, such as a registered psychologist or a doctor.

**Reimbursement:**

You will not be reimbursed for participating in this study.

**Participation:**

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time with no effect on your future care or services you receive from the Grey-Bruce District Health Unit.

**Sponsorship:**

The sponsor and funder of this research is the Canadian Institute of Health Research (CIHR).

**Conflict of Interest:**

I, and the other research team members, have no conflict of interest to declare.
**Consent:**

I have read the Letter of Information, have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction. By signing this form, I agree that:

1. You have explained this study to me. You have answered all my questions.
2. You have explained the possible harms and benefits (if any) of this study.
3. I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my relationship with SickKids and health care at this institution.
4. I am free now, and in the future, to ask questions about the study.
5. I have been told that my medical records will be kept private except as described to me.
6. I understand that no information about who I am will be given to anyone or be published without first asking my permission.
7. I agree, or consent, to take part in this study.

---

**Printed Name of Parent/Legal Guardian**

__________________________

**Parent/Legal Guardian’s Signature & Date**

__________________________

**Child’s Name**

__________________________

**Printed Name of Person who explained the consent**

__________________________

**Signature & Date**

__________________________

**Printed Witness’ name (if the subject/legal Guardian does not read English)**

__________________________

**Witness’s Signature & Date**

---

If you have any questions about this study, please call Irene Zelner at 416-813-7709 or via email at irene.zelner@sickkids.ca or contact Dr. Gideon Koren via phone at 416-813-5781.

If you have any questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call Dr. David Hill, Scientific Director, c/o Lawson Health Research Institute at 519-667-6649.
DATA COLLECTION DOCUMENT

Subject’s ID #:__________

Pregnancy and Delivery Information

1. TWEAK Questionnaire:

T Tolerance: How many drinks can you hold? _______ OR How many drinks do you need to feel high? _____

W Have close friends or relatives Worried or complained about your drinking in the past year? ______

E Eye Opener: Do you sometimes take a drink in the morning when you get up? ______

A Amnesia: Has a friend or family member ever told you about things you said or did while you were drinking that you could not remember?_______

K(C) Do you sometimes feel the need to Cut down on your drinking? ______

2. Information from Antenatal Record 1:

LMP (YYYY/MM/DD): ____ / ____ / ____

GTPAL:

- Gravida _____
- Term_____
- Premature____

Abortuses _____

Living ___

Current Pregnancy Information:

- Nausea/ vomiting? Y / N
- Smoking? Y / N ( ___ cig/day)
- Alcohol, street drugs? Y / N
- Preconceptual folate? Y / N
- Other? _______________________________

Medical History:

- Any concerns? Y / N
- If yes, specify _________________________

Genetic and Family History:

- Any concerns? Y / N
- If yes, specify _________________________

Infectious Diseases:

- Any concerns? Y / N
- If yes, specify _________________________

Psychosocial:

- Any concerns? Y / N
- If yes, specify _________________________
At risk population? Y / N
  o If yes, specify __________________________________________

Physical abnormalities? Y / N
  o If yes, specify __________________________________________

3. Information from Antenatal Record 2:
   Any identified risk factors? Y / N
     o If yes, specify __________________________________________
   Any concerns from subsequent visits? Y / N
     o If yes, specify __________________________________________
   If diabetes is detected, record:
     o 1hr GCT ___________ 2hr GTT__________

4. Delivery Information:
   Gestational age: ___________

   Reason for referral to SJH: __________________________________________

   Alcohol use questions: Parkin _______ Nursing hx_______ Preop_______

   Delivery method (check mark):
     □ Vaginal  (□ Tongs □ Suction)
     □ C-section

   Analgesics used? Y / N
     o If yes, specify : □ oral □ epidural □ local □ systemic □ other ________

   Any complications during delivery? Y / N
     o If yes, specify __________________________________________

   APGAR score
     o at 1min ________ at 5 min _______

   Was the neonate in NICU? Y / N
     o If yes, specify number of days spent in the NICU ______

   Meconium aspiration? Y / N

5. Infant’s Hearing Test Results:
   Left Ear: Pass / Fail     Right Ear: Pass / Fail

   Additional comments (reason for admission, reported alcohol use to nurse, other): ________________________________

   ___________________________________________________________________________________

   ___________________________________________________________________________________
Appendix C. Consent forms and data collection documents for Mt. Sinai Hospital (Chapter 3).

INFORMED CONSENT

PILOT STUDY ON THE AFFECT OF MATERNAL DIABETES ON FATTY ACID ETHYL ESTER LEVELS IN NEONATAL MECONIUM

Primary Investigator:
Dr. Denice Feig
Internal Medicine
Endocrinology and Metabolism
Mount Sinai Hospital
416-586-4800 X 8590

Co-Investigator:
Dr. Gideon Koren
Director, Motherisk Program
The Hospital for Sick Children
416-813-5781

Research Coordinator:
Irene Zelner
Graduate student, University of Toronto
Motherisk, The Hospital for Sick Children
416-813-7709

Sponsor:
Canadian Institutes of Health Research (CIHR).

You are being asked to take part in a research study. Before agreeing to participate in this study, it is important that you read and understand the following explanation of the proposed study procedures. The following information describes the purpose, procedures, benefits, discomforts, risks and precautions associated with this study. It also describes your right to refuse to participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about risks and benefits to be able to make an informed decision. This is known as the informed consent process. Please ask the study doctor or study staff to explain any words you don’t understand before signing this consent form. Make sure all your questions have been answered to your satisfaction before signing this document.
**Purpose of the Study:**

This study is conducted in order to determine if meconium of neonates born to mothers affected by diabetes has higher levels of fatty acid ethyl esters as compared to meconium of neonates who were born to mothers not affected by diabetes. It will also determine whether contamination of meconium with milk stool can affect the accuracy of the test results. This will allow us to improve a test that is designed to identify newborns are risk for neurodevelopmental delays due to prenatal alcohol exposure, and in turn, allow for earlier interventions and significant improvements in their quality of life.

**Description of the Research:**

Information regarding the study will be provided in a pamphlet form, after which time a trained research coordinator will administer the informed consent. This informed consent asks for permission to gain access to your and your child’s health records in order to view the Antenatal 1 and 2 Forms, delivery forms, your glucose and A1C levels throughout pregnancy and delivery as well as your and your child’s dates of birth. Furthermore, you will be asked to fill out a questionnaire which assesses alcohol use and consists of five questions, which would take no longer than 5 minutes to complete. Antenatal 1 and 2 forms consist of health and pregnancy information that is obtained by the obstetrician during prenatal visits and will provide information on any possible complications in pregnancy. We will also obtain your A1C and glucose levels from a computerized laboratory database, as well as, the delivery information that is filled out by the delivering physician which will provide the history of your birthing process, including delivery method and your child’s health at the point of delivery. Additionally, you will be asked when the baby started feeding, as well as the type of feeding given to the baby (i.e., breast-milk, formula, or both).

By agreeing to participate in this study, it is guaranteed that you will not be contacted at any future point in time, as neither the addresses nor the phone numbers are collected for the purposes of this study.
If you agree to participate, you will be asked to provide the infant’s first few bowel movements by placing all the soiled diapers into a plastic bag and recording the date and time of the diaper change and/or asking the nurse to do so. The study coordinator will collect the samples and number-code them in order to protect yours and your child’s identities. The diapers will then be transferred to the Hospital for Sick Children for testing for fatty acid ethyl esters (FAEE) in the samples.

**Potential Harms:**

We know of no harm that taking part in this study could cause you.

**Potential Discomforts or Inconvenience:**

We know of no potential discomforts or inconvenience that taking part in this study could cause you except for the inconvenience of saving your child’s first several diapers. However, in case you experience any anxiety or discomfort please do not hesitate to contact Irene Zelner, the study coordinator, via phone at 416-813-7709.

**Potential Benefits:**

*To individual participants:*

There are no benefits to the individual participants.

*To society:*

Successful determination of the fatty acid ethyl esters (FAEE) levels in the collected samples will allow us to improve a test that is designed to identify newborns at risk for fetal alcohol spectrum disorders. In past studies, we have determined the baseline levels of FAEE that are found in the meconium of babies born to non-drinking mothers due to naturally occurring alcohol in the body and now we need to determine if maternal diabetes can influence the test. Knowing the baseline levels of fatty acid ethyl esters in neonates of diabetic mothers, as well as the effect of contamination with stool, would help in the future to accurately assess infants who were exposed to maternal drinking in pregnancy. This will allow for earlier identification of
children at risk of neurodevelopmental delays due to prenatal alcohol exposure, and in turn, allow for earlier interventions and significant improvements in their quality of life.

**Confidentiality:**

We will respect your privacy. No information about who you are and who your child is will be given to anyone or be published without your permission, unless the law requires us to do this.

Hospital for Sick Children Clinical Research Monitors and regulator of the study, as well as Mount Sinai Hospital’s Research Ethics Board may see the information we collected in order to check on the study.

By signing this consent form you agree to let these people look at the above mentioned information. We will provide you with a copy of this research consent.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members, such as Canadian Institutes of Health Research monitors and the Hospital for Sick Children Research Ethics Board monitors. Following completion and publication of the research study, the data will be kept for five years and then destroyed as required by the Hospital for Sick Children policy. If the results of this study are published, your name will not be used and no information that discloses your identity will be released or published.

**Reimbursement:**

You will not be reimbursed for participating in this study.

**Participation:**

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time with no effect on your future care or services you receive from Mount Sinai Hospital or the Hospital for Sick Children.
Conflict of Interest:

I, and the other research team members, have no conflict of interest to declare.

Consent:

I have read the Letter of Information, have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

Printed Name of Parent/Legal Guardian  
Parent/Legal Guardian’s signature & date

Child’s Name

Printed Name of Person who explained the consent  
Signature & date

Printed Witness’ name (if the subject/legal Guardian does not read English)  
Witness’s signature & date

If you have any questions about this study, please call Irene Zelner at 416-813-7709 or contact Dr. Denice Feig at 416-586-4800x8590 or Dr. Gideon Koren at 416-813-5781.

If you have any questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call the Research Ethics Manger at the Hospital for Sick Children at 416-813-5718 or Dr. R. Heslegrave, Chair of the Mount Sinai Hospital Research Ethics Board at 416-586-4800 x4875. These individuals are not involved with the research project in any way and calling them will not affect your participation in the study.
Subject ID#: __________________

1. Information from Antenatal Record 1:
   
   Current pregnancy:
   
   LMP (YYYY/MM/DD): ____ / ____ / ____
   
   - Alcohol, street drugs? Y / N
   - Preconceptual folate? Y / N
   
   Medical History:
   
   - Any concerns? Y / N
   - If yes, specify ____________________________________________________
   
   Genetic and Family History:
   
   - Any concerns? Y / N
   - If yes, specify ____________________________________________________
   
   Infectious Diseases:
   
   - Any concerns? Y / N
   - If yes, specify ____________________________________________________
   
   Physical abnormalities? Y / N
   
   - If yes, specify ____________________________________________________

2. Information from Antenatal Record 2:
   
   Any identified risk factors? Y / N
   
   - If yes, specify ____________________________________________________
   
   If diabetic, maternal glucose and AC1 levels
   
   - 1hr GCT ___________ 2hr GTT____________

3. Delivery Information:

   Gestational age: ____________
   Birth Weight: ____________
   Sex: ____________
   Date and time of birth: _____________________
   
   Alcohol use questions: Parkin ________ Nursing_______ Preop_________
Delivery method (check mark):

- □ Vaginal
- □ C-section

Analgesics used? Y / N

- If yes, specify: □ oral □ epidural □ local □ systemic

- Type: _________ Time of administration: ____________

Any complications during delivery? Y / N

- If yes, specify:

Any complications during delivery?

APGAR score

- at 1 min _________ at 5 min _________

Was the neonate in NICU? Y / N

- If yes, specify number of days spent in the NICU ________

Meconium aspiration? Y / N

Maternal glucose and AC1 levels ________________

Additional comments (any metabolic, genetic, congenital, or GI disorders? Infection in neonate?)

_____________________________________________________________________

_____________________________________________________________________

4. **Feeding Information:**

- Feeding regimen?
  - □ breast-fed
  - □ formula
  - □ combination

- Feeding commencement (date and time) ________________
Appendix D. Data extraction form for systematic review (Chapter 5)

**DATA EXTRACTION FORM – FAEE synthesis**

**STUDY:**

Authors: __________________________ Year: ___________ Journal/vol(iss/pgs): __________________________

**TYPE**

- [ ] Original Manuscript  
- [ ] Review/ Editorial/ Commentary  
- [ ] Conference proceedings
- [ ] Other ________________

**DESIGN  Human/ Animal / Cell line**

Species/Line: __________________________ Tissue(s): __________________________ Enzyme(s): __________________________

**AIM OF STUDY**

___________________________________________________________________________________________

**DETAILS OF STUDY METHODS**

- [ ] FAEE content or accumulation (In Vitro/ In Vivo)
- [ ] FAEE-synthetic activity (In Vitro/ In Vivo)
- [ ] Inhibitors used
- [ ] Isolation of enzyme/sequencing
- [ ] Other activities
- [ ] FAEE disposition (absorption, distribution, hydrolysis (In Vitro/ In Vivo)

Additional info: ____________________________________________
3. MEASUREMENTS

Purification

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MW</th>
<th>Polypeptides</th>
<th>Associated activities or enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Activity measurements

<table>
<thead>
<tr>
<th>Sample (specify tissue fraction etc)</th>
<th>FAEE synthesis by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions</td>
<td></td>
</tr>
<tr>
<td>- Temp</td>
<td></td>
</tr>
<tr>
<td>- Time</td>
<td></td>
</tr>
<tr>
<td>- pH</td>
<td></td>
</tr>
<tr>
<td>Substrate(s)</td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td></td>
</tr>
<tr>
<td>Km and Vmax</td>
<td></td>
</tr>
<tr>
<td>Effect of Inhibitors</td>
<td></td>
</tr>
<tr>
<td>Proper control (enzymatic)?</td>
<td></td>
</tr>
</tbody>
</table>

**OTHER RELEVANT MEASUREMENTS:**

_____________________________________________________________________________________________________________________________

_____________________________________________________________________________________________________________________________