USE OF MECONIUM AND HAIR FOR DETECTION OF PRENATAL EXPOSURE TO ETHANOL AND OTHER DRUGS OF ABUSE

BY

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A thesis submitted in conformity with the requirements for the degree of
Master of Science
Graduate Department of Pharmacology
University of Toronto

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Use of Meconium and Hair for Detection of Prental Exposure to Ethanol and Others Drugs of Abuse

Master of Science, 2009

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Abstract

In-utero ethanol exposure may result in fetal alcohol spectrum disorder (FASD). Studies have suggested that women who drink ethanol are more likely to consume illicit drugs. Detection of such exposures has been done via meconium and hair testing and can serve to direct needed prevention methods and appropriate management and intervention for the neonate and the mother. This study examined maternal diabetes as a possible confounder for in-utero ethanol exposure testing and determined the trends in drug use associated with heavy in-utero ethanol exposure in a high-risk obstetric Canadian population. It was determined that maternal diabetes does not produce false-positive results in testing for in-utero ethanol exposure. Furthermore, heavy in-utero ethanol exposure was detected in 15.5% of samples and was associated with an increased exposure to amphetamines (OR=3.30) and opiates (OR=2.01), but a decreased exposure to cannabinoids (OR=0.61) when compared to neonates with no heavy in-utero ethanol exposure.
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# TABLE OF CONTENTS

Abstract ........................................................................................................... ii  
Acknowledgement .......................................................................................... iii  
Table of Content ............................................................................................ iv  
List of Tables .................................................................................................. vii  
List of Figures ................................................................................................ viii  
List of Appendices ......................................................................................... ix  
List of Abbreviations ...................................................................................... x

1. Introduction ................................................................................................. 1  

1.1 Statement of Problem .............................................................................. 1  
1.2 Purpose of Study and Objectives .......................................................... 3  
1.3 Statement of Research Hypotheses and Rationale for Hypotheses ....... 4  
1.4 Review of Literature ............................................................................... 5  

1.4.1 Ethanol .................................................................................................. 5  

1.4.1.a Pharmacology of Ethanol ................................................................. 6  
1.4.1.a.1 Absorption and Distribution ....................................................... 6  
1.4.1.a.2 Metabolism and Elimination ....................................................... 7  
1.4.1.b Ethanol Consumption ........................................................................ 10  
1.4.1.c FASD ............................................................................................... 12  
1.4.1.d Variability in the Effects of Ethanol .............................................. 13  
1.4.1.e Importance of Early Detection of In-Utero Ethanol Exposure ....... 14  
1.4.1.f Biomarkers of Chronic Ethanol Consumption ............................... 16  
1.4.1.g Detection of In-Utero Ethanol Exposure via Meconium FAEE ....... 18  
1.4.1.h Meconium FAEE and Neonatal Outcomes .................................... 22  

1.4.2 Confounding Factors in Meconium FAEE Testing ......................... 23  

1.4.2.a Diabetes in Pregnancy ................................................................. 25  

1.4.3 Drugs of Abuse..................................................................................... 28  

1.4.3.a Cannabinoids ................................................................................... 29  
1.4.3.b Stimulants – Cocaine and Amphetamines .................................... 30  
1.4.3.c Opiates ......................................................................................... 32  
1.4.3.d Adverse Effects of Illicit Drugs in Pregnancy ............................... 34  
1.4.3.e Importance of Detecting In-Utero Drug Exposure .................... 38  
1.4.3.f Detection of In-Utero Drug Exposure via Meconium & Hair ..... 38
2. Materials and Methods

2.1 Materials ............................................................................................................. 41
   2.1.1 Chemicals and Reagents ................................................................. 41
   2.1.2 Laboratory Equipment ................................................................. 41

2.2 Methods .............................................................................................................. 42
   2.2.1 Laboratory Analysis of Meconium FAEE ........................................... 42
       2.2.1.a Preparation of FAEE Stock Solution ........................................... 42
       2.2.1.b Preparation of d5-Ethyl Esters Stock Solution ......................... 42
       2.2.1.c Meconium Sample Preparation for FAEE Testing .................... 44
       2.2.1.d Gas Chromatography-Mass Spectrometry Analysis ............... 44
       2.2.1.e Data Analysis ......................................................................... 46
   2.2.2 Laboratory Analysis of Glucose Effect on Meconium FAEE .......... 46
       2.2.2.a Sample Preparation ................................................................ 47
   2.2.3 Ethical Approval for the Use of Clinical Data .................................... 48
   2.2.4 Meconium FAEE Analysis for Prevalence of In-Utero Ethanol Exposure ................................................................. 48
   2.2.5 Laboratory Analysis of Meconium and Hair for Drugs of Abuse ...... 49
       2.2.5.a Sample Preparation ................................................................. 49
   2.2.6 Statistical Analysis .............................................................................. 52

3. Results ...................................................................................................................... 53
   3.1 Internal Standards ......................................................................................... 53
       3.1.1 Purity of d5-Ethyl Esters Stock Solution ....................................... 53
       3.1.2 Ratio of FAEE Standards to Deuterated Standards .................... 54
       3.1.3 Standard Curve ............................................................................. 54
       3.1.4 Interday and Intraday Coefficient of Variation ............................ 55
   3.2 The Effect of Glucose on Meconium FAEE .............................................. 57
   3.3 Use of Drugs of Abuse in High Risk Canadian Population ................. 58
       3.3.1 Rates of Ethanol and Drug Exposures ......................................... 58
       3.3.2 Ethanol Exposure and its Association with Exposure to Others Drugs of Abuse ................................................................. 60

4. Discussion ............................................................................................................... 62
   4.1 The Effect of Glucose on Meconium FAEE .............................................. 62
   4.2 Ethanol Use in High Risk Canadian Population ........................................ 63
   4.3 The Use of Drugs of Abuse in a High Risk Canadian Population .......... 65
   4.4 Ethanol Exposure and Its Association with Exposures to Other Drugs of Abuse in High Risk Canadian Population ................................................................. 68
   4.5 Limitations ................................................................................................... 71
       4.5.1 Meconium FAEE Testing ............................................................ 71
       4.5.2 The Effect of Glucose on Meconium FAEE ................................. 72
       4.5.3 In-Utero Ethanol and Drug Exposure ......................................... 73
   4.6 Summary ...................................................................................................... 73
   4.7 Future Studies ............................................................................................. 74
5. References.................................................................................................................. 76


7. Appendices.................................................................................................................. 95
**LIST OF TABLES**

**TABLE 1.1:** CHARACTERISTICS OF FASD .......................................................... 13

**TABLE 1.2:** BIOMARKERS OF CHRONIC ETHANOL CONSUMPTION .................. 17

**TABLE 1.3:** FAEE USED IN DETECTION OF *In-Utero* Ethanol Exposure .......... 21

**TABLE 1.4:** MECONIUM FAEE STUDIES FOR DETECTION OF *In-Utero* Ethanol Exposure .......................................................... 21

**TABLE 1.5:** MECONIUM FAEE AND ADVERSE NEONATAL OUTCOMES .......... 22

**TABLE 1.6:** *In-Utero* Exposure to Drugs of Abuse and Adverse Neonatal Outcomes .......................................................... 37

**TABLE 3.1:** INTRADAY AND INTERDAY COEFFICIENT OF VARIATION AT 50ng/ml FAEE CONCENTRATIONS ........................................ 56

**TABLE 3.2:** INTRADAY AND INTERDAY COEFFICIENT OF VARIATION AT 100ng/ml FAEE CONCENTRATIONS ............................. 56

**TABLE 3.3:** THE NUMBER OF NEONATES WITH *In-Utero* EXPOSURE TO DRUGS DETERMINED VIA MECONIUM AND HAIR TESTING (n=622) .... 59

**TABLE 3.4:** THE STRENGTH OF ASSOCIATION BETWEEN POSITIVE FAEE TESTS AND OTHER DRUGS EXPRESSED IN ODDS RATIOS ............ 61
LIST OF FIGURES

FIGURE 1.1: ETHANOL METABOLISM AND ELIMINATION ........................................... 9
FIGURE 1.2: FAEE FORMATION ......................................................................................... 19
FIGURE 1.3: EE PRODUCTION ......................................................................................... 24
FIGURE 1.4: THE RELATIONSHIP BETWEEN VARIOUS OPIATES ................................. 33
FIGURE 3.1: GC-MS CHROMATOGRAPH OF D5-ETHYL ESTERS STOCK SOLUTION .... 53
FIGURE 3.2: GC-MS CHROMATOGRAPH OF THE RATIO OF ETHYL ESTERS TO D5-ETHYL ESTERS IN A STOCK SOLUTION ......................................................... 54
FIGURE 3.3: LINEAR REGRESSION COEFFICIENTS .......................................................... 55
FIGURE 3.4: GC-MS CHROMATOGRAPH OF A BOILED MECONIUM SAMPLE WITH 2MOL GLUCOSE SOLUTION .............................................................. 57
FIGURE 3.5: GC-MS CHROMATOGRAPH OF A NON-BOILED MECONIUM SAMPLE WITH 2MOL GLUCOSE SOLUTION .......................................................... 57
LIST OF APPENDICES

APPENDIX A: DOCUMENTATION OF ETHICAL APPROVAL.......................... 95
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-OH-THC</td>
<td>11-hydroxy-THC</td>
</tr>
<tr>
<td>95% CI</td>
<td>95% Confidence Intervals</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol Dehydrogenase</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention Deficit/Hyperactivity Disorders</td>
</tr>
<tr>
<td>AEAT</td>
<td>Acyl-Coenzyme A:Ethanol O-Acyltransferase</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde Dehydrogenase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
</tr>
<tr>
<td>CAD</td>
<td>Canadian Dollars</td>
</tr>
<tr>
<td>CDT</td>
<td>Carbohydrate Deficient Transferin</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
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<tr>
<td>CYP2E1</td>
<td>Cytochrome P450 enzyme 2E1</td>
</tr>
<tr>
<td>EE</td>
<td>Endogenous Ethanol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
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<tr>
<td>EtG</td>
<td>Ethyl Glucuronide</td>
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<tr>
<td>FAEE</td>
<td>Fatty Acid Ethyl Esters</td>
</tr>
<tr>
<td>FAS</td>
<td>Fetal Alcohol Syndrome</td>
</tr>
<tr>
<td>FASD</td>
<td>Fetal Alcohol Spectrum Disorder</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatograph-Mass Spectrometer</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma-Glutamyl Transferase</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantification</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to Charge Ratio</td>
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<tr>
<td>MCV</td>
<td>Mean Corpuscular Volume</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NAS</td>
<td>Neonatal Abstinence Syndrome</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratios</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>R^2</td>
<td>Linear Regression Coefficient</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid Phase Microextraction</td>
</tr>
<tr>
<td>THC</td>
<td>Delta-9-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>THC-COOH</td>
<td>11-nor-9-carboxy-THC</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine Diphosphate-Glucuronosyltransferase</td>
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1. INTRODUCTION

1.1 Statement of Problem

Ethanol is legal and widely available. Canadian data from 2000-2001 estimate that 13.6% of pregnant women consume ethanol at some point in pregnancy, while 4.9% of women drink throughout the entire pregnancy (Dell & Roberts, 2006). In-utero exposure to ethanol may result in fetal alcohol spectrum disorder (FASD), which encompasses a range of physical, behavioral, and cognitive disabilities and in North America affects at least 0.91% of the pediatric population (Sampson et al., 1997).

Early diagnosis of FASD allows the child and mother to receive interventions at an earlier point in time, leading to a decreased risk of secondary disabilities for the child and preventing future ethanol exposed pregnancies for the mother (Burd, 2006; Streissguth et al., 2004). However, due to fear of legal and societal consequences women may not admit to ethanol use in pregnancy, while maternal blood and urine markers may not be sensitive enough to detect in-utero drug exposure due to short half lives of many drugs (Bandstra et al., 2002; Chang, 2001; Lester et al., 2001; Markovic et al., 2000; Nair et al., 1994; Ostrea et al., 1992; Ostrea et al., 1999; Ostrea et al.,
Therefore, fatty acid ethyl esters (FAEE) screening in meconium, a matrix representing the intestinal contents of a fetus before birth, has been used for the detection of heavy in-utero ethanol exposure in late pregnancy (Bearer et al., 2003; Ostrea et al., 2006).

In order to use meconium FAEE testing as a universal screening tool for in-utero ethanol exposure, confounders leading to false-positive test results need to be detected and addressed, as false-positive test results could have devastating social and clinical implications on the family; as such results may lead to child apprehension or mislabeling of a child as being at risk of developing FASD.

One such potential confounder is maternal diabetes, as individuals affected by diabetes have increased endogenous ethanol production (Liebich et al., 1982) and diabetes mellitus is the most common medical complication in pregnancy, affecting up to 14% of pregnancies (American College of Obstetricians & Gynecologists, 2001; Sheffield et al., 2002; Wen et al., 2000). Since a great number of pregnancies are affected by diabetes, it is essential to study this condition as a possible confounding factor in meconium testing for in-utero ethanol exposure.

Ethanol is not the only drug that can damage the developing fetus, as in-utero exposure to illicit drugs has been associated with maternal, fetal and neonatal complications, including prematurity, intrauterine growth retardation, low birth weight, fetal distress, and developmental delays (Chouteau et al., 1988; Schneider & Chasnoff, 1992). Considering that previous studies have shown that women who
abuse ethanol abuse other drugs, such as nicotine, cocaine and opiates (Gladstone et al., 1997; Lester et al., 2001), it is important to determine the association of ethanol exposure and that of drugs of abuse. However, no study has looked into this association in an obstetric Canadian population that is at a high-risk of ethanol and/or illicit drug use, where the drug exposures were confirmed with laboratory testing.

In this study we used meconium, the first few bowel movements of the neonate, and neonatal hair analysis to determine the trends in neonatal exposure to drugs of abuse that are associated with heavy ethanol use in pregnancy. Knowledge of illicit drug exposure, just like in-utero ethanol exposure, can serve to direct needed prevention methods and appropriate management for the neonate and for the mother.

1.2 Purpose of the Study and Objectives

OBJECTIVES:

1. To determine if maternal diabetes could act as a confounding variable in testing of in-utero ethanol exposure via meconium FAEE.

2. To determine the positivity rate for heavy in-utero ethanol exposure during late pregnancy in a high risk obstetric Canadian population.

3. To determine the trends of use for drugs of abuse that are associated with heavy in-utero ethanol exposure during late pregnancy in a high risk obstetric Canadian population.
1.3 Statement of Research Hypotheses and Rationale for Hypotheses

**Hypothesis 1:** Fatty acid ethyl ester levels in meconium will increase with an increase in glucose concentrations. However, the levels will not reach the positive cut-off of 2 nmol FAEE per gram meconium.

**Rationale 1:** Up to 14% of pregnancies are affected by diabetes mellitus. Unless treated, this condition results in elevated glucose levels. Studies have shown that elevated glucose levels in individuals affected by diabetes may lead to increased production of endogenous ethanol. This could potentially result in an increased production of FAEE from the abundant fatty acids and elevated ethanol levels found in the body. However, the levels of endogenous ethanol produced in diabetes-affected individuals are relatively low and hence FAEE production is not expected to exceed the cut-off value of 2 nmol FAEE per gram meconium.

**Hypothesis 2:** The rate of heavy *in-utero* ethanol exposure in late pregnancy among women investigated by Children’s Aid Societies or by physicians who suspect illicit drug use or ethanol in pregnancy will be higher than the rates detected in the general Canadian populations.

**Rationale 2:** The population examined in this study is a high risk obstetric Canadian population where *in-utero* exposure to ethanol and drugs of abuse has been suspected.
Hence, the rate of in-utero exposure to ethanol is expected to be higher than that found in the general Canadian population.

**Hypothesis 3:** Heavy ethanol use in late pregnancy among women investigated by Children’s Aid Societies will be associated with an increased exposure to other illicit drugs.

**Rationale 3:** Previous studies have shown that women who abuse ethanol abuse other drugs, such as nicotine, cocaine and opiates. However, to the best of our knowledge, no previous study has looked into the association of heavy in-utero ethanol use and that of other drugs of abuse in a high risk obstetric Canadian population where ethanol or illicit drug use was suspected in pregnancy and confirmed with laboratory testing. This information is clinically important, as neonatal drug analysis is commonly done for cocaine, amphetamine and other drugs of abuse, but rarely for ethanol, thus missing a potentially devastating form of fetal exposure.

**1.4 Review of Literature**

**1.4.1 Ethanol**

Alcohol compounds are organic molecules that contain one or more hydroxyl groups that are bound to carbon atoms. Ethyl alcohol, also known as ethanol or grain alcohol,
is a two-carbon molecule with one hydroxyl group (C₂H₅OH, Mₙ = 46 g/mol), that is naturally produced as a by product of sugar or starch fermentation by yeasts and other microorganisms (Kalant & Khanna, 2007).

Ethanol consumption is expressed in multiples of one “standard drink”, where the definition of a drink varies from country to country. In Canada and the United States, one standard drink equals 13.6 grams of absolute ethanol, which is equivalent to a 12 oz beer or a cooler (5% ethanol), a 5 oz glass of wine (12% ethanol), or a 1.5 oz spirits (40% ethanol) (Canadian Public Health Association, 2006; Kalant & Khanna, 2007).

The Centers for Disease Control define consumption of no more than two drinks per day for men and no more than one drink per day for women as moderate drinking, while heavy drinking is defined as having more than 2 drinks per day for men and more than 1 drink per day for women. A drinking pattern that increases blood ethanol concentrations to 0.08% or above, corresponding to four or more drinks per single occasion is defined as binge drinking (Centers for Disease Control & Prevention, 2008).

1.4.1.a Pharmacology of Ethanol

1.4.1.a.1. Absorption and Distribution

When ethanol is ingested orally some of it is absorbed through the stomach; however, approximately 80% of the absorption occurs in the duodenum and it is affected by the
rate of gastric emptying (Best & Laposata, 2003; Kalant & Khanna, 2007; Swift, 2003).

Ethanol has a very low lipid-water partition coefficient; is completely miscible with water, is rapidly absorbed through the mucosal surface by simple diffusion and it equilibrates quickly throughout the body with its volume of distribution equaling that of water (Lands, 1998; Swift, 2003; York & Hirsch, 1995). The concentration of ethanol in the tissue depends on the relative water-content and the rate of blood flow to the tissue (Kalant & Khanna, 2007). The total body water content depends on numerous factors, including age, gender, height and weight, where the total body water is approximately 38-46 L in men and 26-33 L in women. This difference in volume of distribution explains why women of the same age and the same weight as men will reach higher ethanol concentration after consuming equal amounts of ethanol (Chumlea et al., 1999; Chumlea et al., 2001; Swift, 2003; Watson et al., 1980).

1.4.1.a.2. Metabolism and Elimination

After absorption by the stomach and intestines, ethanol is carried to the liver via the mesenteric and portal veins (Swift, 2003). Approximately 85% ethanol is metabolized in the liver via enzymatic oxidation. The major metabolic pathway for ethanol metabolism is its conversion to acetaldehyde by alcohol dehydrogenase (ADH) followed the conversion of acetaldehyde into acetic acid (acetate) by aldehyde dehydrogenase (ALDH), where at each of the two steps a nicotinamide adenine
dinucleotide (NAD+) is reduced in the process. The produced acetic acid enters the Krebs cycle where it is rapidly converted to water and carbon dioxide (Kalant & Khanna, 2007; Swift, 2003). (Figure 1.1).

The rate of ethanol metabolism in the liver is best described by Michaelis-Menten kinetics. As the ADH enzymes in the liver get saturated at high ethanol concentrations (above 20 mg/dl), ethanol metabolism becomes zero-order, and no longer depends on ethanol concentration. At this point about 7g of ethanol is metabolized per hour. ADH enzymes are also present in human gastric mucosa, where about 0.9-1.8g ethanol gets converted per hour, a negligible contribution to first-pass metabolism (Haber, 2000; Kalant & Khanna, 2007; Levitt et al., 1997; Levitt & Levitt, 1994; Swift, 2003).

There are two other minor oxidative metabolic pathways for ethanol. Cytochrome P450 enzyme 2E1 (CYP2E1) in the liver oxidizes ethanol and is more active in chronic heavy drinkers due to induction (Ronis et al., 1993; Swift, 2003). The enzyme catalase can also oxidize ethanol to acetaldehyde and water in the presence of peroxides (Kalant & Khanna, 2007; Swift, 2003). (Figure 1.1).
FIGURE 1.1: ETHANOL METABOLISM AND ELIMINATION
(Best & Laposata, 2003; Caballeria, 2003; Kalant & Khanna, 2007; Laposata, 1998; Quertemount & Tambour, 2004; Swift, 2003).

ADH: alcohol dehydrogenase
AEAT: acyl-coenzyme A:ethanol O-acyltransferase
ALDH: aldehyde dehydrogenase
FAEE: fatty acid ethyl ester
NAD+: nicotinamide adenine dinucleotide
PLD: phospholipase D
UGT: uridine diphosphate-glucuronosyltransferase
Ethanol is also biotransformed via non-oxidative pathways where the hydroxyl group reacts with organic acids to form stable compounds. For example, ethyl ester synthase catalyzes the reaction of ethanol with fatty acids leading to the production of FAEE. FAEE formation can also be catalyzed via acyl-coenzyme A:ethanol O-acyltransferase (AEAT). Furthermore, enzymes uridine diphosphate-glucoronosyltransferase (UGT), sulfotransferase, and phospholipase D (PLD) catalyze the reaction of ethanol with glucoronic acid, sulfate and phospholipids, respectively, to form ethyl glucoronide (EtG), ethyl sulfate and phosphotidylethanol, respectively (Diczfalusy, et al., 2001; Swift, 2003). (Figure 1.1).

Lastly, between 2-10% of ethanol is eliminated unchanged via urine, breath and sweat (Best & Laposata, 2003; Kalant & Khanna, 2007).

1.4.1.b Ethanol Consumption

Ethanol is legal and widely available. According to a national Canadian survey conducted between December 2003 and April 2004, almost 80% of Canadians over the age of 15 consumed ethanol in the 12 months prior to the survey, where ethanol was consumed by a greater proportion of males than females, 80.2% vs. 76.8% respectively (Adlaf et al., 2005). Most Canadians consume ethanol in moderation, where 74.2% of men and 53.4% of women reported consuming one or two drinks per day. Of the women who reported ethanol consumption within the past year, 32.8% reported drinking at least once per week, 8.8% drank five or more drinks per sitting,
where 3.3% drank five or more drinks per sitting at least once a week (Adlaf et al., 2005).

Many women reduce or stop their ethanol consumption once pregnancy is detected (Chang, 2001; Fried et al., 1980; Fried et al., 1984); however, many pregnant women do not find out about their pregnancies until later in the first trimester and hence expose the fetus to ethanol. Canadian data from the 2000-2001 Canadian Community Health Survey estimates that 13.6% of pregnant women consume ethanol at some point in pregnancy, while 4.9% of pregnant women drink throughout the entire pregnancy (Dell & Roberts, 2006). Where 75.4% of women who reported drinking during their pregnancy drank less than once per month, 9.7% once per months, 6.5% two or three times per months, 5.3% once per week and 1.3% drank daily (Dell & Roberts, 2006). These estimates are similar to those found in the United States for 2006 and 2007, where 11.6% of pregnant women ages 15 to 44 report current ethanol consumption, with 3.7% reporting binge drinking (6.6% reported binge drinking in the first trimester) (Substance Abuse & Mental Health Services Administraion, 2008).

Due to the fact that ethanol is a very small water-soluble molecule, it readily crosses the placenta, leading to in-utero ethanol exposure. In-utero ethanol exposure could have devastating results on the fetus and may result in FASD (Abel, 1995; Hopkins et al., 2008; Lupton et al., 2004; Randall, 1987; Sampson et al., 1997; Streissguth et al., 2004).
1.4.1.c FASD

FASD is a term describing a range of physical, behavioral, and cognitive disabilities that result from in-utero ethanol exposure. In North America FASD affects at least 0.91% of the pediatric population (Sampson et al., 1997). For the province of Ontario it has been estimated that the prevalence of FASD is 116,480 cases, with 492 new cases arising in 2007 alone (Hopkins et al., 2008).

The primary characteristics of FASD may include facial dysmorphology (such as short palpebral fissure, smooth philtrum, thin vermilion and flattened medial midface), microcephaly, growth retardation, cardiac or skeletal malformations, neurodevelopmental and cognitive abnormalities. Secondary disabilities of FASD may include behavioral problems, mental health problems, substance addiction, disrupted school experience (including suspension, expulsion and drop out), incarceration, dependent living, unemployment, homelessness, inappropriate sexual behavior and premature death (Burd et al., 2007; Burd et al., 2008; Koren & Nulman, 2002; Mattson & Riley, 1998; Sokol et al., 2003; Streissguth et al., 1990; Streissguth et al., 2004). (Table 1.1).

In its most severe form FASD presents as fetal alcohol syndrome (FAS), a pattern of anomalies consisting of growth retardation, characteristic pattern of facial anomalies, and central nervous system abnormalities (Burd et al., 2007; Koren & Nulman, 2002; Mattson & Riley, 1998; Sokol et al., 2003; Streissguth et al., 2004).
<table>
<thead>
<tr>
<th>PRIMARY CHARACTERISTICS OF FASD</th>
<th>SECONDARY DISABILITIES OF FASD</th>
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<tbody>
<tr>
<td>Facial dysmorphology</td>
<td>Behavioral problems</td>
</tr>
<tr>
<td>o short palpebral fissure</td>
<td>Mental health problems</td>
</tr>
<tr>
<td>o smooth philtrum</td>
<td>Learning problems</td>
</tr>
<tr>
<td>o thin vermilion</td>
<td>Disrupted school experience</td>
</tr>
<tr>
<td>o flattened medial midface</td>
<td>Substance addiction</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>Inappropriate sexual behavior</td>
</tr>
<tr>
<td>Growth retardation</td>
<td>Trouble with the law</td>
</tr>
<tr>
<td>Cardiac/skeletal malformations</td>
<td>(including incarceration)</td>
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<td>Neurodevelopmental and cognitive</td>
<td>Dependent living</td>
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<td>abnormalities</td>
<td>Unemployment</td>
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<td>Homelessness</td>
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<td>Unemployment</td>
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<td>Premature death</td>
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</table>

It has been estimated that the lifetime cost to society of each FAS case is over $2 million U.S. (Lupton et al., 2004). In Canada, the annual cost per FASD-affected individual has been estimated at $14,342 CAN, resulting in a total annual cost of $344,208,000 for all FASD-affected individuals between 1 and 21 years of age, where a large proportion of the cost results from secondary disabilities associated with FASD (Stade et al., 2006).

1.4.1.d Variability in the Effects of Ethanol

Ethanol does not appear to have an equal effect on all the exposed fetuses, as in-utero exposure to ethanol affects approximately 40% neonates, where 4% exhibit full FAS features (Abel, 1995). Animal models have suggested that this variability might be attributed to the amounts, patterns and timing of ethanol consumption (Becker, 1994; Bonthius et al., 1988, Bonthius & West, 1988; Coles, 1994; Randall, 1987; West et al., 1990). For example, peak blood ethanol levels, and not necessarily the total
amount of ethanol consumed correlates better with adverse outcomes (Bonthius et al., 1988; Bonthius & West, 1990; Pierce & West, 1986). A study by Bailey and colleagues (2004) showed similar results when testing 7 year old children who were exposed to ethanol in-utero. They have concluded that binge drinking and not the amount of overall exposure in pregnancy had significant negative association with verbal IQ scores and a positive association with teacher-reported delinquent behavior, even after controlling for other prenatal exposures and postnatal environmental factors such as maternal IQ and socioeconomic status (Bailey et al., 2004).

Animal studies also showed that the type of birth defect depends on the fetal systems that are undergoing development at the time of exposure and even a single exposure can be sufficient to produce birth defects and decrease fetal weight in mice (Randall, 1987).

Considering that the brain is one of the first organ systems that begin to develop and the last one to complete, it is no surprise that the central nervous system is sensitive to the effect of ethanol at any point in pregnancy and can form unique neurodevelopmental abnormalities depending on the time of exposure (Becker et al., 1994).

1.4.1.e Importance of Early Detection of In-Utero Ethanol Exposure
It has been estimated that 85% of substance addicted women will have multiple pregnancies, with an average of four pregnancies each, and biological mothers of children affected by FAS have a 47-77% chance of having their next child born with FAS if ethanol use is continued by her (May, 1995; Pepler et al., 2002; Riley & McGee, 2005). Interventions for the mothers after the first pregnancy could potentially prevent three future pregnancies with in-utero ethanol exposure (May, 1995; Pepler et al., 2002; Riley & McGee, 2005). Astley et al. (2000) have estimated that the cost to society associated with prevention of subsequent children affected by FAS is thirty times lower than that associated with raising a child affected by FAS, in turn, benefiting the mothers, their future children and society.

Furthermore, studies on ethanol-exposed animals have shown that enriching the motor and learning environments can lead to improved neurodevelopment (Klintsova et al., 1998; Klintsova et al., 2002). These animal studies, along with some human data, indicate that early diagnosis and intervention for children exposed to ethanol in-utero can lead to better developmental outcomes due to brain plasticity (Burd, 2006; Olson et al., 2007; Streissguth et al., 2004; Sussman & Koren, 2006).

To decrease the risk of secondary characteristics associated with FASD requires early intervention and hence early diagnosis of the affected children (Streissguth et al., 1991; Streissguth et al., 1996; Streissguth et al., 2004). For example, being diagnosed at an earlier age and raised in a good stable environment will increase the odds of escaping secondary disabilities by 2- to 4-fold (Streissguth et al., 2004). Preliminary results from a randomized clinical trial in South Africa also indicate that early
intervention in the form of cognitive control therapy and language and literacy training benefits FASD-affected children (Adnams et al., 2006). O’Connor and colleagues (2006) have also shown that early intervention for FASD-affected children by the use of a Child Friendship Training improves children’s knowledge of appropriate social behavior and leading to improved social skills and fewer behavior problems (O’Connor et al., 2006). However, only 11% of these children are diagnosed before the age of 6, when therapy is most effective at decreasing secondary disabilities that are associated with FASD (Streissguth et al., 1991; Streissguth et al., 1996).

The low rate of FASD-diagnosis can be attributed to the need for a confirmed maternal drinking history during pregnancy or the presence of characteristic facial features unique to FAS (Chudley et al., 2005). These features are present in only 10% of ethanol-affected individuals, while non-anonymous self-reporting is subjective and often unreliable and maternal blood and urine markers could be ineffective due to the lack of sensitivity and specificity (Bearer, 2001; Gareri et al., 2008; Russell et al., 1996; Sampson et al., 1997). Therefore, a reliable biomarker is needed to identify prenatal ethanol exposure.

1.4.1. f Biomarkers of Chronic Ethanol Consumption

There are many biomarkers that have been used for detection of chronic excessive ethanol consumption via testing of urine, blood and breath. These biomarkers include
liver enzymes such as gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Bearer, 2001; Conigrave et al., 2003; Neumann & Spies, 2003; Sharpe, 2001; Swift, 2003). Carbohydrate deficient transferin (CDT), mean corpuscular volume (MCV), acetaldehyde adducts that are produced when acetaldehyde binds tissue proteins and acetic acid are also used (Bearer, 2001; Conigrave et al., 2003; Neumann & Spies, 2003; Sharpe, 2001; Swift, 2003). However, these markers were developed and validated in males and non-pregnant females and are not specific or sensitive enough to be used in obstetric populations (Bearer, 2001; Neumann & Spies, 2003). (Table 1.2).

<table>
<thead>
<tr>
<th>BIOMARKER</th>
<th>TEST</th>
<th>DETECTION OF CHRONIC ETHANOL CONSUMPTION</th>
</tr>
</thead>
</table>
| Acetaldehyde Adducts       | Acetaldehyde adducts are formed when the highly reactive acetaldehyde binds tissue proteins, for example, binding to red blood cell hemoglobin | v Sensitivity 67%, specificity 77% (when consuming >6 drinks per day)  
   v Individual variability in acetaldehyde formation and/or accumulation results in variability formation of acetaldehyde adducts |
| Acetic Acid                | Acetic acid is the major final product of ethanol metabolism.         | v Short half-life and low correlation to ethanol concentration                                                                                               |
| Alanine Aminotransferase   | Serum transaminases are liver enzymes which can be detected in blood and are often raised in alcoholics | v 39% sensitivity and 90% specificity in women                                                                                          |
| Aspartate Aminotransferase |                                                                      | v 54% sensitivity and 96% specificity in women                                                                                                                                                                                      |
| Carbohydrate Deficient     | CDT is a component of serum transferring that has a very high isoelectric point | v Approved by the FDA as a clinical diagnostic test  
   v 49% sensitivity and 90% specificity in women                                                                                          |
| Transferin                 |                                                                      |                                                                                                                                                                                                                                         |
| Fatty Acid Ethyl Esters    | Formed when ethanol reacts with free fatty acids in the blood and tissue | v FAEE are relatively stable and persists in blood and tissue  
   v FAEE are detected in meconium and hair  
   v FAEE cannot be used to determine amount of ethanol consumed, only whether heavy ethanol consumption took place  
   v 100% sensitivity and 98.4% specificity in meconium testing                                                                 |

Table 1.2: Biomarkers of Chronic Ethanol Consumption
(Bearer, 2001; Conigrave et al., 2003; Neumann & Spies, 2003; Sharpe, 2001; Swift, 2003).
FAEE in neonatal meconium, on the other hand, are sensitive and specific and provide a wide window of detection for the identification of heavy in-utero ethanol exposure in late pregnancy, while collection of meconium is easy and non-invasive (Ostrea et al., 2006; Ostrea et al., 1993; Ostrea et al., 2001).

1.4.1.g Detection of In-Utero Ethanol Exposure via Meconium FAEE

Meconium is comprised of water, mucus, bile, enzymes, sugars, lipids, epithelial cells and amniotic fluid. It is free of bacteria or their breakdown products and begins to form in the second trimester with the emergence of fetal swallowing (Kwong & Ryan, 1997; Ostrea et al., 1994b; Rapoport & Buchanan, 1950). Meconium also contains metabolites of ethanol known as FAEE. As discussed earlier, FAEE are formed via non-oxidative pathway of either enzyme-mediated esterification of ethanol and free fatty acids catalyzed by FAEE-synthase or via enzyme-mediated esterification of ethanol and fatty acyl-CoA catalyzed by AEAT (Best & Laposata, 2003; Diczfalusy,
et al., 2001). The FAEE produced depends on the length of the carbon chain and the placement of the double-bond in the fatty acid that is esterified with ethanol. (Figure 1.2).

**Figure 1.2: FAEE formation**

(Best & Laposata, 2003; Diczfalusy et al., 2001).

Perfusion studies have shown that FAEE do not cross the human placenta and FAEE accumulation in meconium is a direct measure of fetal ethanol exposure (Chan et al., 2004b). Various methods were developed in order to determine if the accumulation of single FAEE or the sum of specific FAEE in meconium correlate with maternal ethanol use (Bearer et al., 1999; Bearer et al., 2003; Bearer et al., 2005; Chan et al., 2003; Moore et al., 2003; Ostrea et al., 2006).
The Motherisk Program at The Hospital for Sick Children, Toronto, Ontario, Canada, has developed a method for the use of FAEE as a biomarker for detection of \textit{in-utero} ethanol exposure. This method established a cut-off value of 2 nmol/g total ethyl palmitate (E16:0), ethyl linolate (E18:2), ethyl oleate (E18.1) and ethyl stearate (E18:0) and detects second and third trimesters \textit{in-utero} ethanol exposure with 100% sensitivity and 98.4% specificity and positive and negative predictive values of 62.5% and 100%, respectively (Chan \textit{et al.}, 2003; Hutson \textit{et al.}, 2009). This cut-off value does not distinguish between non-drinkers and social drinkers; a positive test result is obtained in heavy or binge-drinking individuals.

It is also important to note that the correlation of ethanol consumption with FAEE levels has not been established and hence meconium FAEE testing can only determine whether heavy or binge-drinking occurred in late pregnancy (occasional or moderate drinkers will not obtain a positive test result). This can partially be attributed to the fact that FAEE are naturally found in the human body when they arise from food or endogenous ethanol (EE) production (Best \\& Laposata, 2003).

The FAEE detected in the above mentioned studies are listed in Table 1.3 and the studies are summarized in Table 1.4.
### Table 1.3: FAEE Used in Detection of In-utero Ethanol Exposure

(Bearer et al., 1999; Bearer et al., 2003; Bearer et al., 2005; Chan et al., 2003; Moore et al., 2003; Ostrea et al., 2006).

<table>
<thead>
<tr>
<th>FAEE</th>
<th>CONFIGURATION</th>
<th>MOLECULAR FORMULA</th>
<th>MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Palmitate</td>
<td>E16:0</td>
<td>C₁₈H₃₆O₂</td>
<td>284.5</td>
</tr>
<tr>
<td>Ethyl Palmitoleate</td>
<td>E16:1</td>
<td>C₁₈H₃₄O₂</td>
<td>282.5</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>E18:0</td>
<td>C₂₀H₄₀O₂</td>
<td>312.5</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>E18:1</td>
<td>C₂₀H₃₈O₂</td>
<td>310.5</td>
</tr>
<tr>
<td>Ethyl Linoleate</td>
<td>E18:2</td>
<td>C₂₀H₃₆O₂</td>
<td>308.5</td>
</tr>
<tr>
<td>Ethyl Arachidonate</td>
<td>E20:4</td>
<td>C₂₂H₄₆O₂</td>
<td>332.5</td>
</tr>
</tbody>
</table>

### Table 1.4: Meconium FAEE Studies for Detection of In-utero Ethanol Exposure

(Bearer et al., 1999; Bearer et al., 2003; Bearer et al., 2005; Chan et al., 2003; Moore et al., 2003; Ostrea et al., 2006).

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>FAEE USED</th>
<th>CUT-OFF</th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>ETHANOL EXPOSURE LEVEL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bearer et al., 1999,</strong> USA</td>
<td>Total (n=248)</td>
<td>E18:2</td>
<td>1 pmol/g</td>
<td>72%</td>
<td>51%</td>
</tr>
<tr>
<td></td>
<td>Exposed (n=39)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bearer et al., 2003, South Africa</strong></td>
<td>Control (n=6)</td>
<td>E18:1</td>
<td>32 ng/g</td>
<td>84.2%</td>
<td>83.3%</td>
</tr>
<tr>
<td></td>
<td>Exposed (n=21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chan et al., 2003, Toronto &amp; Jerusalem</strong></td>
<td>Control, n=184</td>
<td>Sum of E16:0, E18:0, E18:1, E18:2</td>
<td>2 nmol/g</td>
<td>100%</td>
<td>98.4%</td>
</tr>
<tr>
<td></td>
<td>Toronto (heavy drinkers, n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Moore et al., 2003, Anonymous samples</strong></td>
<td>Hawaii (n=436)</td>
<td>Sum of E16:0, E16:1, E18:0, E18:1, E18:2, E20:4</td>
<td>50 ng/g</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Utah (n=289)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bearer et al., 2005, Cleveland (various exposures, n=248)</strong></td>
<td></td>
<td>E18:2</td>
<td>761 ng/g</td>
<td>88%</td>
<td>64%</td>
</tr>
<tr>
<td></td>
<td>Jordan (control, n=30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ostrea et al., 2006, USA</strong></td>
<td>Control (n=31)</td>
<td>E18:2</td>
<td>0.25 μg/g</td>
<td>27%</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>Exposed (n=93)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sensitivity = true positives/false negatives + true positives
* Specificity = true negatives/false positives + true negatives
* N/A = Not Available
1.4.1 Meconium FAEE and Neonatal Outcomes

Various studies have investigated the ability of meconium FAEE to predict adverse neonatal outcomes, including FASD-related outcome measures such as lower birth weight, smaller head circumference, neurodevelopmental delay and fetal alcohol syndrome or partial fetal alcohol syndrome diagnosis at five years of age (Derauf et al., 2003b; Jacobson et al., 2006; Hutson et al., 2007; Noland et al., 2003; Peterson et al., 2008). These studies have concluded that FAEE are good predictors for neonatal outcomes. Their results are summarized in Table 1.5.

### Table 1.5: Meconium FAEE and Adverse Neonatal Outcomes
(Derauf et al., 2003b; Jacobson et al., 2006; Hutson et al., 2007; Noland et al., 2003; Peterson et al., 2008)

<table>
<thead>
<tr>
<th>STUDY POPULATION</th>
<th>SIGNIFICANT STUDY FINDINGS</th>
</tr>
</thead>
</table>
| Derauf et al., 2003. Honolulu, Hawaii (n=422) | - Lower 1-minute Apgar score  
- Ethyl oleate associated with lower birth weight |
| Noland et al., 2003 Children from a longitudinal neurobehavioral study of a high-risk population (n=316) | - Lower score on executive functioning tasks assessed via tapping inhibition  
- Lower birth weight, birth length, and head circumference |
| Jacobson et al., 2006 Cape Town, South Africa (n=55) | - Elevated ethyl oleate levels in infants with FAS or partial FAS diagnosis at 5 years of age |
| Hutson et al., 2007 Montevideo, Uruguay (n=194)) | - Ethyl linoleate (along with meconium cotinine, maternal body mass index and female gender) are predictors of decreased birth weight |
| Peterson et al., 2008 Cleveland, Ohio (n=190) | - Ethyl linoleate was associated with lower scores on the Mental Development Index at 6.5 months and 2 years of age  
- Ethyl myristate and ethyl oleate were associated with lower scores on the Mental Development Index at 2 years of age  
- Ethyl myristate, ethyl oleate, ethyl linoleate and ethyl archidonate were associated with lower scores on the Psychomotor Developmental Index at 2 years of age |
Hopkins and colleagues (2008) have compared the lifetime benefits of early detection and intervention for neonates with \textit{in-utero} ethanol detection and the cost of such testing via neonatal meconium. The lifetime cost of each FASD-affected individual was $1.3 million CAN, and the cost of the meconium test was $150 CAN. It was concluded that the benefit of early intervention can improve literacy, in turn improving the quality of life and increasing adult lifetime earnings by $26,400 per year. A targeted screening would improve the quality of life of the affected individuals while providing cost-savings to the society (Hopkins \textit{et al.}, 2008).

\subsection*{1.4.2 Confounding Factors in Meconium FAEE Testing}

In order to use meconium FAEE testing as a universal or a targeted screening tool for \textit{in-utero} ethanol exposure, possible confounders that could lead to false-positive test results need to be addressed.

As mentioned earlier, FAEE are naturally found in the human body and are present in non-drinking individuals, as FAEE could arise from food or EE production. Therefore meconium testing, unlike testing for other drugs of abuse, has a cut-off to separate positive and negative results (Best & Laposata, 2003; Chan \textit{et al.}, 2003).

EE is a natural intracellular metabolite that may originate from microbial fermentation of ingested carbohydrates in the gastro-intestinal tract (Blomstrand, 1971; Dahshan &
Donovan, 2001; Krebs & Perkins, 1970) or it can be produced as a final product of glycolysis where the decarboxylation of pyruvic acid produces acetaldehyde which is further reduced to ethanol (McManus et al., 1966). (Figure 1.3).

**Figure 1.3: EE Production**
(Antoshechkin, 2002; McManus et al., 1966; Ostrovsky, 1986; Zeikus, 1980).

EE is generally present in low concentrations in the blood but there is large inter-individual variation. Variations in EE exist for a variety of reasons, including the strain of bacteria and yeast present, bacterial and yeast overgrowth, intestinal motility, and medical conditions such as obesity and diabetes (Baraona et al., 1986; Cope et al., 2000; Galassetti, et al., 2005; Jansson-Nettelbladt et al., 2006; Liebich & Al-Babbili, 1975; Liebich et al., 1982; Nair et al., 2001; Spinucci et al., 2006). It is this latter condition, diabetes, which is of a concern as it may produce false positive results in the FAEE meconium test.
Individuals affected by diabetes have increased levels of EE in both serum and urine, where EE in serum of individuals with diabetes ranges from 0-159 mg/L, with a mean of 10.0 mg/L (Liebich et al., 1982), while EE in individuals not affected by diabetes varies from trace amounts to 39 mg/L with a mean of 6.6 mg/L (Jones et al., 1983, Liebich et al., 1982). Furthermore, Dashe et al. (2000) reported that the glucose concentration in the amniotic fluid of women affected by diabetes is 39±17 mg/dL, which is significantly higher than the 24±11 mg/dL level detected in the amniotic fluid of women not affected by diabetes.

Kapur examined 16 ethanol-positive urine samples that were sent from physicians’ offices for ethanol testing. Glucose was present in eight urine samples, where five of those samples contained no ethyl glucuronide (a direct biomarker for exogenous ethanol exposure). Concluding that the patients were diabetic and the glucose in their urine allowed for endogenous ethanol production (Kapur, 2007). Kapur has also analyzed meconium samples that were incubated with glucose solutions of various concentrations. Trace amounts of ethanol were detected in meconium samples that were incubated at 37°C with 2M glucose solution, while no ethanol was detected when samples were incubated at 4°C or room temperature (personal communication).

1.4.2.a Diabetes in Pregnancy
Diabetes mellitus is a disorder of carbohydrate metabolism. It is manifested by high levels of blood glucose that result from the body’s inability to produce ample amounts of insulin and/or the insulin that is produced is unable to adequately control glucose levels (Centers for Disease Control and Prevention, 2005).

Type 1 diabetes accounts for 5-10% of all diagnosed diabetic cases and it develops when the pancreatic beta cells are destroyed by the body’s immune system. Type 2 diabetes accounts for 90-95% of diabetic cases and usually begins as insulin resistance leading to an increase in insulin production by the pancreatic beta cells that result in a gradual loss in the ability to produce insulin. Gestational diabetes is any form of diabetes with onset or first recognition during pregnancy while pregestational diabetes is any form of diabetes that arises prior to pregnancy (American College of Obstetricians & Gynecologists, 2001; Centers for Disease Control & Prevention, 2005; Sheffield et al., 2002; Wen et al., 2000).

Diabetes is the most common medical complication in pregnancy, with prevalence ranging from 1 to 14%, depending on the studied population, with 2 to 5% being the most common estimated prevalence (American College of Obstetricians & Gynecologists, 2001; Sheffield et al., 2002; Wen et al., 2000). It is important to treat pregestational diabetes, if it is left untreated in the first trimester there is an increased risk of spontaneous abortions and birth defects (Centers for Disease Control & Prevention, 2005; Sheffield et al., 2002). Any form of uncontrolled diabetes in the second and third trimesters may lead to complications including preeclampsia and macrosomia, posing a risk to the mother and baby (American College of Obstetricians
& Gynecologists, 2001; Centers for Disease Control & Prevention, 2005; Kjos & Buchanan, 1999; Wen et al., 2000).

The elevated levels of EE in diabetes-affected individuals may lead to increased levels of FAEE in meconium of their neonates. This in turn may increase the risk of obtaining a false-positive meconium FAEE test result (i.e., >2 nmol/g) in the absence of EE consumption during the last six months of pregnancy.

It has been estimated that one standard alcoholic drink, containing 10 grams of ethanol, per hour raises the blood ethanol levels by approximately 10-30 mg/L, which is equal to or higher than the mean value of 10 mg/L of EE that is present in the serum of individuals affected by diabetes (Liebich et al., 1982). However, the level of EE in serum of diabetes-affected individuals can be as high as 159 mg/L (Liebich et al., 1982), and thus may produce an increase in total FAEE levels present in the meconium of neonates that were born to diabetes-affected mothers.

False-positive meconium FAEE results could have significant legal and clinical implications, as meconium analysis for prenatal ethanol exposure is frequently requested by social services. A false positive result may lead to child apprehension or mislabeling of a child as being at risk of developing fetal alcohol spectrum disorder. Hence, it is essential to rule out maternal diabetes as a potential source of false-positive meconium FAEE results.
1.4.3 Drugs of Abuse

In-utero exposure to illicit drugs is another major concern for the health of the developing fetus. Previously conducted studies have suggested that women who drink ethanol are more likely to consume other recreational and illicit drugs, such as nicotine, amphetamine, cannabis, cocaine, and opiates (Alpert et al., 1981, Chasnoff et al., 2001; Gladstone et al., 1997; Lester et al., 2001). Furthermore, data from Washington’s FAS Diagnostic and Prevention Network shows that 84.5% of children referred to the clinic since 1993 for FASD diagnosis were also exposed to cigarettes and/or illicit drugs (Olson et al., 2007).

In 2002, the rate of illicit drug-exposure in Canadian women within one month prior to taking part in a survey has been estimated at 9.4% (Tjepkema, 2004). American data from 2006 and 2007 is similar, where 9.7% of non-pregnant women and 5.2% of pregnant women between the ages of 15 and 44 used illicit drugs within one month prior to the survey (Substance Abuse & Mental Health Services Administraion, 2008).

Many women who use illicit drugs change their habits during pregnancy, as twenty eight percent of women abstain from illicit drug use after detection of pregnancy, with the abstinence rate increasing to 93% in the third trimester between 1996 and 1998 (Ebrahim & Gfroere, 2003).
1.4.3.a Cannabinoids

Cannabinoids are the most commonly used illicit drugs, as 39.2% of females have reported using it at least once in their lifetimes, and about 10% of women used it in the 12 months prior to taking part in the Canadian Addiction Survey conducted in 2003-4 (Tjepkema, 2004; Adlaf et al., 2005). According to American statistics in 1996-1998 cannabinoids were used by 4.9% of non-pregnant women and by 1.8% of pregnant women (Ebrahim & Gfroere, 2003).

Cannabinoids are derived from the plant *Cannabis sativa*, where the major psychoactive ingredient is delta-9-tetrahydrocannabinol (THC). Various forms of cannabinoids are produced, depending on the component of the plant that was used. These forms vary in potency and onset of the psychoactive effect and include marijuana, hashish and hash oil (Welch & Martin, 2003).

THC is lipid-soluble and is metabolized in the liver into an active metabolite 11-hydroxy-THC (11-OH-THC) that is further oxidized to 11-nor-9-carboxy-THC (THC-COOH), and is the primary urinary metabolite of THC (mainly excreted in the urine in the from of a glucoronic acid conjugate) (Musshoff & Madea, 2006; Welch & Martin, 2003).

The most common route of administration is inhalation (i.e. smoking), where the absorption of THC into the blood stream occurs in the lungs and produces the most rapid and intense effect. Marijuana and hashish can also be ingested, where the onset
of the psychoactive effect with oral administration is slower but lasts for a longer
duration (Hollister, 1986; Welch & Martin, 2003).

In low doses THC’s psychoactive effects include a mixture of depression and
stimulation, while at higher doses effects mainly include central nervous system
depression (Dewey, 1986). Decrease in response rates and locomotor activity is
observed, along with reported relaxation, euphoria and hunger, however paranoia has
been documented in some rare cases (Naditch, 1974; Welch & Martin, 2003).

Various adverse health effects associated with marijuana smoking has been reported,
including increased airway resistance and decreased pulmonary function (Tashkin et
al., 1976; Henderson et al., 1972). Furthermore, some studies immunologic effects of
THC, including decreased production of interleukin 1, T-lymphocytes, macrophage
and natural killer cell activity (Condie et al., 1996; Hollister, 1986; McCoy et al.,
1999).

1.4.3.b Stimulants - Cocaine and Amphetamines

Stimulants, including caffeine, cocaine and amphetamines, are drugs that excite the
central and peripheral sympathetic nervous systems, mainly by enhancing
neurotransmitter activity at catecholaminergic synapses (Gorelick & Cornish, 2003;
Kalant, 2007b; Masand & Tesar, 1996; Plessinger, 1998).
Cocaine can be extracted from either *Erythroxylon coca* or *Erythroxylon novogranatense* plants which vary in their cocaine amounts (Gorelick & Cornish, 2003). Cocaine is the second most widely used illegal drug in the United States, where in 2000 the National Household Survey on Drug Abuse estimated that 11.2% of individuals 12 years old and older have used cocaine at some point in their lifetime and 1.5% had used cocaine within one year prior to the study. The data on cocaine use in the Canadian population is similar, where in 2003-2004 the Canadian Addiction Survey reported 1.9% of the population using cocaine 12 months prior to the study (Adlaf et al., 2005).

Methamphetamine and its metabolite amphetamine are synthetic stimulants. It has been estimated that 5.2% of pregnant women in the United States used amphetamines, and 4.4% of the Canadian student population in 2003 used methamphetamines (Arria et al., 2006; Centre for Addiction and Mental Health, 2006).

In the medical field, amphetamines have been used for attention deficit/hyperactivity disorders (ADHD), narcolepsy, appetite suppression in exogenous obesity, certain cases of depression, decongestion and bronchodilation (Bray, 2001; Gorelick & Cornish, 2003; Greenhill et al., 1999; Masand & Tesar, 1996; Nishino & Mignot, 1999), while cocaine is used clinically only as a local or topical anesthetic, primarily for eye, ear, nose and throat procedures (Gorelick & Cornish, 2003). Stimulant use in therapeutic doses in appropriately diagnosed patients is efficient and safe and does not lead to stimulant abuse (Greenhill et al., 1999; Masand & Tesar, 1996).
However, stimulants have been used in nonmedical settings, leading to abuse and dependence. Initial effects of stimulant use include increased energy and alertness, and decreased appetite and fatigue, and thus, oral stimulants have been used to suppress sleep and as performance-enhancing drugs in endurance sports (Gorelick & Cornish, 2003). With an increase in stimulant use one may develop anxiety, irritability, panic attacks, sleep disturbances, paranoia, impaired judgment, delusions, hallucinations and weight loss (Hando et al., 1997; Williamson et al., 1997; Satel et al., 1991).

Furthermore, cocaine and amphetamine abuse is associated with cognitive impairment (including visuo-motor performance, attention and verbal memory) (Rogers & Robbins, 2001). Cocaine use has also been associated with cerebral vasoconstriction, stroke and cardiac arrhythmias (Boghdadi & Henning, 1997).

1.4.3. c Opiates

Opiates are derived from *Papaver somniferum* plant, and inhibit the electrical activity of the neurons. Opiates are different from opioids, as opioids are substances that are not derived from the *Papaver somniferum* plant, and hence have a different chemical structure, yet they have the same pharmacological properties. As a whole group, opiates and opioids are known as opioid analgesics. In this paper, as in many others, the term opioids will be used to refer to all opiates and opioids as a group.
All opioids, including codeine, morphine, methadone and diacetylmorphine (heroin), produce analgesic effects (Kalant, 2007). Codeine and morphine are natural opioids. Codeine is isolated from opium and is the starting material for opioids such as hydrocodone, and dihydrocodeine. In the liver, codeine is mainly metabolized by CYP2D6 into morphine and codeine-6-glucuronide (Borg & Kreek, 2003; Vree et al., 2000; Srinivasan et al., 1997). Heroin, unlike codeine and morphine, is a synthetic opioid, derived from morphine (Wright, 1941). Heroin is rapidly converted to 6-monoacetylmorphine and then metabolized mostly to morphine. (Figure 1.4). It is a popular drug of abuse due to its rapid onset of action and relatively short half-life (Borg & Kreek, 2003). Methadone is a synthetic opioid agonist that is used primarily in treating heroin addiction (Borg & Kreek, 2003).

**Figure 1.4: The Relationship between Various Opiates**
(Vree et al., 2000; Wright, 1941)
In the medical field, opioids have been used for pain relief, preoperative sedation, cough suppression, treatment of diarrhea symptoms and detoxification and maintenance opioid addiction therapy (Kalant, 2007a).

However, the relaxed and dreamy state produced with the use of some opioids, including heroin, leads to its abuse, tolerance and physical dependence. Opiate use is relatively low in the general Canadian population, where its use has been estimated at 0.3% (Popova et al., 2006).

1.4.3.d Adverse Effects of Illicit Drugs in Pregnancy

Illicit drug use in pregnancy has been associated with maternal, fetal and neonatal complications, including increased infections for the mother and prematurity, intrauterine growth retardation, low birth weight, fetal distress, and developmental delays for the neonate (Bauer et al., 2002; Chouteau et al., 1988; D’Apolito, 1998; Helmbrecht & Thiagarajah, 2008; Schneider & Chasnoff, 1992; Shankaran et al., 2004; Singer et al., 2002b; Smith et al., 2008). (Table 1.6).

In-utero cannabis exposure has been associated with low birth weight, small head circumference and decreased gestational length in some studies (Day et al., 1991; Hatch & Bracken, 1986; Hurd et al., 2005; Fried et al., 1999), while other studies found no such associations (Fried, 1980; Linn et al., 1983). Similar inconsistencies are found in executive functions (i.e. cognition) of neonates with in-utero cannabis
exposure (Fried & Smith, 2001). Some studies indicate that these children perform poorly on various IQ tests (e.g. performance in short-term memory and verbal reasoning subscales) around three and six years of age (Day et al., 1994; Fried & Watkinson, 1990; Goldschmidt et al., 2007). However, others have not detected such associations in children who reach school age (Fried et al., 1992) but have reported behavioral problems around that age, including increased hyperactivity, impulsivity and inattention (Goldschmidt et al., 2000). The variation in results might be attributed to several factors: (1) the definition of cannabis use varied, (2) women were interviewed at different points in pregnancy, and (3) some studies looked at the frequency of use and not the quantity (Day et al., 1991).

The effects of in-utero exposure to stimulants, such as cocaine, increase the risk of placental abruption and premature rupture of membranes that can result in maternal and/or fetal death (Addis et al., 2001; Handler et al., 1991; Boghdadi & Henning, 1997). Furthermore, in-utero cocaine exposure was associated with behavioral problems and various developmental delays, including language and cognitive deficits (Bada et al., 2007; Bandstra et al., 2002; Johnson et al., 1997; Nulman et al., 1994; Singer et al., 2002a; Koren et al., 1998). However, other studies, including a systematic review, could not detect long-term developmental damage due to in-utero cocaine exposure (Frank et al., 2001; Messinger et al., 2004). The conclusion reached by the systematic review is that there is no long-term developmental damage associated with in-utero exposure to cocaine, as many of the effects are correlated to
other factors, including tobacco, cannabis, ethanol and the child’s environment (Frank et al., 2001).

*In-utero* amphetamine and opiate exposure also poses numerous concerns. Amphetamine use in pregnancy may lead to fetal growth restrictions and it may increase the rate of premature delivery and placental abruption (Smith et al., 2003). Furthermore, possible correlation between *in-utero* amphetamine exposure and aggressive and hyperactive behavior has been reported (Billing et al., 1994). Chang et al. (2004) reported lower scores on measures of attention, verbal memory, long term spatial memory and visual motor integration in children with *in-utero* exposure to methamphetamine. Smith et al., (2008) reported that *in-utero* methamphetamine exposure was associated with decreased arousal, increased lethargy and physiological stress, as well as a negative association was detected between frequency of methamphetamine us in third trimester with the quality of movement. This study used meconium testing and self reports in order to determine drug exposures and the effects of methamphetamine use were adjusted for covariates such as exposure to other drugs, socioeconomic status and birth weight (Smith et al., 2008).

It is important to note that medicinal use of amphetamines does not increase the risk of congenital malformations nor are there long-term consequences for the child (Briggs et al., 2008, p.91).

Opiate use in pregnancy has also been linked to intrauterine growth retardation and pre-term deliveries, although methadone seems to be less harmful (Bada et al., 2002;
Chiriboga, 2003). Neonatal abstinence syndrome (NAS) is another concern for the neonate, where symptoms include hyperirritability, tremors, crying, and diarrhea (Chiriboga, 2003; Helmbrecht & Thiagarajah, 2008; Jones et al., 2005). Early studies also raised a concern regarding neurodevelopmental outcomes, including short attention span, hyperactivity, sleep disturbances, perception and memory difficulties (Rosen & Johnson, 1985; Lifschitz & Wilson, 1991).

**Table 1.6: In-Utero Exposure to Drugs of Abuse and Adverse Neonatal Outcomes**

<table>
<thead>
<tr>
<th>DRUG OF ABUSE</th>
<th>STUDIES</th>
<th>SIGNIFICANT STUDY FINDINGS</th>
</tr>
</thead>
</table>
| Cannabinoids  | Day et al., 1991; Hurd et al., 2005; Fried et al., 1999; Day et al., 1994; Fried & Watkinson, 1990; Goldschmidt et al., 2000 | - low birth weight, small head circumference and decreased gestational length  
- poor performance on various IQ tests (e.g. performance in short-term memory and verbal reasoning subscales) around three years of age  
- behavioral problems |
| Cocaine       | Addis, et al., 2001; Bandstra et al., 2002; Handler et al., 1991; Johnson et al., 1997; Koren et al., 1998; Nulman et al., 1994; Shankaran et al., 2004; Singer et al., 2002a; Singer et al., 2002b | - low birth weight, length and head circumference  
- premature rupture of membrane  
- placental abruption that can result in maternal and/or fetal death  
- language deficits (including total, expressive and receptive language deficits) |
| Amphetamine   | Smith et al., 2003; Billing et al., 1994; Chang et al., 2004; Smith et al., 2008 | - fetal growth restrictions  
- increase the rate of premature delivery and placental abruption  
- aggressive and hyperactive behavior  
- lower scores on measures of attention, verbal memory, long term spatial memory and visual motor integration was found  
- decreased arousal, increased lethargy and physiological stress  
- negative association between frequency of thirst trimester exposure and quality of neonatal movement |
| Opioids       | Chiriboga, 2003; Helmbrecht & Thiagarajah, 2008; Jones et al., 2005; Rosen & Johnson, 1985; Lifschitz & Wilson, 1991; | - intratuterine growth retardation and pre-term deliveries  
- neonatal abstinence syndrome  
- short attention span, hyperactivity, sleep disturbances, perception and memory difficulties |
1.4.3. e Importance of Detecting In-Utero Drug Exposure

Most women decrease drug use when pregnancy is detected and continuation of drug use in late pregnancy, long after the mother knew she was pregnant, is indicative of an addiction (Fried et al., 1980; Fried et al., 1984; Koren et al., 2002). Research has shown that maternal addiction is a major challenge for the safety and health of the offspring (Homish et al., 2004; Kaltenbach & Finnegan, 1998; Kerwin, 2005; Regan et al., 1987; Jaudes et al., 1995, Widom & Hiller-Strumhofel, 2001).

Knowledge of illicit drug exposure in-utero, just like in-utero ethanol exposure, allows one to direct needed prevention methods and appropriate management and intervention for the mother and child. However, women may not admit to drug use due to fear of legal and societal consequences, while maternal blood and urine markers may not be sensitive enough to detect in-utero drug exposure due to short half lives of many drugs (Bandstra et al., 2002; Chang, 2001; Lester et al., 2001; Markovic et al., 2000; Nair et al., 1994; Ostrea et al., 1992; Ostrea et al., 1999; Ostrea et al., 2001). Therefore, a reliable biomarker is needed to identify chronic in-utero drug exposure in the neonate. Neonatal hair and meconium can be used as matrices for identifying such exposure in late pregnancy.

1.4.3. f Detection of In-Utero Drug Exposure via Meconium & Hair

Meconium, as mentioned earlier, is comprised of mucus, bile, epithelial cells, and amniotic fluid and begins to form in the second trimester with the emergence of fetal
swallowing (Kwong & Ryan, 1997; Ostrea et al., 1994b). Methods for detection of in-utero drug exposures in the second and third trimesters, including ethanol, have been extensively validated (Bearer et al., 1999; Bearer et al., 2003; Chan et al., 2003; Gareri et al., 2006; Lozano, et al., 2007; Moore et al., 1998; Ostrea, 1999; Ostrea et al., 1994a; Ostrea et al., 1998; Ostrea et al., 2001; Picchini et al., 2003; Pichini et al., 2004; Romero et al., 1993).

Neonatal hair is another matrix that has also been used for the determination of in-utero drug exposure. Systemically circulating drugs and their metabolites are incorporated into the hair during growth, and since neonatal hair growth begins in the last three to four months of pregnancy, it can provide information of exposure in the last trimester (Kintz et al., 2006; Kwong & Ryan, 1997; Lewis et al., 1995; Mieczkowski, 1992; Musshoff & Madea, 2006; Ostrea, 1999; Vinner et al., 2003; Kintz & Mangin, 1993). Neonatal hair testing has been extensively validated and is routinely used (Kintz & Mangin, 1993; Vinner et al., 2003) and just like the meconium tests for in-utero drug exposure, hair testing cannot provide quantitative information on the amount of drug used, it can only be used to determine if a certain drug exposure occurred.

Meconium has been shown to be more sensitive than hair for the detection of certain drug exposures, including cocaine, benzoylecgonine, and cannabis, but this could be partially explained by the fact that meconium begins formation earlier in pregnancy and thus can capture a greater window of possible exposure (Bar-Oz et al., 2003; Ostrea, 1999; Ostrea et al., 2001; Wingert et al., 1994). However, meconium can be
collected only for the first few days after delivery, while hair can be collected up to three months postnatally (Bar-Oz et al., 2003). Another benefit to using meconium is the fact that not all babies are born with hair and some parents may not agree to have their child's hair cut; however, meconium is a discarded material that is collected from the diaper and is passed by all babies, where 94% of healthy newborns pass meconium within 24 hours after birth (Sherry & Kramer, 1955).
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

Ethyl palmitate, ethyl linolate, ethyl oleate, ethyl stearate, palmitic acid, linoleic acid, oleic acid, stearic acid and anhydrous ethanol-d$_6$ (purity $\geq$99%) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Hexane (purity $\geq$99%) was obtained from Caledon Laboratories Ltd., Georgetown, Ontario, Canada. Thionyl chloride, potassium phosphate monobasic ($\text{KH}_2\text{PO}_4$) and disodium hydrogen phosphate dihydrous ($\text{Na}_2\text{HPO}_4*2\text{H}_2\text{O}$) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). NaOH 1M was obtained from Fisher Scientific (Kanata, Ontario, Canada).

Helium and compressed air were obtained from Praxair Canada Inc., (Brampton, Ontario, Canada).

2.1.2 Laboratory Equipment
Supelco® 18mm 36 shore A screw caps with PTFE/Silicone Septa, Supelco® 12x32mm 2mL amber screwtop gas chromatography vials, Thermogreen® 11.5mm injector port septa, glass solid phase microextraction (SPME) vial (10ml, 22.5*46mm) and SPME fiber (65μm polydimethylsiloxane-divinylbenzene) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Teflon/silicone GC vial septa and 50μL glass inserts with plastic bottom spring were obtained from Chromatographic Specialties Inc. (Brockville, Ontario, Canada).

2.2 Methods

2.2.1 Laboratory Analysis of Meconium FAEE

2.2.1.a Preparation of FAEE Stock Solution

Solutions of palmitic, linoleic, oleic and stearic acids were prepared by dissolving the acids in hexane to a concentration of 1 mg/ml. After which the acids were combined to make a stock solution of 1 μg/ml and a stock solution of 50 ng/ml and stored in amber GC vials at -20°C (Hutson et al., 2009).

2.2.1.b Preparation of d5-Ethyl Esters Stock Solution

Deuterated standards of ethyl palmitate, ethyl linolate, ethyl oleate and ethyl stearate were prepared according to the method developed by Pragst et al. (2001). Briefly,
50μl of D₆-ethanol was added to 10mg of free acid that was then cooled on dry ice. Following the cooling, 10μl of thionyl chloride was added and the mixtures were capped and heated for two hours at 40°C. Samples were dried under nitrogen at room temperature.

The remaining traces of reagents were removed by the addition of 1ml of hexane followed by evaporation under nitrogen stream at room temperature. This step was repeated in triplicate.

The remaining residues were weighed and dissolved in hexane to obtain concentrations of 2 mg/ml, and their purities were tested using gas chromatograph-mass spectrometer (GC-MS).

The above deuterated standards were then used to make a stock solution of 1μg/ml of the deuterated fatty acids that were used as the internal standard throughout all the experiments. The ratio of non-deuterated acids to deuterated acids was 1:1 at a concentration of 1ng/μl. This ratio was confirmed by GC-MS analysis.

Furthermore, standard curves were prepared for external (no meconium) and internal (with meconium) samples. Concentrations were 2, 5, 10, 20, 50 and 100 ng/ml. The intraday and interday coefficients of variability were also tested by comparing the peak area ratios of each FAEE to its deuterated standards at 50 and 100 ng/ml. The intraday coefficient of variability was determined by comparing five samples of the same concentration that were run on the same day and the interday coefficient of
variability was determined by comparing five concentrations that were run on three different days.

2.2.1.c Meconium Sample Preparation for FAEE Testing

Samples were prepared according to a method developed by Hutson and colleagues (2009). Briefly, 25μl of deuterated internal standard solution was added to a glass culture tube and the hexane was evaporated with a nitrogen stream. Following evaporation 50mg of meconium was added along with 750μl of 0.1M phosphate buffer (pH 7.6). After vortexing, the mixture was transferred to a headspace SPME vial via glass pipette. The glass culture tubes were then rinsed with 250μl of phosphate buffer and this mixture was also transferred to the same SPME vial using the same glass pipette. SPME vials were then capped with steel screw caps containing PTFE/Silicone septa and inserted into the vial rack of the GC-MS autosampler.

Moreover, a standard curve was prepared for calibration of samples, using 25μl of deuterated internal standard solution and various concentrations of fatty acids stock solution, including 2, 5, 10, 20, 50 and 100 ng/ml. Negative and positive control samples were also run in the same batch. Samples were analyzed by GC-MS.

2.2.1.d Gas Chromatography-Mass Spectrometry Analysis

Meconium FAEE samples were analyzed using a gas chromatograph with a mass selective detector GC/MS-QP2010 that was coupled to an AOC-500 autosampler, both
from Shimadzu (Columbia, MD, USA). A 65μM polydimethylsiloxane-divinylbenzene fiber (Supelco, Bellefonte, PA, USA) and FactorFour Capillary Column (30m x 0.25mm x 0.25 μm) (Varian Inc., Palo Alto, CA, USA) were used for the separation and detection of FAEE.

The autosampler was programmed to process all the experimental steps in sample analysis, including heating, agitation, adsorption and desorption of the sample. In brief, splitless injection mode was used with helium at 1.0ml/min as the carrier gas. The injection temperature was 260°C, and the transfer line was 310°C. The oven temperature was increased from 70°C to 300°C, at a rate of 20 °C. The final temperature was held for two minutes.

The peaks of mass to charge ratio (m/z) for ethyl palmitate, ethyl linoleate, ethyl oleate, and ethyl stearate are 284, 308, 310 and 312, respectively, while for the equivalent deuterated ethyl esters the m/z were 289, 313, 315 and 317, respectively. Mass spectrometer scanned from m/z 80 to 350 on a 0.5s cycle, with the ion source temperature being 230°C (Hutson et al., 2009).

The retention times for ethyl palmitate, ethyl linoleate, ethyl oleate and ethyl stearate were 11.39, 12.22, 12.23, and 12.32 minutes, respectively. The retention times for the equivalent deuterated internal standards were 11.37, 12.20, 12.21, and 12.30, respectively.
The total time per analysis of one sample was 58 minutes. The limit of detection (LOD) for the four FAEE ranged from 0.05 to 0.16nmol/g and the limit of quantification (LOQ) ranged from 0.13 to 0.32nmol/g (Hutson et al., 2009).

2.2.1.e Data Analysis

The resulting chromatograms were analyzed using GCMSsolution software, version 2.21 © 1999-2004 (Shimadzu Corporation, Columbia, MD, USA).

Quantification of FAEE was achieved by comparing the peak areas of each FAEE to the peak area of its deuterated internal standard. A calibration curve was created for each FAEE by spiking meconium with various FAEE concentrations. The created calibration curves were used to extrapolate the concentration of each FAEE in the meconium samples (Hutson et al., 2009).

The FAEE data was then converted to nmol FAEE per gram meconium. Total FAEE concentrations were then calculated by summing the amount of moles of ethyl palmitate, ethyl linoleate, ethyl oleate, and ethyl stearate that were present in one gram of meconium. If the total was above 2 nmol/g then the result was considered positive (Chan et al., 2003).

2.2.2 Laboratory Analysis of Glucose Effect on Meconium FAEE
2.2.2.a Sample Preparation

All stock solutions were prepared using dextrose. Here on dextrose is referred to as glucose. Glucose stock solutions were made by diluting dextrose (molecular weight 180.16g) with double distilled water. The dilutions were 0, 50, 100 and 200 mmol/L and 2mol/L.

Fifty mg of meconium was placed into a 1.5ml eppendorf tube. One hundred and fifty ml of glucose stock solution was added, following which the samples were vortexed for 1 minute and centrifuged for 40 seconds at 400 rpm at room temperature. Eight meconium samples for every glucose stock solution were made; four of which were boiled at 100°C for 10 minutes to denature all enzymes and four samples were not boiled. Afterwards all samples were incubated at 37°C for 12 hours.

After incubation the samples were prepared for GC-MS analysis according to a method developed by Hutson et al. (2009) (section 2.2.1c), with slight modifications. Forty microliters of deuterated internal standard solution at 1.0μg/ml was added to each SPME vial and evaporated under a nitrogen at room temperature. Following evaporation the samples were transferred from the eppendorf tubes to the SPME vial via glass pipettes. The tubes were rinsed with 200μl phosphate buffer (0.1M, pH 7.6). The sample was vortexed for 10 second and transferred to the corresponding SPME vial, with this step being repeated two times with 100μl and 550μl of phosphate buffer, respectively.
SPME vials were then capped with steel screw caps containing PTFE/Silicone septa and the samples were analyzed by GC-MS.

A standard curve was prepared for calibration of samples, using 40μl of deuterated internal standard solution and various concentrations of fatty acids stock solution, including 2, 5, 10, 20, 50 and 100 ng/ml. Negative and positive control samples were added to each GC-MS batch. Furthermore, quality control (QC) samples were included after every 12th sample. The QC samples were prepared with the 0mmol/L glucose solution and the sample was not boiled.

2.2.3 Ethical Approval for the Use of Clinical Data

Ethical approval for the use of clinical data on ethanol and drugs of abuse exposure from meconium and hair samples was obtained from the Research Ethics Board at the Hospital for Sick Children, Toronto, Ontario, Canada. (Appendix A).

2.2.4 Meconium FAEE Analysis for Prevalence of In-Utero Ethanol Exposure

Neonates whose meconium was sent to the Motherisk Laboratories at The Hospital for Sick Children, Toronto, Ontario, Canada, between June 1997 and July 2008 were used for analysis. The samples were sent by Children’s Aid Societies or physicians who suspected in-utero ethanol or drug exposure due to personal or third party reports, evidence of alcohol and/or drug abuse. All neonatal meconium samples were stored at
-80°C and were thawed prior to analyzing. Analyses were conducted within two weeks of receipt of a sample.

Meconium samples were prepared for GC-MS analysis according to a method developed by Hutson et al. (2009) (section 2.2.1c).

2.2.5 Laboratory Analysis of Meconium and Hair for Drugs of Abuse

2.2.5.a Sample Preparation

Neonates who were tested for intrauterine ethanol exposure, using meconium FAEE measurements were identified. All available meconium and hair analyses for those neonates were extracted.

These meconium and hair samples were sent to Motherisk Laboratories at The Hospital for Sick Children, Toronto, Ontario, Canada, between June 1997 and July 2008. The samples were sent by Children’s Aid Societies or physicians who suspect in-utero ethanol or drug exposure. Meconium samples were kept frozen at -80°C and were thawed prior to analyzing, while neonatal hair was kept at room temperature until analysis. Analyses were conducted within two weeks of receipt of a sample.

All drugs of abuse were analyzed by Enzyme-Linked ImmunoSorbent Assay (ELISA). Samples were prepared according to the protocols used in the Motherisk Laboratory based on ELISA kits obtained from Immunalysis Corporation (Pomona, CA, USA).
The kits contain 96-well microplate, enzyme conjugate, negative standard, stop solutions and 3,3’,5,5’-tetramethylbenzidine (TMB) chromogenic substrate. Phosphate buffered saline of 10mM concentration (pH 7.4) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Stock solutions for various drugs were obtained from Cerilliant (Mississauga, Ontario, Canada).

The drugs included in the analysis were: amphetamines (d,l-methylenedioxyamphetamine (MDA) and d,l-3,4 methylenedioxymethamphetamine (MDMA)), barbiturates, benzodiazepines (oxazepam, alprazolam, OH-alprazolam, diazepam, estazolam, flurazepam, halazepam, lorazepam, lormetrazepam and temazepam), cannabinoids (THC-COOH), cocaine (cocaine, and benzoylecgonine), opiates (heroin, morphine, codeine, hydrocodone, and hydromorphone), methadone, oxycodone, phencyclidine, and/or meperidine.

In brief, the meconium samples were thawed prior to analysis and 0.5-1.0gm meconium was used for analysis. To each sample 2ml of methanol was added, and the sample was then vortexed for 15-30 seconds and centrifuged for 5 minutes at 3500 rpm. The supernatant is then transferred to a polypropylene tube and 400μl of phosphate buffer (pH 7.4) was added.

For hair samples, between 5 and 10 mg of hair was used for analysis. The hair was cut into 1-2mm pieces with scissors. Next, 1ml of methanol was added to each sample and the sample was then incubated at 60°C overnight. The supernatant was transferred
to another vial where it was evaporated under nitrogen. Once dry, 400μl 1M phosphate buffer (pH 7.4) was added.

Calibration standard as well as meconium or hair supernatant was transferred to a pre-coated 96-well plate and enzyme conjugate was added. This was followed by incubation after which substrate reagent was added to each well and followed by more incubation. Stop Solution was then added to each well in order to change the color of the sample (from blue to yellow). Absorbance was then measured at 450nm and 650nm with the use of SUNRISE Absorbance Reader (Tecan Group Ltd., Switzerland).

Moreover, a standard curve was prepared for calibration of samples, using 0.1, 0.2, 1, 5, 10, 25, 50 and 100 ng/ml concentrations of stock solutions. Negative and positive control samples are also ran with each ELISA plate.

The LOD for methadone was 0.2 ng/ml and for the remaining drugs it was 0.1 ng/ml. The LOQ for cannabinoids, cocaine, opiates and oxycodone was 0.1 ng/ml, for benzodiazepines it was 0.15 ng/ml, for amphetamine, barbiturates, and methadone it was 0.2 ng/ml. Positive drug samples were sent to another laboratory for confirmation via GC-MS.

Positive drug of abuse test was defined as either a positive meconium test and/or positive hair analysis for the specific drug of abuse.
2.2.6 Statistical Analysis

Descriptive statistics were used to calculate the rate of drug-exposures. The rate of drug exposure was defined as the ratio of positive cases for the specific drug over the sum of the negative and positive cases of that same drug (unknown cases were excluded in these calculations).

Results were plotted in order to detect trends. Odds ratios (OR) and 95% confidence intervals (95% CI) were used in order to determine the likelihood of drug of abuse exposure in combination with heavy *in-utero* ethanol exposure.
3. RESULTS

3.1 Internal Standards

3.1.1 Purity of d5-Ethyl Esters Stock Solution

Purity of deuterated FAEE stock solution was tested by analyzing the amount of non-deuterated FAEE present. No non-deuterated FAEE were detected in these samples upon GC-MS analysis. (Figure 3.1).

**Figure 3.1: GC-MS Chromatograph of d5-Ethyl Esters Stock Solution.** 50mg of blank meconium sample spiked with deuterated ethyl esters at concentration of 2 mg/ml.
3.1.2 Ratio of FAEE Standards to Deuterated Standards

To determine if the dilutions were performed correctly for the deuterated and the non-deuterated standards, the ratio of the peak area concentrations of non-deuterated acids to the peak area concentrations of deuterated acids at 1ng/μl concentration was obtained. The ratio was 0.93 for palmitic acid, 0.99 for linoleic acid, 0.99 for oleic acid, and 0.98 for stearic acid. (Figure 3.2).

**Figure 3.2: GC-MS Chromatograph of the Ratio of Ethyl Esters to d5-Ethyl Esters in a Stock Solution.** 50mg of blank meconium sample was spiked with deuterated ethyl esters and non-deuterated ethyl esters 1:1 ratio at 1ng/μl.

3.1.3 Standard Curve

Linear regression coefficient ($R^2$) was calculated in order to determine the proportion of variability in the data set. The $R^2$ was above 0.992 for each fatty acid in external (no meconium) and internal (with meconium) samples (Figure 3.3).
**Figure 3.3: Linear Regression Coefficients** for palmitic, linoleic, oleic and stearic acids in external sample (no meconium). All $R^2$ were above 0.992

3.1.4 Interday and Intraday Coefficient of Variation

The intraday and interday coefficients of variation were tested to determine the precision of the results at 50 and 100 ng/ml FAEE concentrations.

The intraday coefficient of variation (CV) for 50 ng/ml standards ranged from 1.4 to 5.8 for ethyl palmitate, 7.5-12.8 for ethyl linolate, 7.9-10.6 for ethyl oleate and 6.2-7.6
for ethyl stearate. The interday CV for 50 ng/ml was 11.0, 11.1, 20.5, and 8.4 for ethyl palmitate, ethyl linolate, ethyl oleate and ethyl stearate, respectively (Table 3.1).

The intraday CV for 100 ng/ml was even lower, as it ranged from 3.48-8.64 for ethyl palmitate, 3.4-6.4 for ethyl linolate, 3.3-7.4 for ethyl oleate and 3.2-8.8 for ethyl stearate. The interday CV for 100 ng/ml was 10.9, 7.6, 10.7 and 7.6 for ethyl palmitate, ethyl linolate, ethyl oleate and ethyl stearate, respectively (Table 3.2).

**TABLE 3.1: INTRADAY AND INTERDAY COEFFICIENT OF VARIATION AT 50NG/ML FAEE CONCENTRATIONS**

<table>
<thead>
<tr>
<th>INTRADAY VANRABILITY</th>
<th>INTERDAY VARIABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Palmitate</td>
<td>4.4      1.4      5.8      11.0</td>
</tr>
<tr>
<td>Ethyl Linolate</td>
<td>9.3      7.5      12.8     11.1</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>10.6     7.9      9.8      20.5</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>6.2      7.6      6.7      8.4</td>
</tr>
</tbody>
</table>

**TABLE 3.2: INTRADAY AND INTERDAY COEFFICIENT OF VARIATION AT 100NG/ML FAEE CONCENTRATIONS**

<table>
<thead>
<tr>
<th>INTRADAY VANRABILITY</th>
<th>INTERDAY VARIABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Palmitate</td>
<td>3.5      4.8      8.6      10.9</td>
</tr>
<tr>
<td>Ethyl Linolate</td>
<td>6.4      4.9      3.4      7.6</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>3.3      7.4      6.2      10.7</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>6.9      3.2      8.8      7.6</td>
</tr>
</tbody>
</table>
3.2 The Effect of Glucose on Meconium FAEE

To determine whether glucose could produce false-positive meconium FAEE results, we analyzed meconium samples with added glucose solutions of various concentrations. No increase in FAEE was detected at any glucose concentrations in the non-boiled and all the boiled meconium samples. All samples remained at zero millimoles total FAEE concentrations per gram meconium (Figure 3.4, Figure 3.5).

**Figure 3.4: GC-MS Chromatograph of a Boiled Meconium Sample with 2mol Glucose Solution**

**Figure 3.5: GC-MS Chromatograph of a Non-Boiled Meconium Sample with 2mol Glucose Solution**
3.3 Use of Drugs of Abuse in High Risk Canadian Population

3.3.1 Rates of Ethanol and Drug Exposures

Nine hundred and forty three neonates were tested for *in-utero* ethanol exposure between June 1997 and July 2008. Two hundred and seven were excluded from statistical calculation due to insufficient meconium quantity for FAEE testing. Out of the remaining 736 cases that contained meconium FAEE results, 114 (15.5%) cases had meconium FAEE results of 2 nmol/g (positive test result) or above and 622 (84.5%) cases had meconium FAEE results below 2 nmol/g (negative test result).

Six cases out of the 114 FAEE-positive group and 18 cases out of the 622 FAEE-negative group were excluded from statistical calculations as no test other than the FAEE test were conducted on either hair or meconium of the neonates.

Heavy *in-utero* ethanol exposure was detected in 15.5% of the neonates. Neonates with positive-FAEE results were most frequently tested for cocaine (n=103, 90.4%), cannabinoids (n=95, 83.3%), opiates (n=93, 81.6%), and amphetamines (n=38, 33.3%). The same testing trend was present in neonates with negative-FAEE results, where cocaine was the most commonly tested drug (n=587, 94.4%), followed by cannabinoids (n=567, 91.2%), opiates (n=524, 84.2%), and amphetamines (n=228, 36.7%).
No significant difference was observed in the rates of exposures to drugs of abuse whether the neonates were heavily exposed to ethanol *in-utero* or not; the total rates of exposure to drugs of abuse were 60.5% and 62.7%, respectively.

When all 736 neonates were examined, cannabis was the most commonly used drug, with 37.9% of all neonates testing positive for *in-utero* exposure, this was closely followed by cocaine (35.5%). Narcotic exposure was detected in 14.9% of all neonates, while amphetamine-exposure was detected in 5.6% of all neonates. When the results were examined according to their ethanol exposure, the FAEE-negative group, was most frequently exposed to cannabinoids (39.5%), followed by cocaine (34.8%), methadone (18.6%), opiates (13.4%) and amphetamines (4.4%). The most common co-exposure in the FAEE-positive group was cocaine (39.8%), followed by methadone (30.8%), cannabinoid (28.4%), opiates (23.7%), amphetamines (13.2%) (Table 3.3).

**Table 3.3: The Number of Neonates with In-Utero Exposure to Drugs Determined via Meconium and Hair Testing (n=622)**

<table>
<thead>
<tr>
<th></th>
<th>FAEE POSITIVE NEONATES</th>
<th>FAEE NEGATIVE NEONATES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamines</td>
<td>n=38; 13.2% positive</td>
<td>N=228; 4.4% positive</td>
</tr>
<tr>
<td>Barbiturates</td>
<td>n=18; 0% positive</td>
<td>N=114; 1.8% positive</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>n=19; 5.3% positive</td>
<td>N=111; 4.5% positive</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>n=95; 28.4% positive</td>
<td>N=567; 39.5% positive</td>
</tr>
<tr>
<td>Cocaine</td>
<td>n=103; 39.8% positive</td>
<td>N=587; 34.8% positive</td>
</tr>
<tr>
<td>Opiates</td>
<td>n=93; 23.7% positive</td>
<td>N=524; 13.4% positive</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>n=15; 6.7% positive</td>
<td>N=87; 6.9% positive</td>
</tr>
<tr>
<td>Meperidine</td>
<td>n=2; 0% positive</td>
<td>N=21; 0% positive</td>
</tr>
<tr>
<td>Methadone</td>
<td>n=13; 30.8% positive</td>
<td>N=59; 18.6% positive</td>
</tr>
<tr>
<td>Phencyclidine</td>
<td>n=6; 0% positive</td>
<td>N=43; 0% positive</td>
</tr>
</tbody>
</table>

FAEE; Fatty Acid Ethyl Esters

Obtaining levels of FAEE 2nmol/g or above is considered a positive FAEE test indicative of heavy *in-utero* ethanol exposure in late pregnancy.
3.3.2 Ethanol Exposure and its Association with Exposure to Other Drugs of Abuse

A trend was observed in FAEE-positive group, where neonates in FAEE-positive group were more likely to be exposed to three or more drugs of abuse when compared to FAEE-negative group. However, the results did not reach statistical significance (OR = 2.67, 95% CI 0.99-7.20).

For odds ratio analysis, drugs were grouped in the following categories; stimulants, central nervous system depressants, narcotic opioids. The stimulants contained amphetamines and cocaine. The central nervous system depressants contained barbiturates and benzodiazepines. The narcotic opioids contained opiates (heroin, morphine, codeine, hydrocodone, hydromorphone), oxycodone, methadone and meperidine.

Neonates in the FAEE-positive group, when compared to neonates in FAEE-negative group, were three-times more likely to test positive for amphetamines (OR = 3.30, 95% CI 1.06-10.27), narcotic opioids (OR = 1.90, 95% CI 1.13-3.20), and opiates on their own (OR = 2.01, 95% CI 1.17-3.46). However, neonates in FAEE-positive group were less likely to have been tested positive for cannabinoids (OR = 0.61, 95% CI 0.38-0.98) when compared to neonates in the FAEE-negative group (Table 3.4).

No significant association was detected with the following drugs: stimulants (OR = 1.38, 95% CI 0.91-2.11), depressants (OR = 1.00, 95% CI 0.11-8.75), benzodiazepines (OR = 1.18, 95% CI 0.13-10.68), cocaine (OR = 1.24, 95% CI 0.81-1.91), methadone
(OR = 1.94, 95% CI 0.50-7.46), oxycodone (OR = 0.96, 95% CI 0.11-8.63) (Table 3.4).

No odds ratios were calculated for barbiturates, phencyclidine, and meperidine, as certain sub-groups had no cases of exposures.

**Table 3.4: The Strength of Association Between Positive FAEE Tests and Other Drugs Expressed in Odds Ratios**

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Odds Ratio</th>
<th>Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamines</td>
<td>3.30*</td>
<td>1.06-10.27</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>1.18</td>
<td>0.13-10.68</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>0.61*</td>
<td>0.38-0.98</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1.24</td>
<td>0.81-1.91</td>
</tr>
<tr>
<td>Opiates (heroin, morphine, codeine, hydrocodone, hydromorphone)</td>
<td>2.01*</td>
<td>1.17-3.46</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>0.96</td>
<td>0.11-8.63</td>
</tr>
<tr>
<td>Methadone</td>
<td>1.94</td>
<td>0.50-7.46</td>
</tr>
<tr>
<td>Narcotic Opioids (opiates, oxycodone, methadone, meperidine)</td>
<td>1.90*</td>
<td>1.13-3.20</td>
</tr>
<tr>
<td>Stimulants (amphetamines, cocaine)</td>
<td>1.38</td>
<td>0.91-2.11</td>
</tr>
<tr>
<td>Depressants (barbiturates, benzodiazepines)</td>
<td>1.00</td>
<td>0.11-8.75</td>
</tr>
</tbody>
</table>

*Statistically significant results

FAEE; Fatty Acid Ethyl Esters

Obtaining levels of FAEE 2nmol/g or above is considered a positive FAEE test indicative of heavy in-utero ethanol exposure in late pregnancy.
4. DISCUSSION

4.1 The Effect of Glucose on Meconium FAEE

To determine if glucose could produce a false-positive meconium FAEE result, meconium was spiked with glucose solutions of various concentrations. Some samples were also boiled in order to denature enzymes present in the meconium, in turn eliminating enzymatic activity. It was hypothesized that increase in glucose concentration will result in a slight increase in FAEE, however, no increase in FAEE was detected in any of the samples (Figure 3.4, Figure 3.5).

Klein and colleagues (1999) reported an increase in ethyl linolate in meconium spiked with ethanol, however, they did not test for other FAEE. The highest ethanol concentration that was used by Klein and colleagues was 40mM with a four hour incubation period at 37°C, resulting in the production of 35 ng/g of ethyl linolate (1.13*10^{-4} nmol/g). It is possible that the longer incubation time (12 versus 4 hours) would lead to greater FAEE degradation, as a previous study had determined that FAEE are susceptible to heat and light degradation (Moore et al., 2003; Ostrea et al., 1983). Nonetheless, the concentration detected by Klein and colleagues is very low,
considering that the cut-off value for positive meconium-FAEE result is 2 nmol/g. Such a low level would not have even been detected by the method used in this study, as the LOD for ethyl linolate is 0.05 nmol/g (Hutson et al., 2009).

Chan and colleagues (2003), while determining the cutoff for a positive FAEE test, did not detect a correlation between FAEE accumulation and maternal diabetes as well as maternal microbial infection. However, no information is available on the number of cases affected by these conditions nor the levels of FAEE detected in these cases are provided, but the reader is made aware that the sample size was too small to draw a definitive conclusion and future studies need to be conducted (Chan et al., 2003).

In our study up to 2 moles/L glucose concentrations were used. This concentration is much higher than the 39 mg/dL (2.16*10^{-4} mol/L) that is present in the amniotic fluid of women affected by diabetes (Dashe et al., 2000) and we still did not detect an increase in FAEE production.

These results indicate that an increase in meconium glucose levels do not produce false-positive meconium-FAEE results. This has important implication for the use of the FAEE meconium test in the 7% of women with gestational diabetes and smaller numbers of women with diabetes type I and II.

4.2 Ethanol Use in High Risk Canadian Population
Nine hundred and forty three neonates who were tested for in-utero ethanol exposure were identified. Two hundred and seven were excluded from calculations due to lack of meconium FAEE results. From the remaining 736 neonates, 114 had meconium FAEE concentrations equal to or above 2 nmol/g, indicative of heavy in-utero ethanol exposure in the second and/or third trimesters, while 622 neonates had meconium FAEE concentrations below 2 nmol/g, indicative of no heavy in-utero ethanol exposure in second and third trimesters.

The positivity rate for heavy in-utero ethanol exposure of 15.5% in this study population is slight higher than that observed in the general Canadian population, where in 2000-2001 it has been estimated that 13.6% of pregnant women consume ethanol at some point in pregnancy and 4.9% throughout their pregnancy (Dell & Roberts, 2006). Our is estimate is also higher than the data from the United States, where in 2006-2007 survey 11.6% of pregnant women ages 15 to 44 reported ethanol consumption with 3.7% reporting binge drinking. Neither the Canadian nor the American data indicate at what point in the pregnancy the consumption of ethanol took place, as women in first trimester of pregnancy could have consumed ethanol prior to knowing that they were pregnant (Substance Abuse & Mental Health Services Administration, 2008).

Similar to this study, Moore and colleagues (2003) measured FAEE levels in meconium samples in order to determine the rate of in-utero ethanol exposure. Two different populations were studied and all the samples were anonymized. One study group was obtained from a large, regional perinatal center in Hawaii (n=436) and the
other study group was pooled from six different neonatal intensive care units within the state of Utah (n=289). The positivity rate of heavy in-utero ethanol exposure was 16.7% in the group from Hawaii and 12.1% in the group from Utah (Moore et al., 2003). These results are more similar to the rate detected in our study.

The higher rate of in-utero ethanol exposure that has been detected in this study can be attributed to the fact that these specific meconium samples have been sent to the Motherisk Laboratory at the Hospital for Sick Children, Toronto, Ontario, as there was suspicion that ethanol and/or drug abuse occurred during pregnancy. Hence, our sample population is a high risk Canadian population and not a true reflection of the general Canadian population. This positive rate is good for a screening method, as a very low positivity rate would mean many unneeded tests, whereas a very high positivity rate would mean many missed cases.

4.3 The Use of Drugs of Abuse in a High Risk Canadian Population

Twenty four neonates were excluded from these calculations for drugs of abuse as they were tested only for meconium FAEE and no other drug test was conducted on either hair or meconium.

All neonates, independent of meconium FAEE results, were most frequently tested for cocaine, followed by cannabinoids, opiates and amphetamines. Although the rate of in-utero drug exposure was not statistically different in neonates with in-utero ethanol
exposure and those without, a difference in the rate for specific drugs was detected between the two groups.

This rate of drug exposure is much higher than the 9.4% that has been estimated in Canadian women in 2002 (Tjepkema, 2004), or the 5.2% rate that was estimated in pregnant women between the ages of 15 and 44 in 2006-2007 (Substance Abuse & Mental Health Services Administration, 2008). This study’s results are more closely related to the data from Washington’s FAS Diagnostic and Prevention Network, where 84.5% of children that were referred to the clinic since 1993 for FASD diagnosis were also exposed to cigarettes and/or illicit drugs (Olson et al., 2007).

Cannabis was the most commonly used drug of abuse, followed closely by cocaine, where 37.9% and 35.5% of all neonates tested positive for cannabis and cocaine, respectively. This was followed by opiate (excluding methadone) and amphetamine exposure, where 14.9% and 5.6% of neonates tested positive for opiates and amphetamines, respectively.

Cannabis being the most commonly used illicit drug is consistent with previously published studies, where 39% of females reported using it at least once in their lifetime and about 10% used within 12 months of taking part in the Canadian Addiction Survey in 2003-2004 (Adlaf, et al., 2005; Tjepkema, 2004). Data from the United States in 1996-1998 estimated 1.8% of cannabis use in an obstetric population (Ebrahim & Gfroere, 2003). Both the American and the Canadian rates of cannabis use are lower than the one detected in our study population. However, positivity rate
of 17% was detected in anonymous meconium testing in infants delivered at the University of Maryland Hospital in February and March 1991 (Nair et al., 1994), a rate much higher than the 1.8% that was obtained in the above mentioned survey, but still lower than the rate detected in our study.

Cocaine was the second most common illicit drug exposure in our study population, where 35.5% of all neonates were tested positive for in-utero exposure. This result is consistent with those of high-risk populations, where a 30.7% positivity-rate for cocaine was detected (Ostrea et al., 1992). Nair and colleagues (1994) have also detected a 31% positivity rate for cocaine in neonates delivered at the University of Maryland Hospital. However, the Canadian Addiction Survey reported a 1.9% exposure to cocaine 12 months prior to the conduction of the survey in the years 2003 and 2004, a rate much lower than the one we have detected (Adlaf et al., 2005).

Opiate use in the general Canadian population has been estimated at 0.3% (Popova et al., 2006), a rate that is also much lower than the 14.9% detected in our sample. However, an 18% positivity rate was detected via anonymous meconium testing in neonates delivered at the University of Maryland Hospital (Nair et al., 1994), a rate that is higher than that observed in our study.

The higher rates of drug exposures in this study could be explained by the fact that our study population is a high risk obstetric population, where neonatal meconium and hair samples were sent for testing due to suspected maternal drug abuse.
The rate of exposure to amphetamines, however, is similar to that observed in the general population. In this study the rate of amphetamine exposure was 5.6%, while the rate of exposure in pregnancy in the United States has been estimated at 5.2% and the rate of exposure in a Canadian student population in 2003 was 4.4% (Arria et al., 2006; Centre for Addiction and Mental Health, 2006).

When ethanol exposure was taken into consideration and the rates of drug exposure were examined again, slight differences in drug exposures were observed. Neonates in the FAEE-negative group were most commonly exposed to cannabinoids (39.5%), followed by cocaine (34.8%), methadone (18.6%), opiates (13.4%) and amphetamines (4.4%). The neonates in FAEE-positive group were most commonly exposed to cocaine (39.8%), followed by methadone (30.8%), cannabinoid (28.4%), opiates (23.7%), and amphetamines (13.2%) (Table 3.3).

4.4 Ethanol Exposure and Its Association with Exposure to Other Drugs of Abuse in High Risk Canadian Population

Neonates in the FAEE-positive group, when compared to neonates in the FAEE-negative group, were three-times more likely to test positive for amphetamines (OR = 3.30, 95% CI 1.06-10.27). They were also two-times more likely to test positive for opiates (heroin, morphine, codeine, hydrocodone, and hydromorphone) (OR = 2.01, 95% CI 1.17-3.46), or almost two-times as likely to have been exposed to narcotic opiates (that include all the opiates as well as oxycodone, methadone, and meperidine) (OR = 1.90, 95% CI 1.13-3.20). These results are consistent with previously
published studies where women who drink ethanol were more likely to consume other recreational and illicit drugs, including amphetamine, cocaine, and opiates (Chasnoff et al., 2001; Gladstone et al., 1997; Lester et al., 2001; Smith et al., 2008).

Neonates in the FAEE-positive group were less likely to test positive for cannabinoids (OR = 0.61, 95% CI 0.38-0.98), which is inconsistent with previously published data. Lester and colleagues (2001) detected a four-fold increase rate of cannabis use in women who used ethanol during pregnancy (OR=4.0; 95%CI 3.5-4.7). However, Lester and colleagues (2001) used meconium test results as well as maternal reports in order to detect drug exposures. The use of maternal reports would provide information on first trimester exposures; information that was not available in our study, as the requisitions for testing did not contain information on such exposures, and the meconium and hair samples provide information on second and third trimester exposures only. Considering that the rates of abstinence from illicit drugs increase with progression of pregnancy (Ebrahim & Gfroere, 2003), one could assume that women would decrease cannabinoid use in the second and third trimester when compared to cannabinoid use prior to pregnancy and in first trimester. Hence, it is possible that there is no inconsistency between the two studies.

The negative association between cannabinoids and ethanol exposure might be explained by the common properties of ethanol and cannabinoids. Both drugs affect the same reward pathways, leading to depressive/sedative properties (Mechoulam & Parker, 2003). Thus a pregnant woman who is consuming one of the drugs may be less inclined to consume the other drug in order to reduce fetal exposure to toxins.
When drugs were grouped into stimulants and central nervous system depressants, no significant association was detected with ethanol exposure. The stimulant group contained amphetamines and cocaine, while the central nervous system depressants contained barbiturates and benzodiazepines. Due to the fact that no significant association was detected between cocaine and ethanol, the cocaine samples masked the significant association between amphetamines and ethanol as there were many more samples tested for cocaine. The central nervous system depressants did not reach significance due to the small sample size.

Narcotic opioids (oxycodone, methadone, meperidine and opiates - heroin, morphine, codeine, hydrocodone, hydromorphone), on the other hand, did reach statistically significant positive association with ethanol. This group association reached significance as opiates were significantly associated with ethanol and methadone was also showing a positive association although due to limited sample size that was not statistically significant, while rate of oxycodone exposure was similar in ethanol exposed and non-exposed neonates and thus contributed no weight to the statistics. Hence, the group as a whole reached statistically significant association with ethanol exposure.

No significant association was detected with the use of benzodiazepines, methadone, and oxycodone due to limited sample size.
Chan et al. (2004a) also used the same database as we did in this study for detection of ethanol exposure in neonates whose meconium samples were sent for routine drug screening for cannabis, cocaine, and opiates between March 2000 and March 2003. Samples with sufficient meconium left after testing for illicit drugs were used for FAEE analysis (n=142). A positivity rate of 14% for heavy in-utero ethanol exposure was detected. This rate is similar to the one in our study as would be expected since these samples are not the same used in our study but they are also from a similar high-risk Canadian population. However, a slight higher rate of cannabis exposure (52%) and of exposure to one or more drug of abuse (71%) was detected. This could be due to the fact that the samples in the study by Chan et al. were tested due to suspicion of illicit drugs and not ethanol while in our study the samples were tested due to suspicion of ethanol exposure as well as other drugs of abuse.

Considering that the neonates were more likely to be exposed to multiple other drugs of abuse, the neonatal risks to those exposed in-utero to ethanol may increase in a synergistic and not just an additive fashion (Sokol et al., 1986). For example, evidence exists that co-exposure of ethanol and cocaine gives rise to coca ethylene, a powerful neurotoxin by itself.

4.5 Limitations

4.5.1 Meconium FAEE Testing
The 2 nmol/g cut off line for meconium FAEE testing is 100% sensitive and 98.4% specific. This means that all the neonates (100%) with heavy in-utero ethanol exposure obtain a positive meconium-FAEE test. However, 1.6% of neonates with no heavy in-utero ethanol exposure also obtain a positive meconium-FAEE test (specificity 98.4%). This is a very high specificity rate, but it is important to remember that some of the neonates will obtain a false-positive meconium-FAEE result.

4.5.2 The Effect of Glucose on Meconium FAEE

Meconium samples that were spiked with glucose were incubated for 12 hours. Considering that pregnancy lasts for 40 weeks, 12 hours are insufficient to determine if long-term exposure to elevated glucose levels could lead to an increase in FAEE levels. However, the glucose levels that were used in this study were much higher than those found in diabetes-affected individuals and no increase in FAEE was detected.

It is also important to remember that pregnant women undergo testing for diabetes and are treated if diabetes is diagnosed, in turn decreasing their glucose levels to normal ranges. Hence, there is a low risk of a woman in Canada to have undiagnosed diabetes if she is followed by a physician.
4.5.3 In-Utero Ethanol and Drug Exposure

The studied population is a high risk obstetric Canadian population. The detected ethanol and drug exposures are not generalizable to the general Canadian population.

The data captures only second and third trimester exposures in meconium testing and only third trimester exposure in hair testing. No information is available for first trimester exposures in this population. Therefore, one cannot conclude that similar trends are present in first trimester of pregnancy, when women just discover that they are pregnant.

4.6 Summary

The results suggest that maternal diabetes does not produce a false-positive meconium-FAEE result, and is not a confounding factor in meconium FAEE testing, as hypothesized.

A high rate of in-utero ethanol exposure was detected in our study population. A significant positive association between in-utero ethanol exposure and in-utero exposure to amphetamines and opiates, as well as a significant negative association was detected between in-utero ethanol exposure and in-utero exposure to cannabinoids. Although no significant association was found between in-utero ethanol exposure and in-utero exposure to cocaine, the positivity rate is very high independent of in-utero ethanol exposure.
This study strongly suggests that neonates with in-utero ethanol exposure should also be tested for amphetamine and opiate exposure and vice versa as recent drug use is significantly correlated with being at risk for an ethanol-exposed pregnancy (Project CHOICES Research Group, 2002). Early detection of heavy in-utero ethanol exposure will allow for the detection of neonates at risk of FASD. This will provide the opportunity to deliver the appropriate treatments and support services to the child and mother in question in order to decrease the risk of developing secondary disabilities that are associated with heavy in-utero ethanol exposure.

4.7 Future Studies

It will important to assess the benefit of meconium-FAEE testing as a screening tool. The level of voluntary participation in screening for prenatal ethanol exposure in the general population needs to be established. Furthermore, it is important to determine the degree of benefit children and their families will experience in their quality of life as well as the cost benefit to the society, if children at risk of FASD will be identified, followed up and provided with early interventions.

There are also no data on the dose-response relationship between in-utero ethanol exposure and meconium-FAEE levels. Studies need to be conducted using validated maternal reports in order to correlate maternal drinking with meconium-FAEE levels.
Meconium of neonates born to diabetes-affected mothers should be tested for FAEE presence in order to confirm that maternal glucose levels do not affect FAEE concentrations.
5. References


Kapur, B.M. (2007). Ethyl Glucuronide. International Association of Therapeutic Drug Monitoring and Clinical Toxicology. 6, 7-10.


6. LIST OF PUBLICATIONS AND ABSTRACTS

Abstracts and Manuscripts Published


Abstracts Accepted for Publication


Manuscripts Submitted for Publication that Have Been Peer-Reviewed


7. APPENDICES

APPENDIX A: DOCUMENTATION OF ETHICAL APPROVAL

RESEARCH ETHICS BOARD

September 28, 2007

Dr. Gideon Koren
Clinical Pharmacology & Toxicology
The Hospital for Sick Children

Dear Dr. Koren:

Your study "Presence of Drugs of Abuse in Hair and Meconium: A Database based study."

REB File No.: 1000008229

On behalf of the REB, I am writing to confirm that the above noted study was re-approved by the REB for one year ending in September 2008. The REB approved continuing review at level 1A. As necessary, the Clinical Research Office will be contacting you to arrange follow-up.

Please note that, in accordance with the Personal Health Information Protection Act of Ontario, you are 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. You are also responsible for reporting immediately any privacy breaches to the REB Chair and to Janice Campbell, the Sick Kids privacy officer.

Yours truly,

[Signature]

Melvin Freedman
Chair, Research Ethics Board
RESEARCH ETHICS BOARD

September 12, 2008

Dr. Gideon Koren
Clinical Pharmacology & Toxicology
The Hospital for Sick Children

Dear Dr. Koren:

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REB File No.: 1000008229

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Yours truly,

[Signature]

Richard Sugarman
Chair, Research Ethics Board