Fatty Acid Ethyl Esters (FAEE), A Biomarker of Alcohol Exposure: Hope for a Silent Epidemic of Fetal Alcohol Affected Children

by

Vivian Kulaga

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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Abstract

One percent of children in North America may be affected by fetal alcohol spectrum disorder (FASD). FASD remains difficult to diagnose because confirmation of maternal alcohol use is a diagnostic criterion, and women consuming alcohol during pregnancy are reluctant to divulge this information for fear of stigmatization and losing custody of the child. Consequently, using a biomarker to assess alcohol exposure would provide a tremendous advantage.

Recently, the measurement of fatty acid ethyl ester (FAEE) in hair has provided a powerful tool for assessing alcohol exposure. My thesis fills a translational gap of research between the development of the FAEE hair test and its application in the context of FASD.

The guinea pig has been a critical model for FASD research, in which FAEE hair analysis has previously distinguished ethanol-exposed dams/offspring from controls. My first study, reports a positive dose-concentration relationship between alcohol exposure and hair FAEE, in the human, and the guinea pig. Humans also displayed over an order of magnitude higher FAEE incorporation per equivalent alcohol exposure, suggesting that the test will be a sensitive clinical marker of fetal alcohol exposure. My second study utilized multi-coloured rats to investigate the potential of a hair-colour bias, as has been reported for other clinical hair assays; no evidence of
bias is reported here. My third study is the first to examine the clinical use of the FAEE hair test in parents at high risk of having children with FASD. Over one third of parents tested positive for excessive alcohol use. Parents were investigated by social workers working for child protection services, and my fourth study reports that hair FAEE results agree with social worker reports. Individuals highly suspected of abusing alcohol were at a significantly greater risk of testing positive, whereas individuals tested based on other reasons (such as to cover all bases) were negatively associated with testing positive. The last study of my thesis, confirmed an association between alcohol and drug use by parents at high risk for having children with FASD, posing an added risk to children.

This work helps bridge a gap in translational research, suggesting that the FAEE hair test has potential for use in FASD diagnosis and research.
Acknowledgments

Finally a place to get the last word!!! It is with great joy, and a sigh of relief, I take this opportunity to truly thank everyone who deserves it from the bottom of my heart in helping me to turn my life long dreams and aspirations into reality.

To my supervisor, Dr. Gideon Koren, thank you!! Whenever it mattered, and whenever it counted, you were always there. A truly inspirational mentor and leader, both professionally and personally, you have set a powerful example.

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To my grandmother, Witolda Ciuhak, thanks. I know how much you care.

To my sister, Kasia, you have been my confident and friend, making difficult times easier to bear.

To my dearest friend, Sean, who has made much of this possible. Thank you for your strength, and unwavering support. You made me a better person and able to face the challenges in my life with courage. Thank you from the bottom of my heart. You are sorely missed.
And last but not least, to the little one who cannot read or understand, my dog, Hamlet. Your contribution may seem insignificant to others, but to me you are my best friend and always there; therefore you deserve to be immortalized in my thesis. Who ever said dogs were just man’s best friend!!

If I forgot you, I’m sorry. It was either because you weren’t that important or writing this thesis removed parts of my brain. Just kidding! There are many others who have supported me and contributed to my success, and you know who you are because you are reading this. You are all truly in my heart. Many thanks!!!

Sincerely,

Vivian Kulaga
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADH</td>
<td>Alcohol Dehydrogenase</td>
</tr>
<tr>
<td>AEAT</td>
<td>Acyl-CoA:Ethanol O-Acetyltransferase</td>
</tr>
<tr>
<td>ALDH</td>
<td>Acetaldehyde Dehydrogenase</td>
</tr>
<tr>
<td>ARBD</td>
<td>Alcohol-Related Birth Defects</td>
</tr>
<tr>
<td>ARND</td>
<td>Alcohol-Related Neurodevelopmental Disorder</td>
</tr>
<tr>
<td>AUC</td>
<td>Area-Under-the-Curve</td>
</tr>
<tr>
<td>AUC-BEC</td>
<td>Area-Under-the-Curve of the Blood-Ethanol-Concentration Time Curve</td>
</tr>
<tr>
<td>BEC</td>
<td>Blood Ethanol Concentration</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CAS</td>
<td>Children’s Aid Organizations</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CPS</td>
<td>Child Protection Services</td>
</tr>
<tr>
<td>CV</td>
<td>Co-efficient of Variation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DHI</td>
<td>5,6-Dihydroxyindole</td>
</tr>
<tr>
<td>DHICA</td>
<td>5,6-Dihydroxy-2-Carboxylic Acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EE</td>
<td>Extraction Efficiency</td>
</tr>
<tr>
<td>EtG</td>
<td>Ethyl Glucoronide</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty Acid</td>
</tr>
<tr>
<td>FAE</td>
<td>Fetal Alcohol Effects</td>
</tr>
<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 Disorders</td>
</tr>
<tr>
<td>FAS DPN</td>
<td>Fetal Alcohol Syndrome Diagnostic and Prevention Network</td>
</tr>
<tr>
<td>FPM</td>
<td>First Pass Metabolism</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational Day</td>
</tr>
<tr>
<td>HS-SPME</td>
<td>Head Space-Solid Phase Microextraction</td>
</tr>
<tr>
<td>ICR</td>
<td>Incorporation Rate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>IOM</td>
<td>Institute of Medicine of the National Academy of Sciences</td>
</tr>
<tr>
<td>KS</td>
<td>Korsakoff’s Syndrome</td>
</tr>
<tr>
<td>LE</td>
<td>Long Evans</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantification</td>
</tr>
<tr>
<td>MEOS</td>
<td>Microsomal Ethanol-Oxidizing System</td>
</tr>
<tr>
<td>NCI</td>
<td>Negative Chemical Ionization Mode</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
</tr>
<tr>
<td>PFAS</td>
<td>Partial Fetal Alcohol Syndrome</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
</tr>
<tr>
<td>PtE</td>
<td>Phosphatidylethanol</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Drink</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid Phase Microextraction</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
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</table>
1 Introduction

1.1 Alcohol

1.1.1 Chemistry and Metabolism in Humans

Alcohol, referring to ethyl alcohol (ethanol), is a small polar molecule with a molecular weight of 46 g/mol (Figure 1.1). Ethanol is capable of permeating most membranes in the human body. It is a clear colourless liquid, completely miscible with water, and forms the primary ingredient of alcoholic beverages. Its pharmacological actions include, a central nervous system (CNS) depressant, a local anti-infective agent, and a solvent (National Center for Biotechnology Information, 2008).

![Chemical structure of ethanol](image)

Figure 1.1 The molecular structure of ethanol
Once ingested, the majority of alcohol is rapidly absorbed from the small intestine and distributed through the total body’s aqueous phase, referred to as total body water (Watson et al., 1980). Alcohol undergoes some first pass metabolism (FPM) (Julkunen et al., 1985a; Julkunen et al., 1985b; Frezza et al., 1990) through the stomach and liver before it enters the general circulation. Blood alcohol concentration (BAC) is determined by the extent of FPM, and the rate of absorption, distribution, and elimination. Ninety to ninety-five percent of alcohol is metabolized oxidatively, whereas the remainder is metabolized non-oxidatively and excreted unchanged through the kidney. Alcohol is primarily metabolized in the liver, oxidatively, by alcohol dehydrogenase (ADH), and by CYP 2E1 in the microsomal ethanol-oxidizing system (MEOS), to acetaldehyde, which is then converted by acetaldehyde dehydrogenase (ALDH) to acetate (Figure 1.2) (Lieber and DeCarli, 1970; Matsumoto et al., 1996). Alcohol does undergo some oxidative metabolism in the brain as well primarily by MEOS, and by catalase located in peroxisomes (Zakhari, 2006; Deitrich et al., 2006).
At very low levels of blood alcohol, below 5-10 mg/dl, the rate of elimination of ethanol exhibits Michaelis-Menten kinetics, and follows an exponential decay (Marshall and Fritz, 1953). However, at higher blood alcohol levels that are still considered pharmacodynamically relatively low, 10-20 mg/dl (the Canadian legal limit to operate a motor vehicle is currently below 80 mg/dl), elimination begins to follow zero order kinetics and is linear. This is because ADH, which has a very low Km (0.049 – 4.2 mM) becomes easily saturated resulting in a constant
elimination rate (Riveros-Rosas et al., 1997). Therefore, $V_{\text{max}}$, derived from the slope of the linear portion of the elimination curve, is typically used to estimate the total elimination rate of alcohol.

The average ethanol elimination rate ($V_{\text{max}}$) for men and women ranges between 15 and 18 mg/dL/h, but can vary as widely as 10 to 30 mg/dL/h, with faster rates observed among alcoholics (Dettling et al., 2007; Winek and Murphy, 1984; Mumenthaler et al., 2000; Lieber, 1997). Furthermore, ethanol elimination has been studied in pregnant women and newborn infants, with reported rates ranging between 14 and 24 mg/dL/h for mothers, and a mean rate of approximately 8 mg/dL/h for neonates (Nava-Ocampo et al., 2004; Idanpaan-Heikkila et al., 1972).

Less than 5% of ethanol is metabolized through non-oxidative pathways resulting in the formation of minor metabolites such as fatty acid ethyl esters (FAEE), phosphatidylethanol (PtE), and ethyl glucoronide (EtG) (Figure 1.3). FAEE are the physical conjugates between ethanol and fatty acids in the body and their formation and elimination are discussed in detail in the FAEE section to follow. PtE is a phospholipid formed when phospholipase D catalyses a reaction between phosphatidylcholine and ethanol instead of its primary substrate, water, that would normally lead to the production of phosphatidic acid (Rakhimov et al., 1988). ETG is formed when ethanol is glucoronidated with activated glucoronic acid (UDP-GA) (Schmitt et al., 1995; Kronstrand and Scott, 2007). Studies have shown that the oxidative and non-oxidative pathways of ethanol metabolism are related in an inverse manner. It appears that when oxidative metabolism is decreased or artificially suppressed, non-oxidative metabolism increases resulting
in greater production of non-oxidative metabolites such as FAEE (Werner et al., 2002; Best et al., 2006).

Figure 1.3 Non-oxidative pathways of ethanol metabolism
1.1.2 Deleterious effects

Alcohol has many deleterious effects, from direct effects on cell function to tissue damage produced by metabolic by-products. Alcohol can directly interact with cell membranes, disordering membrane fluidity and disrupting normal cell signaling (Chin and Goldstein, 1977; Chin and Goldstein, 1981). Oxidative metabolism of ethanol can generate substantial oxidative stress leading to tissue damage through the production of reactive oxygen species (ROS). Primary targets of tissue damage are brain, liver, heart, lungs and natural killer white blood cells. The most commonly affected organ is the liver; damage can begin with the appearance of a fatty liver, followed by inflammation, apoptosis, fibrosis, and finally cirrhosis (Zakhari, 2006).

Alcoholism is a physical and psychological addiction to ethanol with serious medical and social repercussions. Many health problems are associated with heavy alcohol use and they include: liver disease; disease of nervous, gastrointestinal, cardiovascular, and respiratory systems; cancers; metabolic and immune system disorders; endocrine disorders; nutritional deficiencies; poisoning; and alcohol-associated injuries and accidents (Hurly and Horowitz, 1990). Furthermore, there are also non-alcohol-related pathological diagnoses associated with alcohol use such as: pancreatic disease, nutritional deficiencies, tuberculosis, various mental disorders, epilepsy, pneumonia and influenza (Hurly and Horowitz, 1990). Alcohol-related mortality is a serious consequence resulting from the above mentioned conditions, in particular alcohol-related mortality involving liver cirrhosis, trauma, motor vehicle accidents, railroad/aviation/boating accidents, drowning, fires, falls, and suicide (Hurly and Horowitz, 1990).

Importantly, heavy alcohol use can also result in permanent neurological damage. Alcoholism can lead to the development of neurological disorders such as Korsakoff’s Syndrome (KS), a
profound memory disorder characterized by short-term memory loss. If consumed during pregnancy alcohol can also cause fetal alcohol spectrum disorder (FASD) in the offspring (described in detail in the FASD section). Alcohol is a potent toxic and terotegenic substance.

1.1.3 Prevalence of Alcohol Use

1.1.3.1 General

Despite alcohol’s toxicity and potential for abuse, it is a legal product, which is widely used across the world. According to the 2004 Canadian Addiction Survey alcohol is the most commonly used psychoactive substance among Canadians, with 79.3% reporting they consumed it in the past 12 months, and 13.5% saying they were former drinkers (Adlaf et al., 2005). Of Canadians who currently consume alcohol, 7.9% said they drink more than 4 times a week, and 12.7% said that their typical usage pattern was more than 5 drinks per occasion. However, those who reported using alcohol more than 4 times a week were not necessarily the same people who reported using more than 5 drinks per typical occasion.

The Canadian Addiction Survey defines heavy drinking as having 5 or more drinks per occasion for men, or 4 or more for women (without reference to a time frame) (Adlaf et al., 2005). The survey reported 20.2% of Canadians participate in heavy drinking on a monthly basis and 4.9% on a weekly basis. Large epidemiological studies conducted in the US (the 2001-2002 National Epidemiologic Survey on Alcohol And Related Conditions, n = 43,093, and the 1991-1992...
National Longitudinal Alcohol Epidemiologic Survey, n = 42,862) have reported rates of alcohol abuse and/or dependence\(^1\) to range between 7.4-8.5 % in the general population (3.0-4.7% for alcohol abuse, and 3.8-4.4 for alcohol dependance) (Grant et al., 1994; Grant et al., 2004).

\[1\]

1.1.3.2 Pregnant Women and Women of Childbearing Age

Several studies have examined the prevalence of alcohol use among pregnant women and women of childbearing age in North America (CDC, 1997; CDC, 2002; CDC, 2004; Floyd et al., 1999; Ebrahim et al., 1999; Ebrahim et al., 1998; Chambers et al., 2005). The majority of these studies (CDC, 1997; CDC, 2002; CDC, 2004; Ebrahim et al., 1999; Ebrahim et al., 1998) have

\[\text{Diagnoses of alcohol abuse and dependence were made according to the DSM-IV criteria. Specifically, “DSM-IV alcohol abuse is manifested by one or more of the following symptoms: recurrent drinking resulting in failure to fulfill major role obligations; recurrent drinking in hazardous situations; recurrent drinking-related legal problems; and continued drinking despite recurrent social or interpersonal problems caused or exacerbated by drinking. DSM-IV alcohol dependence is defined by seven diagnostic criteria: tolerance; the withdrawal syndrome or drinking to relieve or avoid withdrawal symptoms; drinking larger amounts or for a longer period than intended; persistent desire or unsuccessful attempts to cut down on drinking; spending a great deal of time obtaining alcohol, drinking, or recovering from the effects of drinking; giving up important social, occupational, or recreational activities in favor of drinking; and continued drinking despite a physical or psychological problem caused or exacerbated by drinking” (Grant et al., 2004).}\]
relied on data collected from the Behavioural Risk Factor Surveillance System (BRFSS) survey, which is a monthly, state-based, random-digit dialed telephone survey of the non-institutionalized U.S. civilian adult population in all 50 states, District of Columbia, and three U.S. territories. The BRFSS data represents women aged 18-44 years, with approximately 250,000 interviews collected from 1988 to 2002, and an approximate pregnancy rate of 5% across survey years (CDC, 1997; CDC, 2002; CDC, 2004; Ebrahim et al., 1999; Ebrahim et al., 1998). The median response rate from 1988 to 1995 (ratio of completed interviews to completed interviews and refusals) ranged from 80 to 85% (Ebrahim et al., 1998) and the median state/area response rate in 2002 was 58.3% (range 42.2-82.6%) (CDC, 2004).

Among women of child-bearing age (18-44), the prevalence of any alcohol use within the past month ranged from 49.4% in 1991 to 56.3% in 1988, the prevalence of binge drinking (5 or more drinks per occasion) ranged from 10.5% in 1991 (and in 1995) to 12.3% in 1999, and the prevalence of frequent drinking (average of consumption of seven drinks of more per week or one binge episode in the last month) ranged from 12.4% in 1999 to 17.8% in 1988 (CDC, 1997; CDC, 2002; CDC, 2004; Ebrahim et al., 1999; Ebrahim et al., 1998). Among pregnant women, the prevalence of any alcohol use within the past month ranged from 9.5% in 1992 to 22.2% in 1988, the prevalence of binge drinking (5 or more drinks per occasion) ranged from 0.7% in 1991 to 2.9% in 1995, and the prevalence of frequent drinking (average of consumption of seven drinks of more per week or one binge episode in the last month) ranged from 0.8% in 1991 to 3.9% in 1988 (CDC, 1997; CDC, 2002; CDC, 2004; Ebrahim et al., 1999; Ebrahim et al., 1998).

The prevalence of drinking among pregnant women, and women of child bearing age, has fluctuated between the years, sometimes increasing or decreasing. However, it appears that no
overall trend can be discerned since the phrasing and order of the questions regarding alcohol use and pregnancy have changed from survey year to survey year, which may have influenced the results (CDC, 1997; CDC, 2002; CDC, 2004; Ebrahim et al., 1999; Ebrahim et al., 1998). Nevertheless, the fluctuations in prevalence rates, for the most part have been unremarkable, therefore the latest rates (below) can be considered representative.

The latest estimates from the 2002 BRFSS study report that among women of child bearing age (18-44), the prevalence of any alcohol use within the past month was 52.6 %, for binge drinking 12.4 %, and for frequent drinking 13.2 % (CDC, 2004). For pregnant women, the prevalence of any alcohol use within the past month was 10.1 %, for binge drinking 1.9 %, and for frequent drinking 1.9 % (CDC, 2004). Uniquely, the 2002 BRFSS study, for the first time ever assessed prevalence of alcohol use among women who might become pregnant. Women who might become pregnant were defined as those who were not using any type of birth control and provided one of the following reasons: wanted a pregnancy, did not care whether a pregnancy occurred, did not think they would become pregnant, did not want to use birth control, feared the side effects of birth control, thought they were too old to become pregnant, could not pay for birth control, or had lapsed in the use of a method (CDC, 2004). Among women who might become pregnant, the prevalence of any alcohol use within the past month was 54.9 %, for binge drinking 12.4 %, and for frequent drinking 13.1 % (CDC, 2004).

The high prevalence of drinking among women who might become pregnant should be alarming as approximately 50 % of pregnancies are unplanned, and therefore many pregnancies may be accidentally exposed to high levels of alcohol. Floyd et al., investigated the prevalence of periconceptional drinking prior to pregnancy recognition and found similar rates of alcohol use as
found in the BRFSS data (Floyd et al., 1999). Floyd, et al., showed that the majority of women (60%) were unaware of their pregnancy for at least the first 4 weeks of pregnancy and many (30%) were unaware past the sixth week (Floyd et al., 1999). The study used data collected by the National Maternal and Infant Health Survey (NMIHS) across 48 states, the District of Columbia, and New York City (n = 9953). The investigators reported that 48% of respondents admitted to using some alcohol prior to pregnancy recognition, with 14.8% consuming 1 – 5 drinks per week and 5% drinking more than 6 drinks per week (Floyd et al., 1999). Rates of alcohol use did decrease once pregnancy status was known to 21%, with less than 1% of women drinking more than 6 drinks per week.

1.1.3.3 Risk Factors

Studies have reported similar risk factors associated with alcohol use during pregnancy. Those risk factors have included smoking, having a higher level of education, being unmarried, being employed or studying, having a higher annual household income, being white non-Hispanic, and having lower parity (Floyd et al., 1999; CDC, 2004; Ebrahim et al., 1998; CDC, 2002; Chambers et al., 2005). Floyd et al., reported that older women (over 25) were more likely to be frequent drinkers (Floyd et al., 1999), however from the BRFSS data, the CDC reports that frequent drinking and binge drinking are associated with being younger (<30 years) (CDC, 2004; CDC, 2002). Chambers et al., studied drinking in low-income pregnant Latinas in the US and also found that younger maternal age was associated with alcohol use in the peri-conceptional period (Chambers et al., 2005). Therefore, although there is some discrepancy involving maternal age, most studies have reported similar risk factors associated with drinking during and around the time of pregnancy.
1.2 The Fetal Alcohol Spectrum Disorder

1.2.1 History and Etiology

Throughout history it has been suggested that parental alcoholism is detrimental to offspring (see Warner and Rosett, 1975, for review). However, the idea of fetal damage arising from alcohol exposure during pregnancy has only been accepted in scientific communities since the 1970’s (Lemoine, 2003). The first scientific study to describe fetopathies arising from alcohol exposure was that of Jacqueline Rouquette’s thesis entitled “Influences of the parental alcoholic intoxication on the physical and psychological development of young children”, where she described resulting effects for 100 children with confirmed parental alcoholism (Paris 1957) (Lemoine, 2003). Around the same time, Dr. Paul Lemoine, a practicing pediatrician, was independently collecting data on his own patients. In his paper entitled “The children of alcoholic mothers, observed anomalies, discussion of 127 cases”, Dr. Lemoine described a syndrome he was noticing in children born to alcoholic patients. Lemoine characterized children with the syndrome by severe intrauterine growth retardation with lasting hypotrophy and microcephaly, psychomotor retardation with behavioural problems, specific facial dysmorphology, and frequent malformations such as cardiac and skeletal (Lemoine, 2003; Lemoine et al., 2003). However, these French reports went unnoticed in the scientific community until Jones and Smith, published an article in English describing 8 cases in the United States with a similar syndrome; Jones and Smith, described what they called “the first
reported association between maternal alcoholism and aberrant morphogenesis in offspring” (Jones et al., 1973; Koren and Navioz, 2003; Lemoine, 2003). The term “fetal alcohol syndrome” was coined by Jones and Smith in 1973, and they are often mistakenly credited with the syndrome’s discovery (Jones and Smith, 1973; Koren and Navioz, 2003). Nevertheless, their valuable contribution has sparked thousands of research papers on the subject since, which have increased knowledge and awareness regarding this devastating but preventable condition.

A key feature of fetal alcohol syndrome, which was instrumental in its discovery, is a constellation of characteristic facial anomalies consisting of 3 main features: short palpebral fissures, smooth or flattened philtrum, and a thin vermilion border of the upper lip (Figure 1.4) (Chudley et al., 2005). Fetal alcohol syndrome is also accompanied by growth retardation and central nervous system neurodevelopmental abnormalities that can often be severe, such as microcephaly and microencephaly, agenesis of the corpus callosum, impaired fine motor skills, or poor hand-eye coordination, among others. (Chudley et al., 2005).
It is now recognized that the effects of fetal alcohol exposure can be diverse in their manifestation and widespread in their intensity. Furthermore, effects are not necessarily accompanied by the characteristic facial anomalies, often referred to as “the face”. It appears that timing of the exposure plays a role in the differential expression of these effects, with first trimester exposure being crucial for facial malformations and all three trimesters playing roles in CNS dysfunction (Sulik et al., 1981; Sulik and Johnston, 1983; Sulik, 1984; Sulik et al., 1986).

Figure 1.4 Characteristic facial features of Fetal Alcohol Syndrome
1.2.1.1 Nomenclature

Different names have evolved to describe manifestations of fetal alcohol effects; some examples are: partial FAS (PFAS), alcohol-related birth defects (ARBD), and alcohol-related neurodevelopmental disorder (ARND), and fetal alcohol effects (FAE) (Chudley et al., 2005; Streissguth et al., 2004). FAE is a diagnostic term that refers to most manifestations of fetal alcohol affected children that fall outside the diagnostic criteria of FAS. Diagnostic criteria for FAS, PFAS, ARBD, and ARBD are outlined in the “diagnosis and intervention” section.

Recently researchers and clinicians have begun to use the term fetal alcohol spectrum disorder (FASD), an umbrella term used to describe any and all types of effects that result from fetal alcohol exposure (National Organization of Fetal Alcohol Syndrome, 2004). Note that while clinicians, scientists, and many examples of literature (including this thesis) refer to “FASD diagnosis”, FASD is not a diagnostic term. FASD diagnosis refers to diagnoses applied to the variety of disorders grouped under the FASD umbrella term (FAS, PFAS, ARBD, ARBD and FAE, for example). Readers should remain cognizant of this fact.

1.2.1.2 Mechanisms

While FASD is caused by exposure to maternal alcohol during pregnancy, the exact mechanism of teratogenicity is still under study. Numerous mechanisms of toxicity from fetal alcohol exposure have been proposed and are highlighted below, however a comprehensive discussion is outside the scope of this thesis. (For comprehensive reviews please see West et al., 1994, Abel and Hannigan, 1995, or Hannigan et al., 1999). There is likely no one single mechanism for ethanol teratogenicity and the mechanisms proposed likely interact with one another, along with
other contributory factors. Proposed mechanisms include, but are not limited to: fetal hypoxia; toxicity mediated through acetaldehyde and acetaldehyde metabolism; cellular damage resulting from the generation of free radical and reactive oxygen species (ROS); alcohol-induced inhibition of normal cell function and properties; and, contributory effects of alcohol-induced imbalance of prostaglandins, and vitamin/element deficiencies.

Hypoxia is a known cause of cellular damage. Alcohol consumption can result in a contractile response in the umbilical cord blood vessel, resulting in the collapse of vasculature and disruption of blood flow to the fetus (Mukherjee and Hodgen, 1982; Mukherjee and Hodgen, 1982; Savoy-Moore et al., 1989) Alcohol exposure has also been shown to suppress fetal breathing movements, an index of fetal hypoxia (Brien and Smith, 1991). Therefore, it has been hypothesized that fetal hypoxia may be responsible for much of the damage seen in FAS (Abel and Hannigan, 1995).

Exposure to alcohol also results in exposure to its primary toxic metabolite, acetaldehyde. Alcoholics exhibit higher levels of acetaldehyde than social drinkers, most likely due to the induction of the cytochrome p450 system resulting in a faster conversion of ethanol to acetaldehyde (Ronis et al., 1993). Acetaldehyde is a known toxic and highly reactive substance, forming adducts with various proteins such as enzymes, microsomal proteins, microtubules, and neurotransmitters, that have been found in the brain of alcoholics (Zakhari, 2006; Nakamura et al., 2003). There is epidemiological support for the hypothesis that acetaldehyde may play a role in FASD teratogenesis. Hard et al. reported that the incidence of high acetaldehyde levels among alcoholics was found to be in the same range, 43 %, as the incidence of ARBD in offspring born to alcoholics, 34 %, however the levels were not obtained from the same subjects but rather a
metanalysis was performed comparing incidence of levels in alcoholics to the incidence of ARBD observed in different studies (Hard et al., 2001).

Alcohol metabolism also leads to the production of free radicals, including ROS, that can lead to oxidative stress. Oxidative stress has been proposed as one of several mechanisms of alcohol-induced tissue injury, particularly in the brain and liver, and is believed to play a role in the pathogenesis of FAS (Cohen-Kerem and Koren, 2003). Davis et al. demonstrated that injury to neural crest cells, leading to the characteristic facial malformations observed in FAS, is likely the result of ethanol-induced free radical formation (Davis et al., 1990). In fact, the addition of the free radical scavenging enzyme, superoxide dismutase, to the culture medium significantly reversed the deleterious effects of ethanol (Davis et al., 1990; Fridovich, 1978). Low levels of CYP2E1 have been found in fetal brain to produce local ROS (Henderson et al., 1995; Montoliu et al., 1995; Ahmad et al., 1988). Oxidative stress produced by these local ROS can potentially lead to fetal brain damage (Cohen-Kerem and Koren, 2003). Free radicals and ROS readily interact with endogenous molecules leading to lipid peroxidation, cellular damage, and cell death (Sun et al., 1997). Also, exposure to ethanol alters the anti-oxidant defense system of the cell, making it more vulnerable to damage from ROS, and other free radicals (Bailey et al., 2001; Montoliu et al., 1995; Oh et al., 1998). Research is ongoing into ROS as an agent of fetotoxicity.

Furthermore, alcohol has been shown to inhibit normal cell development, function, and to alter cell properties. Alcohol can act directly by disrupting membrane fluidity and function, by altering lipid bilayer components (West et al., 1994; Chin and Goldstein, 1981; Chin and Goldstein, 1977; Beauge et al., 1987). The mechanism by which ethanol alters membrane fluidity is unknown but it has been hypothesized that it may act through weakening non-covalent
forces between membrane components (Hoek and Taraschi, 1988). Ethanol use during gestation has also been observed to inhibit normal neuronal cell-cell adhesion, and disrupt long-term potentiation, an important process for learning and memory in neuronal cells (Ramanathan et al., 1996; Sutherland et al., 1997). In addition, ethanol has been shown to inhibit fetal DNA methylation, resulting in hypomethylation, altering gene activity and function (West et al., 1994; Garro et al., 1991). Consequently, alcohol can act by a variety of mechanisms to induce cellular damage.

Alcohol use also results in an increase of prostaglandins in the fetal brain, which have been thought to play a role in the FAS pathogenesis (Pennington, 1988). Increased prostaglandins have been shown to lead to an increase in cyclic adenosine monophosphate levels, which can reduce the rate of cell division, and consequently interfere with stem cell division and neuronal cell proliferation (Nulman et al., 1998; Pastan et al., 1975). Studies in rats have demonstrated that when prostaglandin synthesis is inhibited by the co-administration of aspirin along with ethanol during pregnancy, that there are dramatic reductions in the incidence of malformations in offspring (Randall et al., 1991a; Randall et al., 1991b).

Alcoholism is also widely known to be associated with poor nutrition. Studies have suggested that deficiencies in vital elements and vitamins, such as zinc, calcium, retinoic acid, vitamin B6, and folate, can act synergistically to potentiate effects resulting from prenatal alcohol exposure (Nulman et al., 1998; Carter et al., 2007; West et al., 1994). Zinc is essential for synthesis of DNA, RNA, and various enzymes involved in fetal brain development; calcium is critical in modulating normal neuronal cell function; retinoic acid is important in mediating morphological development; vitamin B6 extensively influences brain development; folate is vital for synthesis
and methylation of DNA during development (West et al., 1994; Nulman et al., 1998). Deficiencies or imbalances in these vitamins and elements, and potentially others, likely contribute in the etiology of FASD. Furthermore, ethanol can also interfere with placental amino acid transport, potentially leading to fetal malnutrition, which may potentiate or increase vulnerability to toxic effects (Lin, 1981b; Lin, 1981a).

Other risk factors, other than poor nutrition, contributing to the etiology of FASD to consider are peak blood alcohol concentration (BAC), temporal vulnerability, genetic differences, and polydrug exposures (West et al., 1994). Peak BAC is discussed below in the “threshold” section. Temporal vulnerability was touched upon before, when it was discussed that early first-trimester exposures were required for morphological defects to occur, such as the characteristic facial anomalies (Sulik et al., 1981; Sulik and Johnston, 1983; Sulik, 1984; Sulik et al., 1986). Further neuronal loss, and alterations in brain circuitry can result from exposure well into the second and third trimester (West et al., 1994; West and Hamre, 1985; Barnes and Walker, 1981; Miller and Potempa, 1990). Polydrug exposure is discussed in the “Alcohol and other drug use in families involved with child protective services” section and chapter 6.

1.2.1.3 Threshold

There is a positive dose-response relationship between maternal BAC and fetal alcohol effects (Pierce and West, 1986), however the dose of alcohol required to impair normal fetal development is still under debate. Dose-response effects of fetal alcohol are difficult to study in humans, since alcohol is a potent teratogen and cannot ethically be administered to pregnant women. Jacobson and Jacobson, however, have examined results from two of the largest fetal alcohol exposed cohorts ever studied, and used multiple regression analysis to report threshold
levels for neurobehavioral effects and outcomes (Jacobson and Jacobson, 1994). Noteworthy is the fact that the reported threshold levels were not based on statistically significant effects, as the number of children exposed to high levels were too small, but rather as the authors point out, the threshold levels were based on the consistency of data across a large number of neurobehavioral outcomes (Jacobson and Jacobson, 1994).

The first cohort described by Jacobson and Jacobson was that studied by Streissguth and colleagues (Streissguth et al., 1984; Sampson et al., 1994; Baer et al., 2003), who followed approximately 500 children born to predominantly white, middle-class women in the mid-1970’s. The second cohort was that followed by Jacobson and colleagues, which included 480 predominantly economically disadvantaged, black children (Jacobson et al., 1993b; Jacobson et al., 1993a; Jacobson and Jacobson, 1994; Jacobson et al., 1994c; Jacobson et al., 1994b; Jacobson et al., 1994a).

Jacobson and Jacobson, concluded the threshold for neurobehavioural effects seems to be between 7 and 28 standard drinks per week, and that most often these drinks were consumed during fewer occasions, resulting in higher blood alcohol levels (Jacobson and Jacobson, 1994). However, the investigators did report that mental development in the fetus appears to be affected even with the smallest alcohol doses, and consequently that the dose-response pattern appears to be linear with no threshold for mental developmental deficits.

The results presented by Jacobson and Jacobson are only as sensitive and reflective as the measures used in the studies they reviewed to assess neurobehavioral and cognitive function. Consequently, unknown effects may result from exposures below the specified threshold levels. Furthermore, Jacobson and Jacobson state that structural changes in the brain can often be
detected before neurobehavioral deficits. Therefore even if deficits are not apparent, structural changes may still be present and may put children at a disadvantage later in life when, for example, they are stressed or after reaching old age (Jacobson and Jacobson, 1994). As a result, smaller doses can still be detrimental to the offspring in ways that are still not yet fully understood.

1.2.2 Diagnosis and Intervention

Current diagnostic criteria of FASD contain features described in studies from the 60’s and early 70’s. In the 60’s Lemoine described characteristic facial anomalies, substantial growth retardation, increased frequency of malformations, and psycho-motor anomalies in children born to alcoholic mothers, as hallmarks of FAS (Lemoine et al., 2003). In the 70’s, Jones and Smith described FAS as a specific pattern of malformation involving prenatal-onset of growth deficiency, developmental delay, craniofacial anomalies and limb and other birth defects observed in offspring of alcoholic mothers (Jones and Smith, 1975). These common features, including confirmation of maternal alcohol use, are often found in current diagnostic schemes.

Several diagnostic schemes for FASD have evolved based on advances in FASD research. In 1996, the Institute of Medicine of the National Academy of Sciences in the US (IOM) proposed six diagnostic categories: FAS with and without confirmed maternal alcohol exposure, PFAS with and without confirmed maternal alcohol exposure, ARBD, and ARND (Stratton et al., 1996). However, at the time the definitions for diagnostic criteria remained vague and somewhat ambiguous (Hoyme et al., 2005; Manning and Hoyme, 2008).
In 2000 Astley and Clarren described a 4-Digit Diagnostic Coding system based on the medical/research records of 1014 patients of Washington’s FAS Diagnostic and Prevention Network (FAS DPN) (Astley and Clarren, 2000). The code is based on the assessment of four domains: 1) growth deficiency, 2) FAS facial phenotype, 3) CNS damage or dysfunction, and 4) gestational exposure to alcohol. The assessment is structured such that the clinician ranks severity of the four domains using a Likert scale from 1 to 4, 1 representing the least severe to 4 representing the most severe. The case definitions for ranking are clearly defined in Astely et al, 2000. The 4-Digit Diagnostic code can result in 256 possible 4-Digit combinations that are collapsed into 22 diagnostic categories. As presented in table 1.1, only 9 unique diagnostic “outcome” categories exist that are differentiated by differences in alcohol exposure (alcohol exposed, alcohol exposure unknown, no alcohol exposure) into 22 actual diagnostic categories (labeled A-V).
Table 1.1 Summary of 22 diagnostic categories (A-V), based on 4-Digit Diagnostic Code

<table>
<thead>
<tr>
<th>Diagnostic Outcome Categories</th>
<th>Alcohol Exposed</th>
<th>Alcohol Exposure Unknown</th>
<th>No Alcohol Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal Alcohol Syndrome</td>
<td>A</td>
<td>B</td>
<td>n/a</td>
</tr>
<tr>
<td>Atypical Fetal Alcohol Syndrome</td>
<td>C</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Fetal Alcohol Syndrome Phenocopy</td>
<td>n/a</td>
<td>n/a</td>
<td>D</td>
</tr>
<tr>
<td>Sentinel physical findings/static encephalopathy</td>
<td>E</td>
<td>K</td>
<td>Q</td>
</tr>
<tr>
<td>Static encephalopathy</td>
<td>F</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>Sentinel physical findings/neurobehavioural disorder</td>
<td>G</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>Neurobehavioural disorder</td>
<td>H</td>
<td>N</td>
<td>T</td>
</tr>
<tr>
<td>Sentinel physical findings</td>
<td>I</td>
<td>O</td>
<td>U</td>
</tr>
<tr>
<td>No cognitive/behavioural or sentinel physical findings detected</td>
<td>J</td>
<td>P</td>
<td>V</td>
</tr>
</tbody>
</table>

In 2005, the diagnostic categories proposed by the IOM (FAS with and without confirmed maternal alcohol exposure, PFAS with and without confirmed maternal alcohol exposure, ARBD, and ARND) were updated to include less ambiguous definitions of the diagnostic criteria (Table 1.2) (Hoyme et al., 2005; Manning and Hoyme, 2008). In 2005, Chudley and colleagues published the Canadian guidelines for diagnosis of FASD which combined the IOM diagnostic criteria with the 4-Digit code approach (Table 1.3) (Chudley et al., 2005). The Canadian guidelines emphasize the presence of CNS dysfunction in FASD diagnosis over the IOM guidelines, which only require the presence of structural brain anomalies or smaller head circumference, in place of CNS dysfunction, for diagnosis of FAS, PFAS, or ARND. Chudley et al. believe that diagnosis of PFAS under the IOM guidelines could be harmful for an individual, as the diagnosis implies brain dysfunction that may not be present, yet diagnosis may still
stigmatize the child. Another difference between the IOM and Canadian diagnostic guidelines is that a diagnosis of PFAS under the Canadian diagnostic guidelines cannot be made without confirmation of maternal alcohol exposure, as can be made according to the IOM guidelines.

Table 1.2 Summary of IOM diagnostic criteria (adapted from Manning and Hoyme, 2008)

<table>
<thead>
<tr>
<th></th>
<th>FAS*</th>
<th>FAS**</th>
<th>PFAS*</th>
<th>PFAS**</th>
<th>ARBD</th>
<th>ARND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmation of maternal alcohol exposure</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Evidence of characteristic pattern of facial anomalies, at least 2 (Short palpebral fissures, thin upper lip, smooth philtrum)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evidence of growth retardation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evidence of deficient brain growth or abnormality (Structural brain abnormality or small head circumference)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Evidence of a complex pattern of behavioural or cognitive abnormalities that cannot be otherwise explained</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congenital structural defects in specified categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ Indicates required criterion

✓ Indicates required criterion if no other same marked criterion are present

*With confirmed maternal alcohol exposure

**Without confirmed alcohol exposure
Table 1.3 Summary of the Canadian diagnostic guidelines (adapted from Chudley et al. 2005)

<table>
<thead>
<tr>
<th></th>
<th>FAS*</th>
<th>FAS**</th>
<th>PFAS</th>
<th>ARBD</th>
<th>ARND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmation of maternal alcohol exposure</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Evidence of characteristic pattern of facial anomalies, at least 3 (Short palpebral fissures, thin upper lip, smooth philtrum)</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evidence of characteristic pattern of facial anomalies, at least 2 (Short palpebral fissures, thin upper lip, smooth philtrum)</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evidence of growth retardation</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evidence of impairment in 3 or more CNS Domains</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Congenital structural defects in specified categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ Indicates required criterion

*With confirmed maternal alcohol exposure

**Without confirmed alcohol exposure

Regardless of the diagnostic schemes used, criteria for FASD diagnoses overlap and have the commonalities of growth retardation, CNS dysfunction and or structural anomalies, characteristic facial malformations, and behavioural or cognitive abnormalities. Importantly, all diagnostic schemes mentioned above also require confirmation of maternal alcohol exposure for diagnosis in the absence of characteristic facial malformations. These facial anomalies are not always present, as may be the case in children with ARND or FAE, for example. In fact, children with ARND/FAE greatly outnumber children with FAS who have the full constellation of facial characteristics. Therefore, having a reliable biomarker that can independently and retrospectively
assess chronic alcohol use during pregnancy would be a tremendous advantage in the diagnosis of FASD and is the subject of this thesis.

1.2.3 The Silent Epidemic and Gap Towards Intervention

Nearly half of North American women in childbearing age drink alcohol, and since approximately half of all pregnancies are unplanned, the potential number of alcohol-exposed fetuses is staggeringly high. It has been estimated that approximately 40% of heavily exposed fetuses will be affected by FASD, and the reason why some fetuses remain unaffected is still unknown (Jones and Smith, 1975). Nevertheless, the prevalence of FAS and FASD among developed nations has been estimated to be between 1 and 10 per 1000 births (Sampson et al., 1997; Williams et al., 1999; Abel, 1995a). This figure is of epidemic proportions yet is largely unknown by the general public. Furthermore, the prevalence is likely underestimated because many affected individuals are misdiagnosed or never diagnosed, particularly those missing characteristic facial malformations (Williams et al., 1999; Fast et al., 1999; Olson et al., 2007).

Equally astounding, are the estimates of FAS prevalence from developing countries. In a recent study conducted in South Africa, Viljoen et al. reports an alarming rate of 65.2 – 74.2 cases of FAS per 1000 children in the first grade population (Viljoen et al., 2005)! This estimate was based on actively screened students, therefore cases that might otherwise be missed were captured, nevertheless this figure is clearly indicative of a silent FASD epidemic.

The hidden cost of FASD in economic burden reaches epidemic proportions as well. Expenses are generated through medical, educational, societal, and family costs, as well as costs associated
with externalizing behaviours (for example, criminal proceedings, incarceration, etc.) and productivity loses. Estimates of the annual economic burden due to FAS/FASD have ranged from $74.6 million to $9.7 billion in the US (Abel and Sokol, 1987; Abel and Sokol, 1991; Rice et al., 1991; Harwood and Napolitano, 1985) and up to $344 million in Canada (Stade et al., 2006). Yet the greatest cost that cannot be measured is that of the human suffering of the affected individual, family, and caretakers, generated by this tragic and preventable condition.

The origin of burden of FASD can be understood when its behavioural phenotype is described. Kodituwakku stated that, “children with prenatal alcohol exposure exhibit significant deficits in daily functional skills or adaptive behaviour, with deficits in socialization becoming pronounced during adolescence”, and concluded that, “FASD can be defined as a generalized deficit in processing complex information. Diminished intellectual functioning, slow information processing, and relative difficulty with complex tasks are all consistent with a generalized deficit model” (Kodituwakku, 2008). Children with FASD are at a tremendous disadvantage because of their brain-based dysfunction, as they often show maladaptive behaviour that creates conflict between themselves, peers, caretakers, and authoritative figures. As such, it remains critical they
receive early diagnosis in order to gain access to importance resources and sources of intervention.

Streissguth and colleagues describe the tragic circumstances these children are often surrounded by, and the tremendous importance that rearing environment and diagnosis play in their behaviour and prognosis. In a landmark study evaluating the adverse life outcomes and risk factors of 415 patients referred to clinic for FAS/FAE\(^2\) diagnosis, Streissguth et al. reported that 80 % of the patients were not raised by their biological mothers (Streissguth \textit{et al.}, 2004). Out of the adolescents and adults, lifespan prevalence was reported to be 61% for disrupted school experience, 60 % for trouble with the law, 50 % for confinement (in detention, jail, prison, or a psychiatric or alcohol/drug inpatient setting), 49 % for inappropriate sexual behaviour on repeated occasions, and 35 % for alcohol/drug problems. Most importantly, the authors found that the odds of escaping these adverse life outcomes were 2 to 4 fold greater by receiving an early diagnosis of FAS or FAE and by being reared in a good stable environment.

\(^2\) Fetal Alcohol affected (FAE) individuals are those that have heavy prenatal alcohol exposure but do not necessarily manifest the full physical features of FAS (Streissguth \textit{et al.}, 2004).
Equally critical, the study revealed that patients with FAE had higher rates of all five adverse life outcomes compared to those with FAS, and the odds were doubled for them having trouble with the law and alcohol/drug problems. This is an important point as individuals with FAE are often missing characteristic facial features, as mentioned, and are consequently harder to identify. The authors also mention that this result could be because FAE is often treated as a “non-diagnosis”, therefore resulting in these children not receiving adequate attention and resources.

It should be emphasized that the 415 patients in this cohort were referred patients for FAS/FAE diagnosis, therefore they may have been referred for reasons related to adverse life outcomes and therefore are not necessarily representative of all fetal alcohol affected children in general. Nevertheless, the study clearly underscores the fact that early intervention\(^3\), facilitated by early diagnosis, is vital for the child’s success, and helps avoid or mitigate secondary disabilities such as the adverse life outcomes mentioned.

Unfortunately however, there is a gap between the silent FASD epidemic and intervention, and this gap is the failure to diagnose the condition early. Most affected individuals are not diagnosed

\(^3\) For a review on interventions available for FASD please see Kalberg and Buckley, 2007 (Kalberg and Buckley, 2007).
unless actively screened. In an epidemiological study examining hospital records for all live births in 1994 in a regional hospital of northeastern Manitoba (n = 745), Canada, infants approximately 2 years of age were followed up and screened for FAS. It was discovered that only 20 % of FAS cases had been previously identified and diagnosed, therefore 80 % had been missed (Williams et al., 1999). Similarly, in a study investigating the prevalence of FAS/FAE in youth who committed criminal offences and were remanded for psychiatric assessment (n = 287), 67 were discovered to have FAS/FAE, 95.5 % of which were previously undiagnosed (Fast et al., 1999).

If diagnosed, most children are typically not diagnosed until late childhood, missing the opportunity for early intervention. According to the largest clinical database of children born with prenatal alcohol exposure (n = 781), Washington state’s Fetal Alcohol Syndrome’s Diagnostic and Prevention Network (FAS DPN), the average age of referral for FASD diagnosis is 9.5 years, and only about a third of diagnosed children are diagnosed before age 4 (Olson et al., 2007). The most likely reason for the failure to diagnose FASD is the fact that many affected children do not display the characteristic facial anomalies and that in these cases, as in most,

4 The CDC estimates that FASD other than FAS occurs approximately three times more often than FAS (CDC, 2006).
mothers are reluctant to provide confirmation of maternal alcohol abuse during pregnancy, a necessary diagnostic criterion. Therefore, a biomarker assay that can independently confirm maternal alcohol use during pregnancy would provide a tremendous advantage in facilitating diagnosis of FASD and bridging the gap between this silent epidemic and intervention.

1.3 Biomarkers for Alcohol Exposure in Blood and Urine

Alcohol is rapidly metabolized in the body, and can only be measured for several hours following ingestion. Therefore, biomarkers for post-acute ingestion or chronic intake have been developed, many of which are already measured on a routine basis in hospitals. Presented here are the following biomarkers, measured in blood/urine: hemoglobin-associated acetaldehyde (HAA), gamma-glutamyltransferase (γGT), mean corpuscular volume (MCV), aspartate/alanine aminotransferase (AST/ALT), and carbohydrate-deficient transferrin (CDT). However, the majority of these markers either offer low sensitivity and or specificity, and have other limitations associated with the detection of alcohol consumption during pregnancy (Littner and Bearer, 2007; Cook, 2003).
1.3.1 Hemoglobin-Associated Acetaldehyde

Oxygenated hemoglobin can react with acetaldehyde, a main product of alcohol metabolism, to form reversible and irreversible HAA (Stevens et al., 1981; Wickramasinghe et al., 1994). Reversible HAA can be detected up to 48 hours after the last drink, whereas irreversible HAA accumulates and can be detected for at least 28 days post-ingestion of alcohol (Cook, 2003; Peterson et al., 1988). HAA has been reported to have higher clinical sensitivity and specificity than γGT, MCV, and AST/ALT (Cook, 2003).

Hazelett et al. reported sensitivity and specificity for HAA were 67 and 77 %, respectively, in distinguishing patients in alcohol and drug treatment that reported consuming more than 6 drinks per day from those reporting less consumption (n = 182) (Hazelett et al., 1998). Another study evaluated HAA levels in pregnant women (n = 19) visiting an outpatient department for pregnant alcohol abusers, and in non-pregnant (n = 14) controls (social users and abstainers) (Niemela et al., 1991). The study revealed HAA levels were elevated in 63 % of women who delivered babies with FAE (8 in total), and in 28 % of drinkers who delivered healthy babies, indicating that HAA may be useful as a marker for FAE (Niemela et al., 1991). However, the sensitivity of HAA reported in this study is less than desirable, therefore using HAA as a marker for FAE would mean many cases would be missed. Furthermore, the window for detection of alcohol abuse, should the mother decide to quit drinking, would be limited to about a month post-ingestion, and sensitivity would likely decrease with time elapsed since the last drinking episode. Therefore, although HAA can be a useful aid, it is an inadequate biomarker for fetal alcohol exposure on its own.
1.3.2 Glutamyltransferase

γGT is an enzyme produced mainly in the liver and involved in glutathione metabolism and renal reabsorption of amino acids, and has been used as a marker for alcohol use and liver disease for many years (Whitfield, 2001; Rosalki et al., 1970). Although γGT has been used as a biomarker for alcoholism it is not very sensitive, on average detecting 30 – 50% of excessive drinkers in the general community/family practice settings, and in some cases even less than 10% (reviewed by Conigrave et al., 2003). Also, γGT is not a specific biomarker for alcoholism, as it is a marker of liver disease that may or may not be alcohol induced. Concentrations of γGT can increase with the use of certain medications as well, such as anticonvulsants and non-steroidal anti-inflammatory drugs, making results even more difficult to interpret (Conigrave et al., 2003). In a study investigating γGT and MCV in pregnant women referred to clinic for substance abuse, Sarkola et al. reported sensitivity (n = 41) of γGT in detecting heavy drinking and FAE, 31% and 50%, respectively, specificity, 79% and 81%, respectively, and low positive predictive value (PPV), 40% and 40%, respectively (Sarkola et al., 2000). Although the sensitivity of γGT for detecting FAE (50%) may appear high, it should be kept in mind that this figure was derived from a high-risk cohort where 30% of women admitted to heavy alcohol use, and all women were referred to clinic based on suspicions of substance abuse. Therefore, the figure is not likely representative of the general obstetric population. Stoler et al. showed drastically lower sensitivity and PPV of γGT in a much larger cohort of pregnant women obtained from the general population (total n = 529), with less than 1% of drinkers testing positive for γGT, and less than 1% of those testing positive for γGT actually being a true positive (alcohol user) (Stoler et al., 1998). Furthermore, γGT levels are typically decreased in pregnancy (Littner and Bearer, 2007), and γGT has been
found to be of limited value in people less than 30 years of age due to lowered sensitivity and specificity (Nystrom et al., 1993; Conigrave et al., 2003); therefore \( \gamma \)GT, although perhaps useful in conjunction with other biomarkers, is less than optimal for the detection of alcohol exposure during pregnancy.

1.3.3 Mean Corpuscular Volume

MCV is the average estimate of red blood cell size. It can be used as an indicator of macrocytosis of erythrocytes, a common finding in alcoholics drinking more than 80 g of ethanol daily (Wu et al., 1974). The cause of macrocytosis is complex and believed to be caused by the direct toxic effects of alcohol on bone marrow precursors (Larkin and Watson-Williams, 1984). In addition to alcohol use, elevated MCV levels can also result from folate or vitamin B\(_{12}\) deficiencies, bleeding, haematological conditions, bone marrow disorders, hyperthyroidism, and hyperglycemia, consequently elevated levels of MCV are not specific to alcohol exposure (Conigrave et al., 2003). Furthermore, sustained, regular drinking is needed to produce elevated levels of MCV, and because the life span of red blood cells is approximately 120 days, it may take several months for MCV levels to reflect drinking habits (Hasselblatt et al., 2001). In general, MCV has had poor sensitivity in the detection of heavy alcohol users, with sensitivities ranging from less than 20 % to 50 % (Littner and Bearer, 2007; Conigrave et al., 2003; Stoler et al., 1998; Meerkerk et al., 1999). Sarkola et al., reported sensitivity of MCV in pregnant women (\( n = 44 \)) for detecting heavy drinking and fetal alcohol effects (FAE), as 15 % and 25 %, respectively, specificity as 100 % and 100 %, respectively, and positive PPV as 100 % and 100 %, respectively (Sarkola et al., 2000). It should be noted that the sample size of the study was
limited, with only 2 cases testing positive for MCV, nevertheless, the high specificity and PPV, suggest that MCV may be useful in conjunction with other biomarkers, but clearly lacks the sensitivity for use alone. MCV likewise has shown to have poor sensitivity in subjects under 30 years of age (Nystrom et al., 1993). Therefore, it is not recommended MCV be used as a biomarker for alcohol consumption during pregnancy on its own.

1.3.4 Aspartate/Alanine Aminotransferase

AST and ALT are liver enzymes that can become elevated following liver injury, and have consequently been used as biomarkers for heavy alcohol consumption (Littner and Bearer, 2007; Pratt and Kaplan, 2000; Conigrave et al., 2003). However, because they serve as indicators of liver disease, they are not specific to alcohol use alone. In a study examining levels of γGT, MCV, AST, ALT, and AST/ALT ratio, in the blood of 25 pregnant women abusing alcohol, relative to 20 abstinent pregnant controls, AST/ALT ratio along with MCV and urinary dolichol were found to be poor indicators of alcohol abuse (Halmesmaki et al., 1992). Also, AST and ALT markers are not as accurate in patients under 30 (Halvorson et al., 1993), which would likely result in the exclusion of detecting many alcohol exposed pregnancies. Consequently, AST/ALT are not suitable for the detection of fetal alcohol exposure.
1.3.5 Carbohydrate-Deficient Transferrin

Alcohol ingestion is believed to interfere with the normal glycosylation process that occurs during the formation of transferrin, the body’s major iron transfer protein (Cook, 2003). Therefore, CDT is a modified form of transferrin that results from alcohol exposure. Compared to the HAA, γGT, MCV, and AST/ALT, CDT is the most sensitive and specific biomarker for heavy alcohol use (Cook, 2003; Littner and Bearer, 2007; Meerkerk et al., 1999). For alcoholism, its sensitivity ranges from 60 to 91% and specificity from 92 to 100% (Reynaud et al., 1998). However, its use a biomarker in women, particularly in pregnancy, remains somewhat controversial as levels typically increase with pregnancy status, are higher in women, and can vary with hormonal and iron status (Cook, 2003; Littner and Bearer, 2007; Sillanaukee et al., 2000). Therefore, its use in women is not ideal. Also, CDT has a half-life of 17 ± 4 days (Reynaud et al., 1998), which means the window for detection of alcohol abuse is limited. Consequently, it is doubtful that CDT will prove to be a useful marker for fetal alcohol exposure.

1.4 Fatty Acid Ethyl Esters (FAEE)

1.4.1 Production and Accumulation

FAEE are non-oxidative metabolites of ethanol metabolism. They are produced throughout the body in pancreas, liver, heart, brain, white blood cells, and adipose tissue (Laposata and Lange, 1986; Laposata et al., 1987; Ben-Eliyahu et al., 1996). FAEE have been detected in hair,
meconium, blood, adipose tissue and organs affected by alcohol abuse (liver, pancreas, heart, brain) (Laposata and Lange, 1986; Doyle et al., 1994; Doyle et al., 1996; Klein et al., 1999; Klein et al., 2002; Pragst et al., 2001; Wurst et al., 2004; Auwarter et al., 2001). FAEE can be formed spontaneously by the conjugation of ethanol to endogenous free fatty acids, however the reaction is most often catalysed by enzymes such as FAEE synthase, microsomal acyl-coA:ethanol O-acyltransferase (AEAT), carboxylesterase, lipoprotein lipase, cholesterol esterase, or triglyceride lipase (Figure 1.3) (Laposata, 1998b; Laposata and Lange, 1986; Best and Laposata, 2003). These enzymes can also facilitate the breakdown of fatty acyl-CoA, triglycerides, and phospholipids to conjugate liberated fatty acids with ethanol (Best and Laposata, 2003). Non-oxidative metabolism of ethanol appears to be inversely proportional to oxidative metabolism. Studies have demonstrated that when oxidative metabolism is down-regulated experimentally, non-oxidative metabolism increases and consequently results in greater formation of FAEE (Best et al., 2006; Zakhari, 2006; Werner et al., 2002).

FAEE accumulate preferentially in adipose tissue and hair, over other tissues. FAEE enter into adipose tissue from the general circulation where they have a prolonged elimination half-life of 16 hours (four-fold greater than that of alcohol), therefore they can be measured for a longer time following exposure (Laposata et al., 1989). In hair, FAEE are believed to be deposited through sebum. FAEE are incorporated and accumulate in hair indefinitely with minimal loss (Caprara et al., 2004). In fact our laboratory was able to detect measurable levels of FAEE still present in the hair of mummies that were over 750-1000 years old (Caprara et al., 2004). (Effects of weathering and hair treatment are discussed in the “effect of cosmetic treatment and hair care section).
1.4.2 Toxicity of FAEE

Early lines of FAEE research, preceding the investigation of FAEE as a biomarker of alcohol exposure, were involved in the discovery and examination of FAEE as toxic mediators of ethanol-induced damage. FAEE were first discovered in 1963 in total body lipid extracts obtained from rats given radioactively-labeled ethanol (Goodman and Deykin, 1963). Since then several studies reported their discovery in different *in vitro* and *in vivo* systems as well (Newsome and Rattray, 1966; Patton and McCarthy, 1966; Grigor and Bell, Jr., 1973; Johnson *et al.*, 1976). (See Laposata, 1998a for review.) However, it was not until FAEE were discovered in rabbit myocardial tissue (Lange *et al.*, 1981) and in organs most commonly damaged by alcohol, that FAEE were hypothesized to potentially play a role in the toxicity of alcohol-induced organ damage (Lange, 1982; Laposata and Lange, 1986).

Laposata et al. examined FAEE levels in 175 organs from 20 randomly sampled subjects (acutely intoxicated individuals at the time of death, chronic alcoholics, and control subjects) post-mortem. Acutely intoxicated subjects had significantly higher levels of FAEE in organs most commonly damaged by alcohol abuse (pancreas, liver, heart, and brain) compared to controls, and little or no FAEE were detected in organs not damaged by alcohol (Laposata and Lange, 1986). Consequently, FAEE were suspected to play a role in alcohol-induced organ damage.

Indeed there has been evidence to show FAEE to be toxic in heart, liver, and pancreatic tissue. FAEE and FAEE synthase have been documented in human myocardium (Bora *et al.*, 1989), and
myocardial cell damage, and mitochondrial cell dysfunction, has been observed in vitro, and in vivo, in rats injected into the myocardium with ethyl oleate (Bora et al., 1996). FAEE may also be a causative agent in alcohol-induced liver damage; Szczepiorkowski et al. showed that incubation of human hepatoblastoma cells with LDL reconstituted with FAEE decreased cell proliferation and protein synthesis (Szczepiorkowski et al., 1995). Likewise, FAEE may be responsible for alcohol-induced pancreatic damage; Werner et al. showed that rats injected with FAEE developed pancreatic edema, trypsinogen activation, and vacuolization of acinar cells (Werner et al., 1997). In a subsequent study, the group demonstrated that when rats were given ethanol, pancreatic injury was dose-dependant corresponding with FAEE levels, and as oxidative metabolism was inhibited (leading to the greater production of FAEE) pancreatic injury worsened (Werner et al., 2002). Therefore it appears there is evidence to suggest FAEE are toxic and may be mediators of ethanol-induced organ injury.

Laposata et al., also documented non-oxidative alcohol metabolism in the human brain. The group discovered the presence of FAEE synthase in 10 different locations in the human brain, which actively produced FAEE when homogenates were incubated with ethanol (Laposata et al., 1987). FAEE were also found in the brains of individuals who died while intoxicated (n = 5), at significantly higher levels than in controls (n = 5), who had very low or undetectable levels (Laposata et al., 1987). Furthermore, Isenberg et al., using cultured human and rodent neuroblastoma and glioma cell lines, determined that cells with neuronal properties, as opposed to glioma cell lines, contained the highest FAEE synthase activities (Isenberg et al., 1992; Bora and Lange, 1993). Therefore, it is possible that FAEE may be toxic mediators of CNS damage as well.
Moreover, there has been evidence to suggest that FAEE may play a role in alcohol teratogenicity (FAS). Bearer et al. reported significant activity of FAEE synthases in human and mouse placenta, and that mouse heart, liver, placenta, and fetal tissue accumulate significant amounts of FAEE after maternal ethanol consumption (Bearer et al., 1992). Hungund and Gokhale, similarly reported significant accumulation of FAEE in fetal and maternal organs of rats following exposure to ethanol during pregnancy (Hungund and Gokhale, 1994). In the guinea pig, FAEE levels measured in meconium have been found to inversely correlate with fetal body and brain weight (Brien et al., 2006). Likewise, several human studies have shown significant correlations between FAEE levels in meconium and various neonatal outcomes (Noland et al., 2003; Derauf et al., 2003; Chan et al., 2004b; Jacobson, 2006; Hutson et al., 2007; Peterson et al., 2008). Specifically, FAEE levels in meconium have been found to be inversely correlated with birth weight (Noland et al., 2003; Derauf et al., 2003; Chan et al., 2004b; Hutson et al., 2007), head circumference (Chan et al., 2004b; Noland et al., 2003), APGAR score (Derauf et al., 2003), executive functioning (Noland et al., 2003), mental and psychomotor development (Peterson et al., 2008), recognition memory (Jacobson, 2006), processing speed (Jacobson, 2006), and complexity of symbolic play (Jacobson, 2006). (See Koren et al., 2008 for review). Elevated levels of ethyl oleate in meconium have also been measured in children given a diagnosis of FAS or PFAS (Jacobson, 2006).

Although many of the studies mentioned above suggest FAEE are toxic, and may be mediators of ethanol-induced organ injury, and perhaps teratogenicity, many of the studies are associational and do not prove causation. The mechanisms by which FAEE may induce cytotoxicity are still under study. Lange et al. showed that FAEE can disrupt mitochondrial function by uncoupling oxidative phosphorylation in rabbit myocardium (Lange and Sobel, 1983). Hungund et al. found
that FAEE altered membrane anisotropy in mice brain, therefore FAEE disordered membranes (Hungund and Gokhale, 1994). Haber et al. reported that FAEE increase the fragility of isolated pancreatic lysosomes in rat pancreas, perhaps by a similar process (Haber et al., 1993). Therefore, the mechanisms of FAEE toxicity remain to be elucidated, but so far appear to act by interfering with mitochondrial metabolism, and altering membrane structure and or function.

1.4.3 Use as a Biomarker

Numerous studies have investigated the use of FAEE as a biomarker for alcohol and alcoholism in blood, tissue, meconium, and hair (Laposata et al., 1989; Doyle et al., 1996; Bearer et al., 2005; Bearer et al., 2003; Bearer et al., 2003; Bearer et al., 1999; Chan et al., 2004a; Chan et al., 2003; Caprara et al., 2005b; Klein et al., 2002; Klein et al., 1999; Pragst et al., 2001; Pragst and Balikova, 2006). However, FAEE as a biomarker for fetal alcohol exposure will be discussed in the section entitled “biomarkers for fetal alcohol”.

1.4.3.1 Blood and Tissue

Early studies on FAEE as a biomarker focused on blood and tissue. Following the discovery of FAEE in human organs most commonly damaged by alcohol (Laposata and Lange, 1986), Doyle et al. investigated whether or not FAEE would be detectable in serum after ethanol ingestion (Doyle et al., 1994). FAEE were found to be detectable in blood for 24 hours post ethanol ingestion, with elimination appearing biphasic. The initial elimination decay curve resembled that of ethanol, where FAEE had an elimination half-life of 173 minutes, but was followed by a secondary slower terminal phase with a terminal half-life of 693 minutes (Doyle et al., 1996). It
is possible that this secondary phase was a result of redistribution into and out of adipose tissue. As mentioned, FAEE accumulate in fat and have served as post-mortem markers of alcohol intake of chronic alcoholics when blood alcohol was undetectable at the time of autopsy (Laposata and Lange, 1986). Furthermore, human and animal studies demonstrated that FAEE in adipose and liver tissue can be measured at least 12 hours after death (Salem et al., 2001; Refaai et al., 2002). Therefore, FAEE measured in blood and tissue can serve as long-term markers for alcohol intake relative to the measurement of blood alcohol. However, these tools are still limited to hours post-ingestion as opposed to weeks or months, and they are invasive.

1.4.3.2 FAEE in Hair

The advent of measuring FAEE in hair has resulted in a revolutionary non-invasive technique that can uniquely assess chronic alcohol exposure retrospectively days to months post-ingestion. In 2001, Pragst and colleagues established the FAEE hair test that measures cumulative levels of ethyl myristate, palmitate, oleate, and stearate (Pragst et al., 2001). The test has been shown to be a highly sensitive and specific biomarker for chronic excessive alcohol use in adults (Pragst and Balikova, 2006; Wurst et al., 2004). Since the introduction of the FAEE hair test, it has primarily been used in the medical context of assessing alcohol abuse, and the medicolegal context of assessing alcohol use in driving impairment cases (Wurst et al., 2008b).

Low baseline levels of FAEE can exist in non-drinkers, therefore it has been necessary to establish a cut-off level in order to differentiate heavy drinkers from social and non-drinkers. A cut-off level of 0.5 ng of cumulative FAEE per mg hair has been found to be 90% sensitive and specific in the detection of heavy alcohol use (Pragst and Balikova, 2006). Hair FAEE levels between 0.2-0.5 ng/mg have been found to be indicative of social use, typically excluding strict
abstainers, whereas levels in excess of 1.0 ng/mg are nearly 100% specific to heavy alcohol use, but offer lower sensitivity (~75%) than the 0.5 ng/mg cut-off level, consequently many cases may be missed using 1.0 ng/mg (Pragst et al., 2001; Auwarter et al., 2001; Pragst and Balikova, 2006; Pragst and Yegles, 2008). Therefore, 0.5 ng/mg is the level currently used clinically as the cut-off level for heavy alcohol use in both the Mortherisk laboratory in Toronto, Canada, and the Pragst laboratory in Berlin, Germany (the only two laboratories currently measuring FAEE in hair worldwide).

Heavy or excessive alcohol use, as determined by positive FAEE test, has not been explicitly defined. It is an amount that is consistent with that typically reported by alcoholics. More specifically, the studies upon which the cut-off level has been established, define heavy drinkers as alcoholic patients in treatment programs and report consumption between 50-400 g ethanol/day (Auwarter et al., 2004; Auwarter et al., 2001), and or 960-7600 g ethanol in the last month, mean 4440 g (representative of the last 6 months) (Wurst et al., 2004). A standard drink (SD) according to the Canadian low-risk drinking guidelines and the United States’ National Institute on Alcohol Abuse and Addiction’s (NIAAA) is equal to approximately 14 grams of alcohol (Bondy et al., 1999; National Institute on Alcohol Abuse and Alcoholism, 2005). Therefore, if converted into North American standard drinks (SD), heavy drinkers, or excessive alcohol users, according to the 0.5 ng/mg cut-off, are defined as people who consume between approximately 4 - 29 drinks a day, or 70 - 558 drinks (mean 326 drinks) a month.

Despite the test’s many other advantages, FAEE in hair are not currently analyzed by segmental analysis in order to reflect changes in alcohol use patterns retrospectively, as is done with other drugs of abuse. Since it appears that FAEE are primarily deposited into hair by sebum, they have
been found to accumulate within the proximal 5-10 cm of hair, and then to decrease to a plateau afterwards, irrespective of dose; therefore segmental analysis may not be possible (Auwarter et al., 2001); more research is necessary. However, it is possible to confirm a current period of prolonged abstinence if FAEE levels consistent with heavy alcohol exposure are not found in newly grown segments since abstinence commenced.

1.4.3.3 Effect of Cosmetic Treatment and Hair Care

FAEE in hair can be affected by cosmetic treatments and hair care but for the most part only to a minor extent. Research shows that these effects are not likely to be of clinical significance.

Hartwig et al. conducted an extensive study on the effect of hair cosmetics and hair care on FAEE concentrations (Hartwig et al., 2003). FAEE in hair appear to be resistant to the effect of shampooing; no correlation was found between FAEE levels in the hair of 75 individuals in an alcohol treatment program and the frequency of shampooing. Also, no discernable decrease in FAEE could be found following 20 shampoos during a systematic experiment. Shading, bleaching, and perming did decrease internal FAEE concentrations in the hair matrix between 8 to 12 %, and dyeing by 64 %. However, no remarkable differences were observed between alcoholic hair samples that were previously dyed from those not previously dyed, nor was there a clear decrease in FAEE concentration between distal hair segments that were previously bleached from proximal segments that were not bleached within the same patients. Therefore, despite the fact that FAEE may be hydrolyzed by dying, or washed out by bleaching, it appears that these effects do not appear to be of clinical significance. With continued alcohol consumption, FAEE are re-deposited by sebum afterwards, and chronic alcohol users will still likely test positive with high levels of FAEE in their hair.
Forty-nine hair care products were tested for the presence of FAEE and all were found to detect trace amounts, with the highest concentration amounting to approximately 0.003% of the total product ingredients. However, it was shown that when the hair of an alcoholic was incubated with a hair wax containing 10.4 ng/mg of FAEE (the most highly concentrated product), the FAEE were not able to penetrate the hair matrix but rather internal FAEE were extracted from the hair by the wax. Therefore, the use of these products is not likely to generate false positive results. However, the group did discover that the regular use of hair lotions, deodorants, and sprays, containing over 62.5% ethanol were capable of producing false positive FAEE tests of over 1.0 ng/mg in hair. This also suggests, as the authors point out, that FAEE can be produced at the level of the sebaceous gland when it is exposed to alcohol. However, the ratio of the four esters produced from exogenous sources such as deodorants differed than that typically measured, with an unusually low portion of ethyl oleate and high portion of ethyl stearate, making false positives more easily distinguishable (Hartwig et al., 2003). Nevertheless, caution should be applied, and positive results should be interpreted within the context of other clinical evidence of alcohol use.

In addition, Hartwig et al. also found that storing samples in close proximity to open containers of ethanol resulted in dramatic increases of FAEE concentration in hair leading to false positive results. However, good laboratory practices should avoid misidentification of individuals resulting from incorrect storage practices.
1.5 Ethyl Glucuronide (EtG) and FAEE

EtG has been measured in hair and used as a biomarker for alcohol exposure much like FAEE. For this reason it is now briefly discussed, followed by a brief discussion of the combined use of EtG and FAEE in hair.

Less than 0.06% of alcohol entering the body is glucuronidated with activated glucuronic acid (UDP-GA) to produce ethyl glucuronide (EtG) (Figure 1.3) (Pragst and Yegles, 2007). Glucuronidation of alcohol was first described by Neubauer in 1901, and has since been investigated as both a short and long-term marker of alcohol use (Neubauer, 1901). EtG is a minor metabolite of alcohol metabolism formed primarily in the endoplasmic reticulum of liver cells, and to a minor extent in the intestinal mucosa of the lung (Pragst and Yegles, 2007).

In urine, EtG has served as an excellent short-term marker of alcohol exposure. It can be detected up to 80 hours following heavy alcohol use (Wurst et al., 2000; Wurst et al., 2003). Similarly, EtG can be measured in serum up to 8 hours following complete elimination of ethanol (Schmitt et al., 1997). The pharmacokinetic profile of EtG in blood has been characterized and its terminal half-life is between 2 and 3 hours (Droenner et al., 2002; Schmitt et al., 1997). EtG has also been detected in several tissues and bodily fluids suitable for post-mortem analysis, such as muscle, fat, liver, bile, bone marrow, and hair, with highest concentrations found in liver and bile (Schloegl et al., 2006).
Several studies have investigated the measurement of EtG in hair (Skopp et al., 2000; Alt et al., 2000; Janda et al., 2002; Yegles et al., 2004; Jurado et al., 2004; Klyks et al., 2005; Politi et al., 2006; Morini et al., 2006; Politi et al., 2007; Appenzeller et al., 2007a; Appenzeller et al., 2007b; Politi et al., 2008; Pragst and Yegles, 2008). Hair analysis of EtG has been shown to be highly specific (100%) in the detection of heavy alcohol users. Sensitivity of the assay has been reported to be between 50 and 100%, and this variation is likely due to the large range of analytical limits of detection (LOD) reported between laboratories (Skopp et al., 2000; Pragst and Yegles, 2007; Janda et al., 2002; Alt et al., 2000; Yegles et al., 2004). (For a review of the analytical methods used in detecting EtG, see Pragst and Yegles, 2007.)

In general, hair levels of EtG have been undetectable or below the lowest limit of quantification (LOQ) for teetotallers/children, and similarly most social drinkers remain undetected with studies reporting measurable EtG in 0 to 57% of social drinkers (Skopp et al., 2000; Pragst and Yegles, 2007; Janda et al., 2002; Alt et al., 2000; Yegles et al., 2004). However, with improving analytical methods and lower LOD and LOQ, EtG might be detected more frequently in the hair of teetotallers and social drinkers in the near future.

Based on existing data, Yegles and Pragst have proposed the following preliminary cut-off values using GC-MS in negative chemical ionization mode (NCI): < 8 pg/mg is congruent with abstinence but does not exclude heavy drinking, > 8 pg/mg and < 25 pg/mg is indicative of social use but also does not exclude heavy alcohol use, and > 25 pg/mg is 100% specific to chronic alcohol abuse (Pragst and Yegles, 2007).

Absence of EtG in hair does not exclude heavy alcohol use. EtG is highly hydrophilic and easily extracted with water, therefore it is possible that EtG may be washed out with water and frequent
shampooing (Pragst and Yegles, 2007). Also, EtG in hair is more vulnerable to cosmetic treatments than FAEE. Bleaching has shown to result in decreases of EtG up to 78% (Yegles et al., 2004). Consequently, the EtG hair test, although highly specific, can lead to false negatives.

Being a hydrophilic molecule, it is believed that EtG is incorporated into hair primarily through sweat. Therefore, it is unclear whether or not segmental hair analysis can be used to resolve drinking patterns in a time-based manner, and studies have shown conflicting results. Yegles et al. reported no correlation between reported alcohol intake and EtG hair levels in corresponding hair segments of 10 alcoholics in treatment programs (Yegles et al., 2004). On the other hand, Appenzeller et al. reported very good agreement between drinking patterns and segmental analysis in 15 patients in withdrawal (Appenzeller et al., 2007a). Consequently, it appears that segmental analysis may be possible for EtG in hair, however more research is required to resolve this question.

Another question that has been raised is whether using the EtG hair test in addition to the FAEE hair test is of added value. Wurst et al. demonstrated that the combined use of EtG and FAEE hair analysis was able to identify more heavy drinkers (7%) than just either test (4 and 5%, respectively) (Wurst et al., 2008a). Another study by the same group showed that both tests (each separately) identified all alcoholic patients and fatalities with documented excessive alcohol exposure, therefore, the tests acted redundantly (Yegles et al., 2004). However, it is possible that at lower levels of alcohol use, as seen in the former study, each test may detect different heavy alcohol users because of inter-individual variability in alcohol metabolism, deposition of EtG and FAEE, and cosmetic/hygienic hair care habits. The latter study also showed that although both markers identified all heavy alcohol users, neither of the markers
correlated with reported alcohol intake, nor did the biomarkers correlate with each other (Yegles et al., 2004). Again this is likely due to individual difference in alcohol metabolism, biomarker deposition, cosmetic/hygienic hair care habits, and the difference in the method of incorporation (for example, by sweat for EtG, and sebum for FAEE).

### 1.6 Biomarkers for Fetal Alcohol Exposure

Biomarkers for fetal alcohol exposure have only recently become available and are therefore limited. The only current method available that has been validated, and which can assess chronic alcohol exposure during gestation is the measurement of fatty acid ethyl esters (FAEE) in meconium. Other methods to measure fetal alcohol exposure are currently under study and are also described below as well.

#### 1.6.1 Meconium

Meconium is the fecal matter that begins to form during the 13th week of gestation and accumulates until birth when it is discarded during the first 72 hours of life. Therefore, meconium can act as a repository for various drug exposures to the fetus during pregnancy.

Mac et al, first detected significantly higher levels of fatty acid ethyl esters (FAEE) in the meconium of fetal alcohol exposed infants relative to non-exposed infants in 1994 (Mac et al., 1994). Since then several groups have used the measurement of FAEE in meconium as a biomarker for fetal alcohol exposure (Bearer et al., 2003; Bearer et al., 1999; Chan et al., 2003;
Chan et al., 2004a; Klein et al., 1999; Moore et al., 2003; Moore and Lewis, 2001; Ostrea et al., 2006). Some groups have applied the measurement of a single FAEE to distinguish exposed infants from non-exposed, such as that of Bearer et al., whereas others, such as our laboratory (Chan et al.,) have advocated the use of cumulative FAEE because of increased clinical sensitivity. Either way a sufficient body of evidence has emerged validating the use of FAEE in meconium as a biomarker for fetal alcohol exposure. (For a comprehensive review of studies involving FAEE in meconium please see Chan et al, 2004a, and Littner and Bearer, 2007).

Noteworthy, a method for the measurement of EtG and Ethyl Sulphate (EtS) in meconium, as a biomarker for fetal alcohol exposure has been developed (Morini et al., 2008). EtS is minor metabolite of ethanol that is formed when ethanol undergoes sulfate conjugation with 3'-phosphoadenosine 5'-phosphosulfate through the action of cytosolic sulfotransferase (Helander and Beck, 2005). Studies are underway and preliminary results show that EtG and EtS are measurable in the meconium, however more research needs to be conducted before the utility of these assays can be evaluated (Morini et al., 2008).

Despite the utility of these methods, the disadvantage in using meconium as a biomarker matrix is that it is only available for the first 72 hours of life, therefore limiting the time available for detection of exposure.

1.6.2 Neonatal and Maternal Hair

Hair offers an alternative matrix for the measurement of biomarkers to meconium. Neonatal hair is available for at least the first 3 months after birth, and since it begins to form at the end of the
second trimester/beginning of the third trimester, it is representative of exposure during the last trimester of pregnancy (A more detailed discussion surrounding the embryology of hair growth is provided in the “synthesis and growth cycle section”). Maternal hair can also be used if neonatal hair is not available or in conjunction with neonatal hair, and it is often more readily available.

Animal studies measuring FAEE in guinea pig hair have shown potential for the use of neonatal and maternal hair as a biomarker for fetal alcohol exposure (Caprara et al., 2005a; Kulaga et al., 2006). The latter study is a work of this thesis and will be discussed in the second chapter. The former is a study involving pregnant guinea pigs that received alcohol throughout gestation (Caprara et al., 2005a). Following gestation exposed dams and offspring were successfully distinguished from non-exposed controls by the measurement of FAEE in their hair; exposed dams and offspring had 10, and 15-fold, significantly higher levels of FAEE relative to non-alcohol exposed controls, respectively.

Preliminary studies involving humans have also suggested that the FAEE hair test is a sensitive method in detecting alcohol exposure using maternal and neonatal hair. Our laboratory conducted a baseline study testing hair from 56 infants of non-alcoholic women that showed that FAEE were detectable in almost all neonatal hair samples at very low concentrations with a mean level of 0.32 pmol/mg (Caprara et al., 2005b). This demonstrates that FAEE are measurable in neonatal hair. Additionally in our laboratory, we also had a case of a mother, an admitted gestational drinker, and her infant, with measurable levels of FAEE of 2.6 and 0.4 pmol/mg, respectively, demonstrating that FAEE are detectable in the hair of an exposed mother and child (Klein et al., 2002).
In addition, a recent pilot study investigated the use of maternal hair, in addition to a battery of other biomarker tests, to investigate their performance in assessing alcohol use in the general pregnant population (n = 103) (Wurst et al., 2008a). The study was able to identify 7 women using heavy amounts of alcohol by hair analysis (EtG and FAEE), 3 of which were identified by FAEE hair analysis alone, and which were not detected by self-report questionnaire nor any other measure (Wurst et al., 2008a). Therefore, there is evidence that the measurement of FAEE in hair may be useful for assessing fetal alcohol exposure, and its use in this context is the topic of this thesis.

1.7 Hair Structure, Growth, and Drug Incorporation

1.7.1 Structure and Chemical Properties of Hair

There are generally two types of hair, vellus and terminal hairs. Vellus hairs are shorter, lighter, and grow all over the body; whereas terminal hairs are stronger, grow longer, are pigmented, and grow on scalp, axilla, eyebrow, beard, and pubic area. The remainder of the section will focus on terminal hairs as vellus hairs are not used for hair testing at present.

Hair is comprised of 65-95 % proteins, 1-9 % lipids, 0.1-5 % melanin (Harkey, 1993). Its structure contains four units (Figure 1.5). The outer cuticle consisting of scale-like cells surrounding the cortex. The cortex, which is made up of spindle-shaped cells, contains the fibrous proteins of the hair, keratin. The medulla, a loosely packed porous region that is usually
present only in thicker hair, is often absent from finer hair, particularly that of animals (Robbins, 2002). The final unit is called the cell membrane complex, and it is the substance that binds all the other cells together and forms the major pathway for diffusion within the hair matrix.

Hair has an isoionic point of pH near 6.0 (5.2-6.2) and an isoelectric point of pH approximately 3.7 (Robbins, 2002). Robbins defines the isoionic point as “the point at which a protein or particle has an equivalent number of total positive and negative charges as determined by proton exchange; it is a whole fiber property of hair and is reflected in the equilibrium acid-base
properties of the total fiber”. Robbins defines the isoelectric point as “the pH at which a protein or a particle does not migrate in an electric field, and is related to acid-base properties of the fiber surface”. Therefore, because the pH of hair is so low it is predisposed to interact with basic or cationic compounds at physiological pH, a topic that will be discussed in the section entitled “Melanin and Bias in Hair Testing”.

### 1.7.2 Sebum

Sebum is produced by sebaceous glands located across most of the body, wherever hair grows. Its production is regulated by androgens, and consequently is lower in females than in males (Imperato-McGinley et al., 1993). Sebum production and composition is related to age, sex, and hormonal status, (Strauss and Pochi, 1963), and can vary seasonally, and even daily (Kligman and Shelley, 1958; Gloor, 1978). Output increases during puberty and in the early twenties, and begins to decline by the fourth decade of life, particularly in females after menopause (Strauss and Pochi, 1963). More than half of human sebum is composed of triglycerides and free fatty acids, with saturated and unsaturated fatty acids that have been found to range from 5 to 22 carbon atoms in length; other main components include: free cholesterol, cholesterol and wax esters, paraffins, and squalene (Robbins, 2002; Nikkari, 1974; Gloor, 1978). Also, the amount of paraffinic hydrocarbons is higher, and the concentrations of squalene and cholesterol lower, in children’s hair compared to that of adults (Robbins, 2002). (For a detailed review of sebum composition please see Robbins, 2002, and Nikkari, 1974.). Therefore, the constitution of sebum and its production can vary greatly between individuals, and even within individuals across time.
1.7.3 Synthesis and Growth Cycle

Hair synthesis begins at the base of the bulb of the hair follicle. The hair follicle is comprised of the papilla, located at the base of the bulb, and which is instrumental in controlling the growth cycle, an external root sheath (Henle’s layer), a middle layer (Huxly’s layer), and an internal root sheath that is continuous with the growing hair fiber located in the middle. Associated with hair follicles are also arrector pili muscles, sebaceous glands, and apocrine sweat glands (Figure 1.6) (Paus and Cotsarelis, 1999).

There are three main zones of growth in hair formation (Robbins, 2002). They are the zone of differentiation and biological synthesis, the keratinization zone, and the region of permanent hair. Cells are formed at the base of the bulb and then migrate upwards where they differentiate and elongate to form permanent hair cells, and part of the inner root sheath. In the zone of keratinization, disulfide bonds are formed through a mild oxidative process to create permanent hair that is stronger and more resistant to degradation and breakage. Melanocytes are located at the apex of the bulb and are responsible for producing and transferring hair pigment, melanin, to the hair cells. This process is described in more detail under the section entitled “melanin”.

There are three growth phases, anagen, catagen, and telogen (Paus and Cotsarelis, 1999; Tobin, 2005). Anagen is the phase of active growth, and in adults generally lasts between 2-6 years (Robbins, 2002). Catagen is the transition stage where metabolic activity slows down and generally last several weeks. Telogen is the resting stage when growth has stopped and the hair bulb has atrophied. A new hair begins to form underneath and eventually pushes the telogen hair out, and the process starts again. In humans each hair follicle cycles independently through the three growth phases irrespective of what the neighboring follicle is doing, which is referred to as
a mosaic pattern of growth. This mosaic pattern of growth also occurs uniquely in the guinea pig, unlike the pattern seen in many other small mammals, such as the rat, is not mosaic but rather a wave pattern where regions of hair enter the same growth phase simultaneously.

In humans, hair growth begins at the end of the third gestational month (Tobin, 2005). Referred to as prenata l hair, neonatal hair, or lanugo, it originates from the malpighian layer or the stratum germinativum of the epidermis (Robbins, 2002). Neonatal hair typically grows to a maximum of 15 cm when it is generally shed within the first 3 months of life and replaced by children’s hair. Children’s hair, sometimes called prepubertal or primary terminal hair, is longer and coarser and can grow to a maximum of 60 cm. Adult hair begins to form during adolescence and typically grows to a maximum of 100 cm, however, there have been many documented cases of hair exceeding 150 cm in length.

Scalp hair grows at a rate of approximately 0.35 mm per day, but can vary greatly between 0.07-0.78 mm/day, with 80% of the population having a rate between 0.32-0.46 mm/day (Kronstrand and Scott, 2007). Hair grows fastest on the vertex of the scalp region, followed by the temporal regions, and then slowest on other parts of the body (Robbins, 2002).

1.7.4 Routes of Drug Incorporation

Drugs can be incorporated into hair by three routes, blood, sweat, and sebum (Figure 1.6). The route of incorporation is primarily dependant on the molecular and chemical properties of the substance, which will determine whether or not it is present in blood, sweat, and or sebum.
Drugs that are present in blood can migrate from nourishing blood vessels located near the base of hair follicles and be incorporated into the hair at the root as it is formed. Therefore, drugs that are incorporated from blood are incorporated as the hair grows, and consequently leave a chronological record of exposure. Basic drugs that are cationic at physiological pH are favoured for incorporation by this method because the acidic properties of hair attract them. Similarly,
studies have shown that melanin, the pigment of hair, prefers to bind to such drugs because of melanin’s acidic properties, however these studies are discussed in the “melanin and bias in hair testing” section.

Hydrophillic substances, such as EtG for example, may be preferentially incorporated through sweat at the surface of the scalp. As discussed in the “ethyl glucoronide” section, it is still not yet known if incorporation by this method leaves a chronological record of drug exposure, as drugs that are primarily incorporated from blood. Furthermore, it is difficult to determine the relative contribution of incorporation of drugs from sweat or blood.

Lipophillic drugs can be incorporated through sebum, and in fact it is believed that this is the primary route of incorporation for FAEE. In a study investigating segmental analysis of FAEE, no relationship could be shown between hair segments and reported alcohol intake for the corresponding time frame (Auwarter et al., 2001). Auwarter and colleagues reported that the pattern of FAEE accumulation was similar regardless of reported ethanol intake, with concentrations steadily increasing within the proximal 5-10 cm from the scalp and then slightly decreasing to plateau thereafter (Auwarter et al., 2001; Yegles et al., 2004). The most likely explanation for this is that FAEE are distributed along the hair shaft by sebum, after which they diffuse into the matrix and become incorporated. Some are likely lost or degraded through weathering in distal parts of the hair, however a significant amount becomes irreversibly incorporated and remains stable within the hair matrix. Therefore, segmental analysis of xenobiotics incorporated through sebum may be difficult to interpret.
1.8 Melanin and Bias in Hair Testing

Several studies have investigated the issue of bias in hair testing because of differences among hair types, and because of the serious legal and social consequences that may result from testing positive for drugs of abuse. The majority of studies have focused on hair colour as a source of bias; however, there are many factors that can influence bias in hair testing.

This issue of bias in hair testing received great attention when Kidwell and Smith illustrated what they called “cultural bias” in hair testing, by showing a 31-fold difference in the amount of cocaine absorbed between the Asian-Caucasian male population and the African American female population (Kidwell and Smith, 2007). Originally, misperceived as the result of hair colour differences, the authors explained how hair characteristics can combine along with the clinical management of data, and population characteristics, to affect hair-testing bias. These important points are summarized below in brief; a detailed presentation is outside the scope of this thesis.

Kidwell and Smith review the main components associated with drug uptake and retention in hair, and how they relate to bias (Kidwell et al., 2000; Kidwell and Smith, 2007). The permeability of hair and its ability to bind substances can be influenced by genetic and cultural differences. For example, morphological differences that vary between different ethnic groups can affect permeability to drugs in hair, and put populations with certain hair types at greater likelihood of testing positive. FAEE are incorporated primarily through sebum, therefore strand permeability may affect FAEE incorporation, however this has never been investigated and is
beyond the scope of the current thesis. Alternatively, drugs that bind heavily to melanin may accumulate in darker hair to a greater extent than lighter hair, therefore people of ethnic backgrounds with darker colour hair may be disfavoured. (The present thesis investigates the possible influence of hair colour on FAEE incorporation, and hence the effect of melanin on drug incorporation will be discussed in greater detail in the following section.) The use of cosmetic treatments and hair habits, which may or may not be culturally associated, can likewise influence drug incorporation. For example, grooming that increases strand breakage can result in higher drug incorporation rates. Kidwell and Smith describe how many of these factors combine to put African Americans at a disadvantage because of an increased likelihood of testing positive based on hair colour, morphological characteristics, and grooming habits. Hygiene, and routes of drug administration, including passive exposure, are also important aspects to consider in the uptake and retention of drugs.

Kidwell and Smith also review how bias can be produced or removed by the selection of different laboratory cut-off levels for what constitutes a positive test. Cut-off levels are often selected in order to differentiate between passive exposure and active drug use. The authors explain how the amount of drug use in a given population, and the shape of the distribution curve of drug hair concentration in populations with different hair types, can have a significant impact on hair testing bias. The issues are too complex to discuss in detail here, however, the authors do explain how a sufficiently low cut-off level will likely avoid bias by having all drug users test positive even if certain populations are incorporating drugs to a greater extent than others. However, if passive exposure is an issue this may create the problem of mislabeling passive users as active users. Consequently, the selection of cut-off levels needs to be considered as a potential source of bias.
Unlike with many other drugs of abuse, passive exposure of alcohol is not a significant issue, as the route of administration is oral. However, false positives generated from exogenous sources such as hair sprays or improper storage, although unlikely, are possible, and have been discussed in detail in the “FAEE in hair” section.

1.8.1 Melanin

Melanin is a pigment found in hair and skin. Different types of melanins exist and in combination they form different colours and shades in hair. The primary forms of melanin are eumelanins, responsible for dark brown or black pigments, formed from 5,6-dihydroxyindole (DHI) and 5,6-dihydroxy-2-carboxylic acid (DHICA), and pheomelanins, responsible for fiery/carrot red colour pigments, composed of benzothiazine, benzothaizole, and isoquinoline units (Castanet and Ortonne, 1996). More recently it has been discovered that oxidative products of the pigment monomers exist called oxyeumelanin, responsible for brown/chestnut colours, and oxypheomelanin, responsible for other red hues (Prota, 2000).

Melanins are formed by melanocytes located at the apex of the papilla in the hair bulb (Figure 1.6). Melanogenesis takes place in cytoplasmic organelles called melanosomes, located within the melanocytes (Kronstrand and Scott, 2007). From tyrosine, dopaquinone, the precursor for melanins is formed under the action of tyrosinase (Ozeki et al., 1996). Dopaquinone is then reduced to form eumelanins, or in the presence of cysteine leads to the formation of benzothiazine derivatives and ultimately pheomelanins (Ozeki et al., 1996; Kronstrand and Scott, 2007) (Figure 1.7). As melanins are formed, the melanosome transforms into a uniformly dense
melanin particle that is transferred by a phagocytic mechanism to cortical and medullary keratinocytes (Robbins, 2002). They are then part of the growing hair fiber.

Figure 1.7 Simplified schema of melanin synthesis from tyrosine precursor
Importantly, melanins have acidic properties. Melanin polymers contain many carboxylic acid residues, which provide ionic binding sites for substances such as drugs (Potsch et al., 1997b). For this reason, as will be discussed in the next section, melanins in hair typically interact with basic drugs, as oppose to non-basic drugs, through their cation exchange properties (Borges et al., 2001b; Borges et al., 2003; Borges et al., 2002; Nakahara et al., 1998; DeLauder and Kidwell, 2000; Nakahara and Kikura, 1996; Nakahara et al., 1995; Gygi et al., 1997).

1.8.2 Studies on Melanin in Hair and Drug Incorporation

Numerous animal studies have shown that certain drugs preferentially accumulate into pigmented hair over non-pigmented hair (Hubbard et al., 2000; Slawson et al., 1998; Slawson et al., 1996; Borges et al., 2001b; Wilkins et al., 1998; Potsch et al., 1997a; Gygi et al., 1997; Slawson et al., 1996; Gygi et al., 1996; Green and Wilson, 1996; Boyd et al., 1991; Uematsu et al., 1992; Nakahara et al., 1998). The majority of these studies have been controlled-dose experiments typically involving multi-coloured rat stains such as the Long Evans rat, and or several stains of rat with different hair colours. In all cases where differences in drug accumulation between pigmented and non-pigmented hair have been observed, drugs have always been found to accumulate preferentially into pigmented hair.

Acid/base properties, lipophilicity, pKa, and molecular size and shape of a drug all play a role in drug-binding into hair, and not all drugs accumulate to a measurable extent in hair (Kikura and Nakahara, 1998; Potsch, 1996; Nakahara and Kikura, 1996). As mentioned, drug incorporation is also directly proportional to the drug’s basicity, therefore studies have shown that basic drugs
accumulate more into hair than non-basic drugs (Borges et al., 2001b; Borges et al., 2003; Borges et al., 2002; Nakahara et al., 1998; DeLauder and Kidwell, 2000; Nakahara and Kikura, 1996; Nakahara et al., 1995; Gygi et al., 1997). For example, basic drugs such as cocaine, amphetamine, phencyclidine, and codeine have been found to accumulate substantially into pigmented hair (Potsch et al., 1997a; Gygi et al., 1997; Slawson et al., 1996; Borges et al., 2001b; Hubbard et al., 2000; Slawson et al., 1998), whereas neutral or weakly acidic drugs, such as the non-basic analogue, N-acetylamphetamine, or phenobarbital, incorporate to a much lesser extent into hair. Also, concentrations of these latter non-basic drugs, although measurable in hair, have not been found to differ between pigmented and non-pigmented hair (Gygi et al., 1997; Borges et al., 2001b).

In-vitro studies have also confirmed the strong ability of melanins to bind basic drugs (Gautam et al., 2005; Borges et al., 2003; Borges et al., 2002; Potsch et al., 2002b; Potsch et al., 2002a; Testorf et al., 2001; Joseph, Jr. et al., 1997). However, Potsch et al. caution that in-vitro studies in drug-melanin binding provide limited information as these interactions are representative of only those that occur at the surface of melanin granules, which seem to be of minor importance; the authors suggest that drug-melanoprotein loading during melanogenesis is of primary importance in determining the influence of hair pigment on drug accumulation in hair fibers (Potsch et al., 1997b).

Controlled animal studies, in-vitro studies, and human studies, have also shown that the pigments, eumelanin and pheomelanin, have different binding capacities. Eumelanins appear to bind drugs more strongly than pheomelanins, accumulating more drug in darkly pigmented hair (Borges et al., 2003; Slawson et al., 1998; Potsch et al., 1997a; Gygi et al., 1996; Joseph, Jr. et
al., 1996; Rollins et al., 2003). Different colours of human hair contain different proportions of these pigments. Black hair contains approximately 99% eumelanin and 1% pheomelanin, brown and blond hair contain approximately 95% eumelanin and 5% pheomelanin, whereas red hair contains approximately 67% eumelanin and 33% pheomelanin (Borges et al., 2001a). However, as mentioned earlier, numerous factors besides hair pigment can affect incorporation and accumulation into hair.

Controlled dose studies in humans involving cocaine and codeine administration, have shown increased levels of drug in darkly pigmented hair, and a positive linear relationship between melanin content and hair drug concentration (Scheidweiler et al., 2005; Rollins et al., 2003; Kronstrand et al., 1999; Henderson et al., 1998). Similarly, studies investigating differences in drug concentration between pigmented and non-pigmented hairs in gray-haired individuals have found a preference for drugs to accumulate in pigmented hairs (Kronstrand et al., 2003; Sato et al., 1993; Reid et al., 1996).

Although a multitude of evidence suggests hair colour can influence the incorporation of certain drugs, there have also been mixed reports, or negative results, regarding associations between hair colour and drug accumulation (Mieczkowski and Kruger, 2007; Mieczkowski, 2003; Mieczkowski, 2000; Kelly et al., 2000; Mieczkowski and Newel, 1993; Mieczkowski and Newel, 2000). However, as Kidwell and Smith commented, many of these studies had study design limitations, mostly because many were secondary analyses, and therefore the data could not be appropriately assessed in the manner required by the studies; secondly, the studies focused either only on positive results or highly positive users, which as explained earlier can lead to the failure to find a bias even if one exists (Kidwell and Smith, 2007).
Consequently, it appears that there is sufficient evidence to state that hair pigment, melanin, can influence drug incorporation and accumulation for certain drugs. The clinical significance of this effect remains to be determined, nevertheless, drugs that are to be assessed by hair analysis need to be examined for their potential to be influenced by hair colour, and this is a key topic of the current thesis (chapter 3).

1.9 Animal Models for Alcohol Exposure

Ethical issues concerning research in pregnant women are highly complex, as there are two participants, mother and baby, one of which whom does not have the ability to consent. Exposures that may be harmless to adults may be toxic to the fetus, as is the case with alcohol. Therefore, research involving toxic/teratogenic substances is difficult to conduct in humans. Furthermore, accurate reporting of alcohol use during pregnancy is notoriously hard to obtain. Consequently, the use of animal models for controlled dose-response studies is an invaluable tool.

Below I have briefly outlined relevant points regarding hair growth and alcohol metabolism in the guinea pig and rat, as these are the models that I have applied in my thesis, and which are discussed in chapters 2 and 3. When available, information specific to Dunkin-Hartley, “albino”, guinea pig, and or the pregnant guinea pig, as well as the Long Evans rat, has been used, as these
are the models of relevance. Likewise, discussion of ethanol elimination in the rat has been limited to that administered by intraperitoneal injection for the same reason.

1.9.1 The Guinea Pig

There are at least five different types of hair in the guinea pig ranging from 3 to 25 mm in length (Dawson, 1930). The longest are thick, broad, slightly flattened hairs ending in a sharp point. The next longest hairs are finer, with a very fine tip, followed by two types of thin hair, and the last type, a very short, thick-based hair. Hair growth in the guinea pig mimics that in the human in that it occurs in a mosaic pattern. Each follicle enters the relative phases of growth (anagen, catagen, and telogen) independently of one another. Hair growth rate is directly related to hair length and rest period, in which there is no growth. The average growth period is 4 weeks long (ranging 2-7 weeks), whereas the resting period can vary 2 to 22 weeks in males, 12 to 22 weeks in females, and 3 to 5 weeks in pregnant females (Dawson, 1930).

The hair coat of guinea pigs comes in two principal patterns, a solid uniform colour, or spotted. The Dunkin-Hartley strain is an “albino” guinea pig with a solid white coat. It is an outbred variety of the English stock, whose coat is relatively smooth and short. Importantly, guinea pigs are one of the only small mammals to be born with a full coat of hair. At birth their hair averages a length of 18 mm long, growing rapidly to 25 mm in the first week, then slowing down to reach a final average length of 33 to 35 mm (Cooper and Schiller, 1975).

Like in the human, alcohol elimination in the guinea pig follows Michaelis-Menten kinetics, and it is primarily metabolized by ADH in the liver. The Km for ADH in the adult guinea pig (0.42
mM) has been reported to be in the same range as that for humans (0.049 - 4.2 mM) (Riveros-Rosas et al., 1997; Zahlten et al., 1981). The average ethanol elimination rate in our guinea pig model is higher than the average rate reported for humans in general, but is comparable to that reported for alcoholics. The pregnant guinea pig eliminates alcohol at approximately 26 mg/dl/h (Litvin and Switzer, 1988), whereas humans eliminate on average between 15 – 18 mg/dl/h, but can vary as widely as 10-30 mg/dL/h, with faster rates observed among alcoholics (Dettling et al., 2007; Winek and Murphy, 1984).

1.9.2 The Rat

Rats have several types of hair: guard hairs that include bristle and awn hairs; and, underhairs. Most rat hairs (excluding tactile or sinus hair located around the lips and nose) are between 0.5 and 2.5 cm in length, with underhairs being about 1/3 of the length of bristle hairs, and awn hairs being about 1/2 to 3/4 (Hebel and Stromberg, 1976).

Unlike the human or guinea pig, only around 10 % of rat hair follicles are growing at any given time, while the rest remain in the telogen state. The pattern of hair growth in the rat is like most rodents, except the guinea pig, and occurs in a wave pattern where growth is synchronized, and the growth of each follicle affects that of neighbouring follicle’s. (Human and guinea pig hair grows in a mosaic pattern). New cycles of growth begin ventrally and spread dorsally, and also follow a rostral to caudal direction (Durward and Rudall, 1949). The cycle typically repeats every 35 days, with resting and growing periods each lasting approximately 17 days, and with the venter growing approximately 4-5 days in advance of the dorsum (Greene, 1968). Also, as rat
hair grows, they do not necessarily push out old hairs, as in human follicles, therefore, compound follicles form that contain several hairs, and comprise 20% of the follicles located on the back, and 40% of those on the abdomen (Hebel and Stromberg, 1976).

Rats come in various colours and colour patterns. The Long Evans rat is coloured black and white. Black hair is found primarily on the head and top of the back (also called a hooded pattern). White hair can be found on the sides, arms, and abdomen.

Alcohol metabolism in the rat, like the human and the guinea pig, also follows Michaelis-Menten kinetics and is primarily metabolized in the liver by ADH. Elimination rates for ethanol administered by intraperitoneal (i.p.) injection have been reported in the Wistar (~25 – 40 mg/dl/h) (Zidi et al., 2003; Traves and Lopez-Tejero, 1994) and Sprague-Dawley rats (~22 mg/dl/h) (Livy et al., 2003). It has also been demonstrated that the rate of elimination is higher in animals administered ethanol by the i.p. route as compared to intragastric lavage (Livy et al., 2003). Also important, higher elimination rates have been observed in rats chronically treated with ethanol, as opposed to those who have received acute administration (Traves and Lopez-Tejero, 1994). Therefore, it appears that alcohol elimination in the rat is in the order of that of alcoholics and higher, particularly in rats who have been chronically exposed to alcohol.
1.10 Alcohol and Other Drug Use in Families Involved with Child Protection Services

Alcohol and other drug use are common issues in parents investigated by child protection services (CPS) (also commonly referred to as children’s aid organizations, CAS). In a large-scale survey conducted in the US by the Child Welfare League of America, 234 member agencies across 10 US states reported that 37% of the children they served (n = 305,716) were affected by problems associated with alcohol and other drugs (Curtis and McCullough, 1993). Similarly, a large US epidemiological study that interviewed adults over the age of 18 in their homes (n = 42,862) reported that 1 in 4 children in America is exposed to alcohol abuse or alcohol dependence⁵ in the family (Grant, 2000).

⁵ Alcohol abuse or alcohol dependence was ascertained using diagnoses of alcohol use disorders according to DSM-IV guidelines, derived from the Alcohol Use Disorders and Associated Disabilities Interview Schedule (Grant, 2000).
Freisthler et al., also found that the number of alcohol outlets (i.e. bars, liquor stores) and arrests for drug use and sales in a given area, was associated with increased referrals to CPS (Freisthler and Weiss, 2008; Freisthler et al., 2007). Specifically, an average decrease of one off-premise alcohol outlet per year across 579 zip code units across the state of California (total child population 4,890,813) was found would reduce referrals to CPS by 1040 cases, substantiated allegations by 180 cases, and foster care entries by 93 cases, and an average reduction of one bar across the units would decrease foster care entries by 153 cases (Freisthler et al., 2007).

Furthermore, the Quebec Incidence Study of Reported Child Abuse, Neglect, Abandonment, and Serious Behavioural Problems of 1998, reported that alcohol and drug use was the second most important factor in differentiating neglected children from other children reported to child welfare authorities (Mayer et al., 2004). Therefore, there is sufficient evidence to suggest that substance abuse, including polydrug use, seems to be a major issue in families involved with CAS/CPS.

Without question, mothers abusing alcohol can result in inadvertent fetal exposures to alcohol. Therefore, the prevalence of FASD is expected to be higher in families involved with CPS because the prevalence of alcohol use is higher. In fact, a recent study that 34 % of children with disabilities in the care of child welfare services in Manitoba were diagnosed with FASD, representing 11 % of all children in care (Gough and Fuchs, 2006). This figure is staggeringly high; general population estimates of FASD have been conservatively estimated to be around 1 % (Sampson et al., 1997; Williams et al., 1999; Abel, 1995a). Therefore, it appears the prevalence of FASD is over 10-fold higher in families involved with CPS.
Polydrug exposure during pregnancy can compound risks to the fetus, and exacerbate FASD, as well as complicate issues surrounding treatment and intervention. With the risk of FASD and polydrug use being so high in families involved with CPS, the question arises, to what extent do parents who abuse alcohol also abuse other drugs? This question is in fact addressed in the current thesis in chapter six.

Biomarker studies have been performed to examine fetal exposures to alcohol and drugs of neonates investigated by CAS. Two studies, conducted in our laboratory, investigated the prevalence of fetal exposure to excessive alcohol and illicit drugs by meconium analysis (total n = 878) (Chan et al., 2004b; unpublished results). The studies report approximately 16 % of babies were positive for excessive alcohol exposure, 39 % for cannabis, 36 % for cocaine, 6 % for amphetamine, and 5 % for opiates. Alcohol use was significantly associated with drug use (n = 736); specifically, neonates exposed to alcohol were three times more likely to be exposed to amphetamines, and twice as likely to opiates, as compared to those who were not exposed to alcohol (unpublished results). Similar associations should be observed in parents, however until the present (discussed in chapter six) this has never been investigated.
1.11 Thesis Rationale

FASD is a devastating disorder that has reached epidemic proportions. It is a silent epidemic because the majority of cases remain unidentified and undiagnosed. Confirmation of maternal alcohol use during pregnancy is a diagnostic criterion for FASD, however it remains difficult to obtain. Often mothers are reluctant to acknowledge their drinking habits due to fears of stigmatization or losing custody of the child. Furthermore, mothers are not always available as many babies are in the care of child protective services, and or family members, or friends.

The FAEE hair test is a powerful biomarker for heavy alcohol use that could be used in the context of FASD diagnosis and research. However, a major gap exists between previous FAEE research, and research applying the FAEE hair test in the context of FASD. Translational research, bridging laboratory findings with clinical use is needed, and the current thesis strives to address this issue.
1.12 Study Question, Objectives, Hypothesis

The present work encompasses five scientific studies based on the main study question, objective, and hypothesis of this thesis. Each study targets specific questions and has corresponding specific objectives and hypotheses that are outlined below.

1.12.1 Main Study Question:

Does the FAEE hair test hold potential for use in the context of Fetal Alcohol Spectrum Disorder (FASD) diagnosis and research?

1.12.2 Specific Study Questions:

Does the ethanol dose-FAEE concentration relationship and rate of FAEE incorporation in our most relevant animal model for researching hair FAEE in the context of FASD, parallel that of humans?

Is there a significant possibility that the FAEE hair test is susceptible to hair colour bias?

What is the prevalence of testing positive for heavy alcohol use, as measured by FAEE hair analysis, in families at high risk of having children with FASD?
Does the FAEE hair test agree with social worker reports?

To what extent are parents who use alcohol excessively, and who are therefore at risk of having children with FASD, at risk for using other recreational drugs, as measured by objective FAEE and drug hair analysis?

1.12.3 Main Objective and Hypothesis

**Objective:** To investigate the use of FAEE in hair as a biomarker of chronic heavy alcohol exposure in the context of Fetal Alcohol Spectrum Disorder (FASD) diagnosis and research.

**Hypothesis:** The FAEE hair test holds significant potential for use in the context of FASD diagnosis and research.

1.12.4 Specific Study Objectives and Hypotheses

1) **Knowledge Gap:** The guinea pig is an excellent model for fetal alcohol research involving hair FAEE. It is the only small mammal born with neonatal hair, and the timing of its brain growth spurt during fetal development most closely resembles that of the human compared to any other rodent. A previous study involving the guinea pig, demonstrated that guinea pigs exposed to alcohol throughout gestation can be distinguished from controls by FAEE hair analysis. However, to date no one has examined the dose-concentration relationship between
systemic exposure to alcohol and hair FAEE in both the human and guinea pig, and evaluated the relative rates of FAEE incorporation between the two species.

**Specific Objective (1):** To assess the relationship between systemic ethanol exposure and FAEE concentrations in the hair of humans and guinea pigs, and to compare the relative rates of FAEE incorporation.

**Hypothesis (1):** A positive dose-concentration relationship between exposure to ethanol and FAEE in hair is expected in both species, and the relative rate of FAEE incorporation into hair between species is likely to differ, however the magnitude or direction of that difference is not yet known.

2) **Knowledge Gap:** Studies have shown that hair pigment has the potential to affect drug incorporation into hair, however the effect of hair pigment on the incorporation of FAEE has never been studied.

**Specific Objective (2):** To determine whether FAEE incorporation is affected by hair pigmentation.

**Hypothesis (2):** FAEE incorporation will not be influenced by hair pigment.

3) **Knowledge Gap:** Alcohol has long been documented to be a major issue in families involved with child protection services, putting these families at higher risk of having children with FASD; however never before has there been an objective biomarker available to assess chronic alcohol consumption in these families.
Specific Objective (3): To document the use of the FAEE hair test, as a biomarker of excessive alcohol use by parents at-risk for having children with FASD, and quantify the prevalence of testing positive for excessive alcohol use in this cohort.

Hypothesis (3): The prevalence of excessive alcohol use in this cohort is expected to be higher than that of the general population.

4) Knowledge Gap: Every year child welfare organizations across Canada investigate thousands of parents for alcohol abuse. Starting in October 2005, the Motherisk Program established a diagnostic program using FAEE hair analysis to detect excessive parental drinking in clients involved with children’s aid. In the previous study it was discovered that over a third of parents tested positive for excessive drinking. However, not all parents were tested based on suspicions of alcohol use but rather some were tested for other reasons, for example to confirm abstinence. Therefore, whether or not social worker reports are in agreement with FAEE test results is unknown.

Specific Objective (4): To examine the relationship between social worker reports and the novel FAEE biological marker.

Hypothesis (4): The FAEE hair test will agree with social worker reports.

5) Knowledge Gap: An association between alcohol and other drug use has long been known, and concern has been expressed about concomitant exposure during pregnancy. Illicit drug exposures in addition to alcohol can potentially exacerbate manifestations of FASD, and certainly compromise rearing environment. Several studies have reported that alcohol and other
drug use problems occur frequently among parents involved with child protection services. The extent of co-use of illicit recreational drugs and alcohol by parents has never been studied using an objective measure that can assess chronic use such as hair analysis.

**Specific Objective (5):** To determine the risk of other drug use among parents testing positive for excessive alcohol exposure, by hair analysis.

**Hypothesis (5):** Parents who use alcohol heavily will be at greater risk for using other recreational drugs than those who do not.
2 Fatty Acid Ethyl Esters (FAEE); Comparative Accumulation in Human and Guinea Pig Hair as a Biomarker for Prenatal Alcohol Exposure (Kulaga et al., 2006)

2.1 Abstract

Aims: To compare the incorporation rate (ICR) of FAEE in hair between guinea pigs and humans, and to assess the relationship between ethanol exposure and FAEE concentrations in hair. Methods: Published data from pregnant guinea pigs, including maximum blood ethanol concentration, dosage regimen, and total hair FAEE concentration, were compared to published data from alcoholic patients, where dose of ethanol consumed, and total hair FAEE concentration were reported. Mean values of ethanol Vmax for pregnant guinea pigs and humans were obtained from published data (26.2 mg/dl/h, and 24 mg/dl/h, respectively). Results: Total and individual FAEE ICRs, defined as the ratio of hair FAEE to the area under the blood ethanol concentration-time curve (total systemic ethanol exposure), were found to be on average an order of magnitude lower in the guinea pig than in the human. The profiles of ester incorporation also differed slightly between species, with ethyl stearate being highly incorporated in guinea pig hair, and less so in human hair. The results may reflect in the human, greater FAEE production, greater FAEE deposition in hair, slower FAEE catabolism, differential sebum production and composition, or a combination thereof. Also, ethyl oleate was found to correlate with total systemic ethanol exposure for both guinea pigs and humans, correlation coefficients equaling
0.67 (P < 0.05), and 0.49 (P < 0.05), respectively. No other ethyl esters, nor total FAEE, were found to correlate to systemic ethanol exposure. Conclusion: When extrapolating FAEE concentrations in hair from guinea pigs to humans, an order of magnitude difference should be considered, with humans incorporating more FAEE per unit of ethanol exposure. Also, the results suggest caution should be taken when interpreting values of single esters because of their differential incorporation among species. Lastly, our findings suggest ethyl oleate may be of keen interest in FAEE hair analysis, particularly across species.
2.2 Introduction

FAEE are direct biomarkers of ethanol exposure. FAEE are formed during non-oxidative metabolism of ethanol by the conjugation of ethanol to endogenous free fatty acids and fatty acyl-CoA. FAEE formation can be spontaneous, but is most often catalyzed by microsomal AEAT, which utilizes ethanol and acyl-CoA as its substrates, or cytosolic FAEE synthase that is found ubiquitously throughout the body, which uses ethanol and free fatty acids as its substrates (Laposata, 1998b; Laposata and Lange, 1986). FAEE can be detected in hair, meconium, blood, and various organs (Laposata and Lange, 1986; Doyle et al., 1994; Doyle et al., 1996; Klein et al., 1999; Pragst et al., 2001; Auwarter et al., 2001; Wurst et al., 2004). However, it is the unique ability to measure FAEE in hair as a means of assessing chronic or long-term ethanol exposure that is of most interest to our group.

The ability to assess ethanol exposure by FAEE hair analysis is a remarkable tool with many applications. First developed by Pragst et al., 2001, the FAEE hair test is useful for forensic applications because it provides an unbiased means of assessing long-term alcohol usage, and potentially alcoholic status. The test is also useful in toxicological settings, and holds particular promise for use in pediatric patients to assess prenatal exposure to ethanol (Klein et al., 2002). The potential to use hair FAEE to screen for infants at high risk for fetal alcohol associated effects would revolutionize care for such patients who are often either misdiagnosed, or not diagnosed at all (Williams et al., 1999; Fast et al., 1999; Olson et al., 2007; Streissguth et al.,
2004). Therefore, our laboratory’s goal is to further develop the hair test in order to be able to reliably quantify FAEE concentrations in neonatal hair, assessing in-utero ethanol exposure.

The guinea pig has been our choice of experimental animal for recent studies of FAEE as a biomarker of fetal ethanol exposure (Brien et al., 2006; Caprara et al., 2005a) because of the guinea pig’s long history as a validated animal model for FAS (Reynolds and Brien, 1995; Cudd, 2005; Kimura et al., 2000), and most critically because the guinea pig is the only small mammal born with neonatal hair. Unlike the rat or mouse, the guinea pig undergoes substantive prenatal brain development that closely mimics that of the human (Dobbing and Sands, 1979). Furthermore, the manifestations of ethanol neurobehavioural teratogenicity in the guinea pig are similar to the brain dysfunction and dysmorphology of fetal alcohol affected humans (Reynolds and Brien, 1995; Cudd, 2005; Kimura et al., 2000). Recently, it has been shown in pregnant guinea pigs that were treated with ethanol throughout gestation that total FAEE concentrations in fetal meconium, neonatal hair, and maternal hair were significantly higher than isocaloric-sucrose/pair-fed nutritional control (Brien et al., 2006; Caprara et al., 2005a). However, before we can accurately interpret how FAEE concentrations in guinea pig hair relate to concentrations found in humans it is important to know if a difference exists in the FAEE hair incorporation rate between the two species, that is the ratio of total hair FAEE to total systemic exposure to ethanol. Therefore, the objective of the present study is to utilize recently published data in order to compare FAEE incorporation rates into hair between guinea pigs and humans, as well as to assess the relationship between systemic ethanol exposure and FAEE concentrations.

2.3 Methods
2.3.1 Guinea pigs

Published data from female Dunkin-Hartley strain Albino guinea pigs (n = 9) were used (Caprara et al., 2005a). The pregnant guinea pigs received chronic oral administration of 4 g ethanol/kg maternal body weight/day from gestational day (GD) 0, defined as the last day of full vaginal membrane opening, until GD 67. Maternal hair samples of full fur length and weighing approximately 20 mg, were obtained on GD 57 and 65, and quantitatively analyzed for ethyl myristate (E14), ethyl palmitate (E16), ethyl oleate (E18:1), and ethyl stearate (E18). Analytical methods are described in Caprara et al., 2005a, but briefly: samples underwent an overnight liquid-liquid extraction adapted from Pragst et al., 2001, using dimethylsulfoxide and hexane. Hexane layers were then separated, evaporated at 35°C under nitrogen, and reconstituted with hexane. Solid phase extraction was then performed using aminopropyl columns and the samples were then again evaporated and reconstituted two more times to yield final reconstitution volumes of 50 µl. Samples were analyzed using a Varian Saturn 2100T GC/MS/MS with ion trap mass spectrometer in GC/MS mode, using a CPSil-8 low-bleed/MS fused silica chrompack capillary column (30 m X 0.25 mm X 0.25 µm) with helium carrier gas (1.0 mL/min). The temperature program applied was: 2 min at 100°C, ramp up 20°C/min up until 300°C. Chemical ionization mode was used with isobutene as the ionization gas. The temperatures of the injector, transfer-line, manifold, and injector trap were 260°C, 300°C, 50°C, and 220°C, respectively. The concentration of total FAEE (E14, E16, E18:1 and E18) was expressed as picomoles of total FAEE per milligram of hair (pmol/mg). Maternal blood samples were collected on GD 58 at 1 h after the daily ethanol dose to determine the apparent peak blood ethanol concentration (BEC).
For our analysis, maternal hair FAEE concentrations for each pregnant guinea pig were recorded as the average of the total FAEE concentration values for the samples taken on GD 57 and 65. Total maternal systemic ethanol exposure was determined for each pregnant guinea pig by calculating the area-under-the-curve for the maternal blood ethanol concentration-time curve (AUC-BEC) (Figure 2.1).

**Figure 2.1 Sample figure of the blood ethanol concentration-time curve showing the area-under-the-curve (AUC-BEC)**

The latter task was accomplished using zero-order kinetics, measured peak maternal blood ethanol concentration (BEC), and a reference value Vmax of 26.2 mg ethanol/dl blood/h specific to the pregnant guinea pig (Litvin and Switzer, 1988). Zero order kinetics model was chosen because pregnant guinea pigs exhibit apparent zero-order kinetics of ethanol elimination at the
dose level investigated (Litvin and Switzer, 1988). The ICR of FAEE into hair is defined as the ratio of FAEE in hair to total systemic exposure to ethanol (AUC-BEC) to (Figure 2.2).

\[
\text{ICR} = \frac{\text{FAEE}^*}{\text{AUC-BEC}^{**}}
\]

** Concentration of FAEE (pmol/mg)

**Total systemic ethanol exposure (mg/dl/h)

**Figure 2.2 Ratio defining the incorporation rate (ICR) of FAEE into hair**

The ICR represents how much FAEE is incorporated per standard unit of ethanol exposure. The inverse of the ICR is the amount of systemic ethanol exposure required to produce one standard unit of FAEE. Therefore, for each pregnant animal, the value of total FAEE was divided by the AUC-BEC in order to obtain the ICR of total FAEE into hair. The median ratio was used to determine the guinea pig’s average FAEE ICR because the data were not normally distributed. Furthermore, the incorporation rate of each ester was also investigated by calculating the ratio of
each ester to the AUC-BEC separately. Once again the median ratio for each ester was used to define the average of each incorporation rate because the data were not normally distributed.

2.3.2 Humans

Data from 18 alcoholic patients in a detoxification program (14 male; 4 female) with a mean age of 44, and mean body-mass-index (BMI) 21.7, were obtained from Wurst et al, 2004 (Wurst et al., 2004). Alcohol intake was reported as grams of ethanol consumed during the last month. Hair was collected from these patients for analysis of total FAEE (E14, E16, E18:1, E18) on day 7 of hospitalization. Analytical methods have been previously described in Pragst et al., 2001, and are briefly as follows: 6 cm of hair from the root end, weighing approximately 50 mg, was used for analysis. Hair was externally decontaminated by n-heptane, followed by liquid-liquid extraction using dimethylsulfoxide and n-heptane. The n-heptane layer was then separated and evaporated at 40°C under nitrogen. Residues underwent solid phase micro-extraction and were analyzed using Hewlett-Packard 5973 GC, 5973 MS, with deuterated standards for each ester. An Hp5-MS capillary column (28 m X 0.25 mm X 0.25 µm) was used with helium (1 ml/min) as the carrier gas. The temperature program applied was: 2 min at 100°C, ramp up 20°C/min up to 300°C. The temperatures of the injector, the interface, the ion source, and the quadrupol were 260°C, 280°C, 230°C, and 106°C, respectively. The concentration of total FAEE (E14, E16, E18:1 and E18) were expressed as nanograms of FAEE per mg of hair but were converted for our purposes to picomoles of total FAEE per milligram of hair (pmol/mg).
Patients’ weight was estimated at 58 kg for women, and 66 kg for men, using mean BMI scores and population specific statistics for height (Grandjean, 1988). Peak BEC levels were estimated in the following manner. Daily dose (g/day) was calculated by dividing the reported monthly intake of ethanol by 30. The daily dose was then divided by reference values of volume of distribution, 0.59 L/kg for women, and 0.73 L/kg for men (Marshall et al., 1983). AUC-BEC was calculated using zero order kinetics, with peak BEC as the y-intercept, and a reference Vmax value of 24 mg/dl/h specific to patient age group and alcoholic status was used for the slope (Adachi et al., 1989) (Figure 2.1). These calculations are a crude estimate, as we assumed that the daily dose is consumed all at the same time. In practice, drinking may be distributed across time in various patterns, which may lead to a decrease of AUC-BEC in zero order kinetics. In a similar way, our estimates could not address varying rates of absorption. Zero order kinetics were used because this method has been shown to provide accurate and acceptable estimates of systemic exposure to ethanol given the high doses patients were consuming (Crow and Batt, 1989; Hardmann et al., 2001). Similar to the guinea pigs, each value for total FAEE was then divided by the respective value of AUC-BEC. The median ratio was used to determine the average human FAEE ICR because like in the guinea pig the data were nonparametric. Furthermore, the incorporation rate of each ester was also investigated by calculating the ratio of each ester to AUC-BEC separately. Once again the median ratio for each ester was used to define the average of each incorporation rate because the data were likewise nonparametric.
2.3.3 Statistical Analysis

All statistical tests were performed using Sigma Stat, version 2.0. Total hair FAEE per total systemic ethanol exposure (AUC-BEC) ratios, as well as the individual FAEE per AUC-BEC ratios were compared between the pregnant guinea pig and the alcoholic human using the Mann-Whitney Rank Sum test because the data were not normally distributed. The relationship between AUC-BEC and total hair FAEE within species, as well as the relationship between AUC-BEC and individual FAEE within species, was also investigated using the Spearman Rank Order correlation test because the data were not normally distributed.

2.4 Results

In the pregnant guinea pig, the mean level of total hair FAEE was $0.43 \pm 0.33$ pmol/mg (range 0.05 - 1.07 pmol/mg). The most predominant esters were ethyl palmitate, ethyl oleate, and ethyl stearate, whose mean concentrations respectively were $0.14 \pm 0.14$ pmol/mg, $0.14 \pm 0.11$ pmol/mg, $0.14 \pm 0.13$ pmol/mg. The mean concentration of ethyl myristate was $0.01 \pm 0.01$ pmol/mg. The median AUC-BEC was 2253.29 mg/dl/h (range 1632.99 - 3345.01 mg/dl/h). The incorporation rates for individual ethyl esters, as well as total FAEE are displayed in table 2.1. For convenience, the inverse ICRs and their ranges are displayed in table 2.2. The ratios between guinea pig and human ICRs (column 4, Table 2.1) are equal to the ratios of the inverse ICRs, and therefore are omitted in table 2.2.
### Table 2.1 Guinea pig and human hair FAEE incorporation rates

<table>
<thead>
<tr>
<th>Fatty Acid Ethyl Ester</th>
<th>Guinea Pig ICR*</th>
<th>Human ICR*</th>
<th>Guinea Pig ICR/ Human ICR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Myristate</td>
<td>0.000000</td>
<td>0.000130</td>
<td>**</td>
</tr>
<tr>
<td>Ethyl Palmitate</td>
<td>0.000071</td>
<td>0.000650</td>
<td>9.2***</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>0.000057</td>
<td>0.000982</td>
<td>17.1***</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>0.000119</td>
<td>0.000171</td>
<td>1.4***</td>
</tr>
<tr>
<td>Total FAEE</td>
<td>0.000151</td>
<td>0.001909</td>
<td>12.6***</td>
</tr>
</tbody>
</table>

* Units of ICR are pmol/mg of FAEE per mg/dl/h of systemic ethanol exposure

** Interspecies ICR difference could not be assessed

*** Interspecies difference in ICR were significant
Table 2.2 Guinea pig and human hair inverse FAEE incorporation rates and their ranges

<table>
<thead>
<tr>
<th>Fatty Acid Ethyl Ester</th>
<th>Guinea Pig ICR(^{-1}*)</th>
<th>Human ICR(^{-1}*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Myristate</td>
<td>0 (0-208081.5)</td>
<td>7674.2 (785.5-207603.3)</td>
</tr>
<tr>
<td>Ethyl Palmitate</td>
<td>14152.9 (0-47048.6)</td>
<td>1539.2 (55.6-10470.7)</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>17417.0 (9802.4-253599.0)</td>
<td>1018.4 (64.9-5349.1)</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>8384.4 (0-59801.1)</td>
<td>5859.5 (239.3-22300.4)</td>
</tr>
<tr>
<td>Total FAEE</td>
<td>6605.2 (3008.9-17417.0)</td>
<td>523.9 (25.7-2726.21)</td>
</tr>
</tbody>
</table>

* Units of inverse ICR are mg/dl/h of systemic ethanol exposure per pmol/mg of FAEE

The mean level of total hair FAEE among alcohol detoxification patients was 4.40 ± 2.30 pmol/mg (range 1.29 - 10.96 pmol/mg). The most predominant esters were ethyl oleate and ethyl palmitate. Mean concentrations of ethyl oleate, palmitate, stearate and myristate were 1.91 ± 0.92 pmol/mg, 1.69 ± 1.01 pmol/mg, 0.47 ± 0.17 pmol/mg, and 0.32 ± 0.42 pmol/mg, respectively. The median AUC-BEC was 2390 mg/dl/h (range 91.90 - 11417.83 mg/dl/h). The incorporation rates for individual esters, as well as total FAEE are displayed in table 1, and for convenience their inverse is displayed in Table 2.2.
The FAEE incorporation rates of guinea pigs were significantly lower than those found in humans (P < 0.05) for all individual esters, and total FAEE, except for ethyl myristate whose median ratio was zero (Table 2.1). Six out of the nine guinea pigs had undetectable levels of ethyl myristate in their hair; therefore incorporation rate could not be accurately assessed for this ester. Guinea pigs required on average over an order of magnitude more exposure to ethanol in order to produce equivalent levels of FAEE (Table 2.1); the one exception was ethyl stearate in which guinea pigs only required 1.4 times the ethanol exposure required by humans to produce equivalent FAEE levels. Ethyl oleate was the only ester found to correlate to systemic ethanol exposure (AUC-BEC) in either species, and it was found to correlate in both species; correlation coefficients for guinea pig and human were 0.67 (P < 0.05), and 0.49 (P < 0.05), respectively (Figure 2.3 and 2.4).
* Spearman correlation statistically significant (P<0.05)

**Figure 2.3** Relationship between ethyl oleate and total systemic ethanol exposure in guinea pigs
2.5 Discussion

After standardization for systemic ethanol exposure, cumulative levels of ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate were approximately 13 times lower in guinea pigs than in humans. This result indicates that there is an order of magnitude difference in the average FAEE incorporation rate into hair between guinea pigs and humans, with guinea pigs requiring
an order of magnitude more exposure to ethanol in order to produce equivalent FAEE concentrations. Analysis of individual FAEE incorporation rates revealed that different ethyl esters incorporate at different rates for each species but are still largely of an order of magnitude lower in guinea pigs, with the exception of ethyl stearate. The ICR of ethyl stearate was only 1.4 times lower than that of the humans, but this difference was still statistically significant. This result can be explained by the fact that although ethyl stearate was found to be equally prevalent as ethyl oleate, and palmitate, it is incorporated at a much higher rate in the guinea pig (almost double the rate of either of the other two), coupled with the fact that ethyl stearate is incorporated at a relatively low rate in humans (Table 2.1). Ethyl oleate and palmitate, in addition to ethyl stearate, did however contribute largely to the total FAEE incorporation rate in guinea pigs. Conversely, in humans ethyl stearate contributed less than 10% to the total FAEE incorporation rate, but the rate was dominated by the most prevalent and highly incorporated esters, ethyl oleate and palmitate. This difference in ethyl ester profiles of incorporation rates is likely the result of a natural species difference. FAEE are found throughout several matrices: meconium, hair, blood, organs most commonly affected by alcohol abuse, and in adipose tissue (Laposata and Lange, 1986; Doyle et al., 1996; Pragst et al., 2001; Chan et al., 2004a). FAEE are actively produced in most of these media when exposed to ethanol, with some matrices showing slightly different ester profiles even within the same species tissue (Laposata and Lange, 1986; Doyle et al., 1996; Pragst et al., 2001; Chan et al., 2004a; Bearer et al., 1999; Bearer et al., 2003; Chan et al., 2003). Lange, 1982, showed that FAEE produced in rabbit myocardial tissue were made primarily from their non-esterified fatty acid precursors, and that the most highly incorporated FAEE into myocardial tissue were ethyl linoleate, and oleate (Lange, 1982). Hair FAEE are believed to be primarily deposited by sebum, and sebum composition between guinea pigs and
humans differs drastically (Auwarter et al., 2001; Nikkari, 1974). It is likely that the difference in ethyl ester ICR profiles is the result of differences in fatty acid composition at the source of FAEE production (the sebaceous gland for example), and perhaps differential enzymatic specificities.

However, differences in ICR ester profiles between species do not account for the major finding that on average guinea pig produce an order of magnitude less FAEE for equivalent ethanol exposure. There can be several reasons for this interspecies difference; the first being FAEE production or degradation. Guinea pigs may produce or degrade FAEE at a different rate than humans. In humans, FAEE are formed through non-oxidative ethanol metabolism primarily by AEAT and FAEE synthase. In the guinea pig, the metabolic route by which FAEE are formed has not been confirmed; however if FAEE synthase and AEAT are involved they may have different levels of activity. The guinea pig’s higher rate of ethanol metabolism in general could also result in faster degradation of FAEE, and consequently result in less opportunity for incorporation into hair. Another explanation for the 13-fold difference could stem from differences in FAEE deposition. Lipophilicity, membrane permeability, and melanin affinity are key factors affecting drug deposition into hair (Nakahara et al., 1995). The first two factors are unlikely to be responsible for our finding since we are discussing deposition of the same species, FAEE, into two different mammals. As for melanin affinity, certain drugs are known to bind to melanin, affecting drug concentrations in differently pigmented hair. The guinea pigs in the present study were Albino pigs, therefore it is possible that if FAEE bind to melanin, which remains unknown, that a large difference of FAEE incorporation between guinea pigs and humans could occur. However, drug-melanin interactions that occur in melanin granule formation during melanogenesis are much more important that drug-melanin interactions that
occur on the surface of granules such as would occur from sebum (Potsch et al., 1997b); therefore if FAEE deposited by sebum were binding to melanin, this interaction would not likely affect FAEE hair concentrations. Furthermore, FAEE are non-polar so it is doubtful that they would interact with the cation exchange properties of melanin that provide most of the ionic binding sites for drugs (Potsch et al., 1997b). The most likely explanation for species difference in total FAEE ICR is the composition of sebum of between species. The composition of sebum is highly species-specific with human sebum containing twice as much saponifiable material as nonsaponifiable material, and more than half of human sebum is composed of triglycerides and free fatty acids, whereas the seba of guinea pigs, other rodents, rabbits, and sheep contain less than 10% free fatty acids and virtually no triglycerides (Nikkari, 1974).

The present study found a significant correlation between systemic ethanol exposure and ethyl oleate for both guinea pigs and humans, 0.67 (P < 0.05), and 0.49 (P < 0.05), respectively. This indicates that oleic ethyl ester may be a truly important ester in hair for assessing ethanol exposure, even across species. Interestingly, oleic ethyl ester has been found to be one of the most, or the most, prevalent esters in: the meconium tested positive for ethanol exposure, the blood of acutely intoxicated individuals, the organs and adipose tissue of alcoholics, myocardial tissue of rabbit ethanol perfused hearts, and the adipose tissue of acutely exposed rats (Lange et al., 1981; Lange, 1982; Laposata and Lange, 1986; Doyle et al., 1996; Moore and Lewis, 2001; Bearer et al., 2003; Chan et al., 2004a; Salem et al., 2001). Also, Doyle et al., 1994, reported that ethyl oleate, along with ethyl stearate, palmitate, and linoleate, correlated significantly with blood ethanol levels from a group of emergency room patients (Doyle et al., 1994). The fact that the correlation in the present study was found despite significant limitations in assessing the systemic ethanol exposure of the humans is encouraging. We were not able to account for
consumption patterns of individuals, and perhaps if systemic ethanol estimates were more accurate an even stronger correlation would have been found. However, encouraging is the fact that Soderberg et al., 1999, who found total FAEE blood concentrations to correlate with blood ethanol concentration, also found that the rate of alcohol consumption did not affect the FAEE concentration (Soderberg et al., 1999). The lack of correlation of the other esters with systemic ethanol exposure may be due to the sample size of the population and the large variability associated with these esters.

It is true that the current study was limited by the fact that previously published data from different studies was used. Consequently, not all variables sought in the current study were available, such as peak blood ethanol concentrations or weight of human subjects, and therefore they had to be estimated. Also, the guinea pigs’ dosage regimen was controlled and invariable, whereas human ethanol consumption was recorded from the subjects’ memory. However, given the magnitude of our findings it is doubtful that the above limitations greatly influenced the results.

The current study has shed light onto the FAEE ICR of guinea pigs and humans, and the differences that exist between them. We have discovered that an order of magnitude difference exists between them, that the contributions of the esters vary slightly, and that ethyl oleate may be an especially important biological marker of ethanol exposure, particularly across species. These findings suggest that the guinea pig is a good and highly sensitive model for FAEE in hair, because the levels are still measurable and a dose response relationship exists. This study furthers our laboratories goal of developing a neonatal hair test for fetal ethanol exposure because it amplifies our previous finding that we were able to distinguish in-utero exposed
guinea pig pups from controls by FAEE hair analysis. The current study suggests FAEE hair analysis is highly sensitive in humans, and coupled with our previous findings, that it holds significant potential for discriminating fetal-alcohol exposed neonates.
3 The Effect of Hair Pigment on the Incorporation of Fatty Acid Ethyl Esters (FAEE) (Kulaga et al., 2009)

3.1 Abstract

Aims: The objective of the current study was to determine whether FAEE incorporation is affected by hair pigmentation. Methods: Black hooded LE rats were injected intraperitoneally daily with ethanol. Prior to dosing, black and white patches of fur were shaved and analyzed for baseline levels of FAEE using an adapted extraction procedure and GCMS method. Once the shaved “patches” had grown back they were re-sampled along with hair outside the “patches”, referred to as “no patch” hair, and tested for post-treatment FAEE levels in the same manner. Blood was also sampled for pharmacokinetic analysis of ethanol. Results: Total FAEE levels were significantly higher in post-treatment hair (black and white) compared to baseline (pre-treatment) levels. Total FAEE levels were also significantly higher in post-treatment “patch” hair (black and white) compared to “no patch” hair. No significant differences were found between post-treatment black and white hair. The FAEE profiles were similar between black and white hair, with FAEE levels being highest for ethyl myristate, followed by ethyl stearate, palmitate, and then oleate. Conclusion: FAEE incorporation into hair does not appear to be affected by hair pigment, which is in congruence with what is known about the chemistry of drug-melanin
interactions. This is important in avoiding potential bias and discrimination in the interpretation of alcohol abuse based on hair colour.
3.2 Introduction

In recent decades hair testing as a method for the detection of drugs of abuse has been gaining popularity because of its unique ability to reliably measure chronic and or past substance use. Lately, there has been an increased need for the development of such a biomarker for alcohol exposure. A prominent example is the case of diagnosing fetal alcohol spectrum disorder (FASD). FASD is a prevalent cause of neurocognitive handicap among children in North America (Abel and Sokol, 1987; Abel and Sokol, 1986). One of the most serious challenges in diagnosing FASD is the need to establish evidence of excessive maternal drinking during pregnancy as a major diagnostic criterion (Chudley et al., 2005). However, maternal self-reports of alcohol use are often unreliable (Russell et al., 1996; Ernhart et al., 1988; Wurst et al., 2008a; Alvik et al., 2006; Alvik et al., 2006; Alvik et al., 2006), therefore a reliable objective biomarker that can detect past chronic alcohol use would prove invaluable in this area.

In 2001, Pragst and colleagues established a biomarker in hair that can reliably assess chronic heavy alcohol use by the measurement of non-oxidative metabolites of ethanol, fatty acid ethyl esters (Pragst et al., 2001). Since its introduction, the FAEE hair test has been increasingly used in the medical context of alcohol abuse and the medicolegal context of “driving under the influence” (Wurst et al., 2008a; Pragst and Yegles, 2008) because of its high sensitivity and specificity in the detection of excessive drinking in adults (Pragst and Balikova, 2006; Wurst et al., 2004). Recent advances in research have also suggested the FAEE hair test has potential for use as a new diagnostic tool for FASD and FASD research, further expanding its utility.
The increase of the FAEE hair test’s applications has prompted us to ask whether FAEE incorporation is susceptible to hair colour bias, as has been the case with certain other drugs. Several studies have demonstrated that some drugs can accumulate preferentially in pigmented hair (Hubbard et al., 2000; Wilkins et al., 1998; Gygi et al., 1997; Kintz, 2007; Slawson et al., 1996; Gygi et al., 1996; Green and Wilson, 1996; Scheidweiler et al., 2005; Kronstrand et al., 2003; Reid et al., 1996). The issue of whether or not a hair colour creates bias in interpreting drug exposure is serious and may discriminate individuals with dark hair. The interactions of most significance are those that occur between basic/cationic drugs, such cocaine or methamphetamine, with the anionic centers of the melanin granules (Borges et al., 2001b; Gygi et al., 1997; Nakahara et al., 1992; Borges et al., 2003; DeLauder and Kidwell, 2000; Nakahara and Kikura, 1996; Nakahara et al., 1995). Neutral drugs have not been found to accumulate substantially in hair or to preferentially accumulate in pigmented hair because of this fact. The objective of the present study was to determine whether FAEE incorporation is affected by hair pigmentation, as the test is becoming more widely used. We hypothesize that FAEE incorporation will not be influenced by hair pigment because of their neutral, lipophilic, nature.

### 3.3 Materials and Methods
3.3.1 Animals and Treatment Protocol

The study protocol was approved by the Animal Care Committee of the Hospital for Sick Children in Toronto. Hooded black and white LE rats (n = 6) were obtained from Charles River Canada Inc., St. Constant, Quebec, and housed together in groups of 3 with a 12-hour day/night cycle. Beginning on the first experimental day, two 3 x 3 cm “patches” of fur were shaved from each animal, one black and one white, and tested for baseline levels of FAEE. Prior to dosing, each animal was weighed and then dosed to achieve a peak blood ethanol concentration of 200 mg/dl. Rats were administered 16% w/v ethanol in saline injections by i.p. route according to a protocol developed by Bloom et al., 1982, that dictates the volume of injection in order to correct for the inadequacy of g/kg formulations, which do not produce uniform blood ethanol concentrations because they do not take into account the fact that developmental increases in liver weight and function do not keep pace with developmental increases in body weight (Bloom et al., 1982).

The rats were dosed daily for 3 weeks (Monday to Friday) when it was determined that a sufficient amount of hair (approximately 1.00 cm in length) had grown back in the shaved “patches” to be re-sampled for FAEE analysis. Black and white fur outside of the “patches” was also sampled for analysis. On the last day of dosing, blood samples were taken at 30, 60, 90, 120, 180, and 240 minutes post-dose through a catheter implanted in the jugular vein and analyzed for ethanol. The animals were anesthetized with 3% isoflurane for all procedures except weighing.
3.3.2 Blood Ethanol Analysis

Blood ethanol levels were measured by Headspace Gas Chromatography (Agilent 6890N GC with Headspace Sampler G1888). Samples of 10 ul of serum were diluted to 100 ul and mixed briefly (3-5 seconds) on the vortex with 1.0 ml of internal standard (n-propanol in water). Samples were then analyzed by the headspace GC-FID against known calibrators and quality control solutions.

3.3.3 Pharmacokinetic Analysis

Total systemic exposure to ethanol per dose, area under the blood-versus-ethanol-time curve (AUC), Vmax, and Km were estimated for ethanol, by fitting plasma concentrations to time using Modkine v.1.2.2 (Biosoft, Cambridge, UK). Initial parameters were estimated with Scientist for Windows v. 3.0 (Micromath, Saint Louis, Missouri). Michaelis-Menten elimination kinetics was used as the kinetic model and the maximum enzyme velocity (Vmax; mg×dL⁻¹×min⁻¹) and the Cp at which Vmax is half or Michaelis-Menten constant (Km; mg×dL⁻¹) were obtained; fitting procedure was repeated three times. The area under the blood-versus-ethanol-time curve (AUC₀⁻₄; mg×dL⁻¹×min) was then estimated by non-compartmental procedures.

3.3.4 Chemicals and Reagents

Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, dimethylsulfoxide (DMSO), and n-Heptane were obtained from Sigma Aldrich. Deuterated internal standards of D-5 ethyl
myristate, D-5 ethyl palmitate, D-5 ethyl oleate, and D-5 ethyl stearate were obtained from the laboratory of Dr. Fritz Pragst, Berlin, Germany. Salts for phosphate buffer, potassium dihydrogen phosphate (KH2PO4), and disodium hydrogen phosphate x 2 H2O (Na2HPO4 x 2 H2O), were obtained from Fischer Scientific, and BDH Laboratory Supplies, respectively.

3.3.5 Hair Extraction

A method adapted from the published method, Pragst et al., 2001, was used for FAEE extraction and GCMS analysis. Briefly, 20-25 mg of hair was weighed for each sample into 4 mL extraction vials and chopped finely into 1-3 mm pieces. A multipoint standard curve with concentrations ranging from 0.0125 ng/mg hair (0.25 ng/sample) to 2.5 ng/mg hair (50 ng/sample) was prepared by spiking naïve rat hair with fresh stock solutions of FAEE standards (ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate). Deuterated FAEE (D-5 ethyl myristate, D-5 ethyl palmitate, D-5 ethyl oleate, and D-5 ethyl stearate) were used as internal standards for each sample. Extraction solvents, 500 µL DMSO and 2 ml heptane, were added to each sample. The samples were then placed in a VWR mini shaker at 1200 rpm for 15 hours. The samples were then cooled at 4°C for 30 minutes to freeze the DMSO phase, and then the heptane phase was decanted into 10 mL solid phase microextraction (SPME) vial. Heptane was then evaporated at 40°C under nitrogen gas. To the residues, 1 mL of phosphate buffer (0.1 M, pH 7.6) was added, and the vials were capped and put into the AOC 500 Shimadzu autosampler to undergo headspace solid phase microextraction (HS-SPME).
3.3.6 HS-SPME Conditions

Samples were preheated for 5 minutes at 90°C and 250 rpm agitation. Headspace absorption occurred for 30 minutes at 90°C at 150 rpm agitation, and desorption was for 15 minutes at 260°C. The agitation mode was 60 s right, 30 s interval, 60 s left, 30 s interval, etc.

3.3.7 GC/MS Conditions

A Shimadzu GCMS-QP2010 was used for gas chromatography-mass spectrometry (GCMS) analysis. For chromatographic separation a Varian Factor Four, VF-Xms, capillary column (30 m x 0.25 mm x 0.25 μm) was chosen. The temperature of the injector, interface, ion source and quadrupole were at 260°C, 310°C, 230°C, and 70°C, respectively. The temperature program was as follows: 2 min at 70°C, then 20°C/min up to 300°C, hold 0.5 min at 300°C. Analytes were quantified based on their molecular weight ion. Retention times and \( m/z \) values of molecular weight and qualifier ions of FAEE and D5-FAEE that were used for quantification and identification of the species are shown in table 3.1. Final results are reported as cumulative FAEE (total ethyl myristate, palmitate, oleate, and stearate) per mg hair.
Table 3.1 Retention times and m/z values of molecular weight and qualifier ions of FAEE and D5-FAEE.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention Time (min)</th>
<th>Molecular Weight Ion</th>
<th>Qualifier Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D5-Ethyl Myristate</td>
<td>10.35</td>
<td>261</td>
<td>93, 106, 162</td>
</tr>
<tr>
<td>Ethyl Myristate</td>
<td>10.38</td>
<td>256</td>
<td>88, 101, 157</td>
</tr>
<tr>
<td>D5-Ethyl Palmitate</td>
<td>11.38</td>
<td>289</td>
<td>93, 106, 162</td>
</tr>
<tr>
<td>Ethyl Palmitate</td>
<td>11.40</td>
<td>284</td>
<td>88, 101, 157</td>
</tr>
<tr>
<td>D5-Ethyl Oleate</td>
<td>12.22</td>
<td>315</td>
<td>93, 106</td>
</tr>
<tr>
<td>Ethyl Oleate</td>
<td>12.24</td>
<td>310</td>
<td>88, 101</td>
</tr>
<tr>
<td>D5-Ethyl Stearate</td>
<td>12.30</td>
<td>317</td>
<td>93, 106, 162</td>
</tr>
<tr>
<td>Ethyl Stearate</td>
<td>12.33</td>
<td>312</td>
<td>88, 101, 157</td>
</tr>
</tbody>
</table>

3.3.8 Analytical Precision

The method was evaluated for its reproducibility prior to sample testing. Intraday co-efficient of variation (cv) values were calculated from six replicate analyses of direct injection of three
different concentration levels (5, 10, 20 ng/µL) of pure standard on the same day. Interday cv values were calculated by single analysis repeated over three days using the same concentration levels. All intraday and interday cv values were below 3% and 5% for each FAEE, respectively. Reproducibility was similarly evaluated in extracted white and black rat hair samples. Six replicates at three different concentration levels (2, 20, 50 ng/mL), for each white and black hair samples, were spiked with FAEE standards, extracted, and quantified. Intraday and interday cv values for all esters were below 18 % and 16 %, respectively.

3.3.9 Efficiency of Extraction

Efficiency of Extraction was estimated by spiking both white and black rat hair with 3 different concentrations of FAEE (6 replicates each), followed by extraction. Analogous concentrations of pure standards were then directly injected by syringe into the GCMS, with 6 replicates each. Direct area counts were then averaged among the 6 extracted replicates processed by HS-SPME, and compared to the average of 6 directly injected replicates. Percent extraction efficiency values for the four esters in both white and black hair ranged between 8-76 %, and 5- 55 %, respectively.
3.3.10 Data Analysis

FAEE levels between categories and the effect of initial hair weight on efficiency of extraction were evaluated using paired t-tests, and Pearson product moment correlation tests using Sigma Stat software, version 3.1.

Upon analyzing the data, a significant effect of initial hair sample weight on efficiency of extraction was detected using Pearson correlation. There was a significant inverse relationship between mg hair used in the extraction and FAEE per mg hair quantified (Figure 3.1). After separating the data by white hair, black hair, and treatment category (baseline hair, post-treatment “patch”, and post-treatment “no patch”), the effect of sample weight on extraction efficiency was significant in all categories for black hair ($r = -0.82$, $p = 0.05$, $r = -0.90$, $p = 0.02$, $r = -0.90$, $p = 0.01$, respectively), and in post-treatment “patch” category for white hair ($r = -0.95$, $p = 0.01$), however the effect was not significance in baseline, or post-treatment “no patch” categories for white hair ($r = -0.66$, $p = 0.16$, $r = -0.59$, $p = 0.22$). Therefore, white samples were not adjusted for comparison between baseline and post-treatment levels of FAEE, whereas all black samples were standardized to 20 mg to correct for the effect of initial hair weight on efficiency of extraction. For comparisons of FAEE levels between black and white hair, all black and white post-treatment “patch” samples were standardized to 20 mg and compared.
Figure 3.1 Initial sample weight (mg) of all black and white hair samples plotted against their cumulative FAEE results per mg hair

3.4 Results

3.4.1 Ethanol Pharmacokinetics and FAEE Profile

Ethanol disposition exhibited Michaelis-Menten kinetics (Figure 3.2). The average Vmax, Km, and AUC$_{0-t}$ (area-under-the-blood-versus-ethanol-time-curve) values were $0.86 \pm 0.19$ mg/dL/min, $3.23 \pm 1.58$ mg/dL, and $27,897 \pm 5,434$ mg/dL/min, respectively ($n = 4$). No
significant correlations using the Pearson test were found between hair FAEE (cumulative FAEE, ethyl oleate, or cumulative FAEE corrected to 20 mg) and blood ethanol AUC.

![Blood Ethanol Pharmacokinetics](image)

**Figure 3.2 Blood ethanol concentrations in Long Evans rats (n = 5)**

The FAEE profile was similar in both black and white fur, with average levels of FAEE being highest for ethyl myristate, followed by ethyl stearate, palmitate, and then oleate.
3.4.2 Baseline and Post-Treatment FAEE

Using paired t-tests, cumulative FAEE levels were significantly higher in rat hair after ethanol treatment for both black and white hair (Figure 3.3 and 3.4). Significantly higher levels of FAEE were found when comparing baseline (pre-treatment) levels to both the “patch” hair that grew back post-treatment, and the surrounding “no patch” hair (the hair that was not shaved), and in the “patch” levels compared to in the surrounding “no patch” post-treatment hair.

* Indicates statistical significance as measured by paired t-test

Figure 3.3 Mean cumulative FAEE levels in white rat fur, before and after ethanol treatment, not standardized to 20 mg (paired comparison)
* Indicates statistical significance as measured by paired t-test

Figure 3.4 Mean cumulative FAEE levels in black rat fur, before and after ethanol treatment, standardized to 20 mg (paired comparison)

3.4.3 Effect of Hair Pigment on FAEE

Using the paired t-test, no significant difference could be found between FAEE levels in white and black post-treatment “patch” hair (Figure 3.5).
Figure 3.5 Mean cumulative FAEE concentrations in white and black hair after standardization to 20 mg (paired comparison)

3.5 Discussion

The purpose of the present study was to determine whether FAEE incorporation is affected by hair pigmentation, and our results suggest that it is not. The current study did reveal an effect of initial sample weight on efficiency of extraction, which required standardization of samples to 20 mg for the comparison of FAEE levels between black and white hair. This effect has been noted previously in human hair by Pragst et al., 2001, who reported decreasing extraction yields as
initial sample amount was increased (Pragst et al., 2001). Because we were only able to collect between 10-15 mg of hair for some of the post-treatment “patch” samples in white hair in the present study, standardization to 20 mg was of particular importance, but not all samples (baseline and post-treatment “no patch” white hair) could be standardized, as the effect size was not statistically significant in those treatment groups. Therefore, only post-treatment “patch” samples were compared between black and white hair. However, post-treatment “patch” hair accumulated the highest amount of FAEE, significantly higher than pre-treatment levels and post-treatment “no-patch” levels, therefore making it the most useful treatment category for comparison.

Comparison of post-treatment “patch” hair revealed no significant difference in FAEE incorporation between pigmented and non-pigmented hair, supporting our hypothesis that FAEE incorporation is not affected by hair pigmentation. FAEE are neutral, lipophilic molecules, and melanin granules favor interactions with basic/cationic substances, consequently such an interaction between FAEE and melanin was not expected (Nakahara et al., 1995; Nakahara and Kikura, 1996; Borges et al., 2003; Kikura and Nakahara, 1998). Similar results have been reported by colleagues for the hydrophilic molecule, EtG (Appenzeller et al., 2007b). EtG is a minor metabolite formed during ethanol metabolism when ethanol is glucuronidated with activated glucoronic acid instead of water (Pragst and Yegles, 2007). Its use as a biomarker in hair for the assessment of excessive alcohol use has recently been investigated, and Appenzeller et al demonstrate no significant difference in the incorporation of EtG between pigmented and non-pigmented hairs of humans with graying hair (n = 21) (Appenzeller et al., 2007b). In fact, the investigators report a correlation of 0.99 between EtG results from pigmented and non-
pigmented hair. Therefore, it appears that substances can be largely incorporated into hair yet not be influenced by hair colour bias, as appears to be the case for FAEE.

The present study is the first to describe the use of the LE rat as a model for hair FAEE research. The LE rat has some differences from the human that need to be acknowledged, although altogether provides a suitable model for this research. Firstly, LE rat hair contains only eumelanins (the pigment responsible for dark brown or black hair tones) (Slawson et al., 1998), whereas human hair contains both eumelanins and pheomelanins (the pigment responsible for red tones) (Borges et al., 2001a). Pigment granules comprise less than 3% of total hair fiber mass (Robbins, 2002), and the concentration of eumelanin in LE rat hair has been measured to be 17.56 ± 0.61 µg/mg in pigmented hair (undetectable in non-pigmented hair) (Slawson et al., 1998), and to range from 2-15 µg/mg in human hair of varying colours from individuals of different ethnicity (Borges et al., 2001a). Different colours of human hair contain different proportions of these pigments. Black hair contains approximately 99% eumelanin and 1% pheomelanin, brown and blond hair contain approximately 95% eumelanin and 5% pheomelanin, whereas red hair contains approximately 67% eumelanin and 33% pheomelanin (Borges et al., 2001a). However, the fact that LE rat hair may contain a higher concentration of eumelanin than human hair, and that it does not contain pheomelanin, does not affect the primary end point of the present study, to determine if FAEE incorporation is affected by hair pigment. Studies have shown that although some drugs interact with pheomelanins, those interactions are typically much weaker than those with eumelanins, and consequently of less importance (Gygi et al., 1996; Potsch et al., 1997; Slawson et al., 1998; Mars and Larsson, 1999; Rollins et al., 2003; Borges et al., 2003). Furthermore, if FAEE were interacting with eumelanin during incorporation leading to increased incorporation in pigmented hair, the fact that LE rats have a greater
concentration of this pigment present in their hair relative to humans would result in a pronounced effect of higher FAEE levels in pigmented hair using the LE model. However, this was not observed in the present study confirming the hypothesis that FAEE incorporation is not affected by hair pigment.

Another difference between the LE rat and humans is the metabolic rate of the LE rat is much higher, with Vmax values of $0.86 \pm 0.19 \text{mg}\times\text{dL}^{-1}\times\text{min}^{-1}$, compared to $0.47 \text{mg}\times\text{dL}^{-1}\times\text{min}^{-1}$ in the human (Gubala and Zuba, 2003). Therefore, rats may require longer periods of exposure to achieve comparable hair levels of FAEE. In the current experiment FAEE hair levels in the rat were much lower than those typically seen in humans, but this is non-consequential as levels were still measurable to examine our primary endpoint.

A relationship between blood ethanol and hair FAEE was not observed in the current study as has been seen in a previous study for both the guinea pig and humans (Kulaga et al., 2006). The present experiment was tightly controlled to achieve equal blood ethanol levels among laboratory animals, therefore there may not have been enough variation in blood ethanol concentration in order to detect such a correlation.

Also noteworthy, is the fact that the profile of FAEE incorporation in hair did differ in rats as compared to humans, FAEE levels being highest for ethyl myristate, followed by ethyl stearate, palmitate, and then oleate in the rat, whereas in the human levels of ethyl oleate predominate followed by ethyl palmitate, stearate and myristate (Kulaga et al., 2006). The difference in FAEE profile among species, likely reflects a natural difference in fatty acid composition. Such differences also occur between matrices such as meconium, blood and various organ tissues.
(Doyle et al., 1994; Laposata, 1998b; Laposata and Lange, 1986; Bearer et al., 2005; Bearer et al., 2003; Moore et al., 2003; Klein et al., 1999; Chan et al., 2004a), and may also occur across different ethnic groups in humans. This finding stresses the importance of measuring cumulative levels of multiple FAEE as oppose to a single FAEE. Altogether, however, the LE rat appears to be a suitable model for hair FAEE research as it is sensitive enough to detect chronic alcohol use after a brief 3-week administration period.

Furthermore, an interesting finding was uncovered when we examined the relationship between post-treatment FAEE levels in “patch” and “no patch” hair that may contribute towards our knowledge of the mode of FAEE incorporation. For both the standardized black hair samples, and non-standardized white hair samples, cumulative levels of FAEE were found to be significantly higher post-treatment in both the “patch” hair (hair that grew back post-treatment), and the surrounding “no patch” hair (hair that was not shaved), however levels in the “patch” hair were also significantly higher compared to in the surrounding “no patch” hair. This suggests that FAEE preferentially accumulate in actively growing hair over hair in the resting phase.

Substances are incorporated into hair through blood, sweat, and sebum, and the amount of incorporation is affected by the amount of exposure the hair receives from each of these sources, the pH of the surrounding mediums, physicochemical properties of the analyte, opportunity and ability of the analyte to penetrate the hair cells, and binding sites for the analyte within the hair matrix (Kikura and Nakahara, 1998; Kintz, 2007). Previously, FAEE have been described to primarily incorporate into hair through sebum (Auwarter et al., 2001; Pragst and Balikova, 2006). Our results are not in opposition to this hypothesis because as the rat hair grew it had the opportunity to interact with sebum close to the skin’s surface, and this was likely a contributing source of incorporation. However, our results also suggest that blood may have been an
important additional source of incorporation. Actively growing hair had higher levels of FAEE compared to the surrounding hair that had not been shaved, it did not have as much opportunity to be exposed to sebum as static hair that was present on the rat from the beginning of the experiment. Consequently, an additional source of incorporation likely facilitated higher FAEE accumulation in actively growing hair, and this source was likely blood. Supporting this hypothesis is the fact that vascularity surrounding rat hair cells is known to dramatically increases during the hair growth phase (Durward and Rudall, 1949). Therefore, increased vascularity and blood exposure could provide a substantial increase in FAEE exposure to hair in growing follicles relative to non-growing follicles. However, this increased vascularity may also have enhanced FAEE supply to accompanying sebaceous glands, thereby also increasing FAEE exposure to growing hairs from sebum. Consequently, sebum and blood probably both act as important sources of incorporation for FAEE, a phenomenon that has not been previously documented.

In conclusion, we established the LE rat as a model to investigate the mechanism of hair accumulation of FAEE. Our results indicate that hair accumulation of FAEE is independent of melanin, thus obviating potential bias in interpretation of chronic alcohol abuse, an issue that has marred the interpretation of certain other drugs in various ethnic groups.
4 Hair Analysis of Fatty Acid Ethyl Esters (FAEE) in the Detection of Excessive Drinking in the Context of Fetal Alcohol Spectrum Disorders (FASD) (Kulaga et al., 2009)

4.1 Abstract

A serious challenge in diagnosing FASD is the need to document alcohol use during pregnancy. Maternal/paternal alcohol abuse affect the likelihood of fetal alcohol exposure, and hence the occurrence of FASD. The objective of the current study was to document the use of the FAEE hair test, a biomarker of excessive alcohol use, in parents at-risk of having children with FASD, and quantify the prevalence of testing positive for excessive alcohol use in this population. Hair samples submitted for FAEE testing between October 2005 and May 2007 were evaluated (n = 324). Subjects consisted of at-risk children’s parents. Samples were analyzed using a previously published method. Briefly, samples underwent a liquid-liquid extraction, followed by HS-SPME, and were then analyzed by GC-MS using deuterated FAEE as internal standards. LOD and LOQ values were between 0.01 - 0.04 ng/mg, and 0.04 – 0.12 ng/mg, respectively. Positive levels for excessive drinking were ascertained using a cut-off level of 0.5 ng/mg, offering 90% sensitivity and specificity. The rate of positive hair samples for excessive drinking was 33.3 % (32.4 %
among women, and 35.4% among men) (n = 324). The majority of samples (62%) had
cumulative FAEE levels above a level that excludes strict abstinence (0.2 ng/mg) and many (19
%) were highly positive (above 1.0 ng/mg). Out of 26 FAEE hair tests where women were
reported to be pregnant, 38% had FAEE hair levels above 0.2 ng/mg, and 19% tested positive
for excessive drinking with levels above 0.5 ng/mg, and 12% had levels above 1.0 ng/mg. The
high rate of positive FAEE results demonstrates that the FAEE hair test corroborates the clinical
suspicion of alcohol use in parents of children at-risk for FASD. Our results suggest that FAEE
hair analysis may be a powerful tool in detecting heavy alcohol use in the perinatal period.
4.2 Introduction

FASD is the most prevalent cause of neurocognitive handicap among children in North America (Abel and Sokol, 1987; Abel and Sokol, 1986). The frequency of this debilitating condition has been estimated to occur as high as 1 in 100 births (Abel, 1995a; Sampson et al., 1997), but the majority of these cases remain undiagnosed until school age or later, if diagnosed at all (Streissguth et al., 2004; Olson et al., 2007). One of the most serious challenges in diagnosing FASD is the need to establish evidence of excessive maternal drinking during pregnancy as one of the major diagnostic criteria (Chudley et al., 2005). However, maternal self-reports of alcohol use are often unreliable due to fears of stigmatization, embarrassment, shame or guilt (Russell et al., 1996; Ernhart et al., 1988; Alvik et al., 2006). Therefore, establishing a biological marker that can detect chronic excessive alcohol use during pregnancy would provide a tremendous advantage in detecting children at risk for FASD and in diagnosing FASD in affected children.

In 2001, Pragst and colleagues established a hair test measuring fatty acid ethyl esters (FAEE), a group of non-oxidative metabolites of ethanol (Pragst et al., 2001). FAEE have previously been measured in pancreas, liver, heart, brain, and white blood cells (Laposata and Lange, 1986; Laposata et al., 1987; Ben-Eliyahu et al., 1996). FAEE have also been shown to be useful intermediate markers of alcohol exposure in blood (Doyle et al., 1994; Doyle et al., 1996), and post-mortem markers of pre-mortem alcohol use in adipose and liver tissue (Refaai et al., 2002). Furthermore, FAEE have been used as markers of fetal alcohol exposure in meconium (Chan et
al., 2004a), with the test offering high sensitivity and specificity, however being limited by the fact that meconium is only available for the first 2-3 days of infant life. Hair testing offers the unique advantage over conventional matrices such as blood and urine, because it provides cumulative information regarding past and chronic substance use. In the neonate, hair is available for the first 1-3 months of life, or longer, and is representative of exposures during the last trimester, as this is when the hair is formed. In the mother, hair is available for longer periods, and the proximal 6 cm to the scalp, which is measured, is representative of the 6 months prior to collection.

The FAEE hair test measures cumulative levels of four FAEE, ethyl myristate, palmitate, oleate, and stearate, in hair, and has been shown to be highly sensitive and specific in the detection of excessive drinking in adults (Pragst and Balikova, 2006; Wurst et al., 2004). Low baseline levels of FAEE can exist in non-drinkers, therefore it has been necessary to use a cut-off level to differentiate heavy drinkers from social and non-drinkers. A cut-off level of 0.5 ng of cumulative FAEE per mg hair offers 90% sensitivity and specificity, with respect to the identification of heavy drinking (Pragst and Balikova, 2006). Levels between 0.2-0.5 ng/mg are indicative of social use and typically exclude strict abstainers, whereas levels in excess of 1.0 ng/mg are nearly 100% specific to heavy alcohol use, but offer lower sensitivity so that many cases may be missed (Pragst et al., 2001; Auwarter et al., 2001; Pragst and Balikova, 2006; Pragst and Yegles, 2008). Since its inception, the FAEE hair test has been increasingly used in the medical context of alcohol abuse and the medicolegal context of “driving under the influence” (Wurst et al., 2008a; Pragst and Yegles, 2008), however the test is now being expanded into other areas.
More recently, a pilot study in pregnant women with low alcohol consumption, was performed employing the FAEE hair test, among other biomarkers, to evaluate the additional benefit alcohol biomarkers would provide over maternal self report for alcohol use in pregnancy (Wurst et al., 2008a). The study demonstrated a substantial benefit of the combined use of several biomarkers over maternal self-report in detecting heavy drinking during pregnancy. The study was conducted in a relatively low-risk population with respect to FASD, and accordingly 3 % were found positive for excessive alcohol use by FAEE hair test, and 7 % by combined FAEE and ethyl glucuronide hair tests (Wurst et al., 2008a). EtG is another minor metabolite of alcohol metabolism formed when alcohol is glucuronidated with activated glucoronic acid. Studies investigating the measurement of EtG in hair have demonstrated the test to be highly specific in the detection of heavy alcohol users, however appears to lack sensitivity in detecting alcohol users that use moderate to heavy amounts, unlike the FAEE hair test that detects most social drinkers (Skopp et al., 2000; Alt et al., 2000; Janda et al., 2002; Yegles et al., 2004; Jurado et al., 2004; Klys et al., 2005; Politi et al., 2006; Morini et al., 2006; Politi et al., 2007; Appenzeller et al., 2007a; Appenzeller et al., 2007b; Politi et al., 2008; Pragst and Yegles, 2008). Therefore, the FAEE hair test may be better suited in the detection of alcohol use during pregnancy, when women may curb their typical alcohol intake.

To the best of our knowledge, the tremendous advantages of the FAEE hair test have not been previously applied in the detection of excessive alcohol use in the context of populations at high risk for having children with FASD. Alcohol use has been demonstrated to be a major problem in many families involved in the child welfare system, putting this population at high risk for producing offspring with FASD (Curtis and McCullough, 1993; Freisthler et al., 2007; Freisthler and Weiss, 2008). A recent Canadian study revealed that out of children with disabilities in the
care of child welfare services in Manitoba, 34 % were diagnosed with FASD, representing 11 %
of all children in care (Gough and Fuchs, 2006). The objective of the current study was to
document the use of the FAEE hair test as a biomarker of excessive alcohol use by parents in an
at-risk population for FASD, and to quantify the prevalence of alcohol abuse in this population.
It is our hypothesis that the rate of excessive alcohol use in this at-risk population will be much
higher than that which has been found in the general obstetric population (Wurst et al., 2008a),
or in the surrounding general Canadian population.

4.3 Subjects and Methods

4.3.1 Study Sample

Starting in October 2005, we established a diagnostic program for hair analysis of FAEE to
diagnose excessive parental drinking. For this study, all samples ordered for FAEE testing at the
Motherisk Laboratory by any children’s aid society (CAS) between October 2005 and May 2007
were evaluated (n = 324). CAS clients that underwent hair testing consisted of children’s parents,
and all CAS organizations were based in Canada with the majority residing in Ontario and
British Columbia. Data regarding common reasons for requesting hair FAEE testing are
presented in Table 4.1.
Table 4.1 Common reasons for social workers requesting FAEE hair testing (reasons are not mutually exclusive, therefore several reasons may have been indicated for a given case).

<table>
<thead>
<tr>
<th>Reason</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third party reports alleging alcohol abuse</td>
<td>35</td>
</tr>
<tr>
<td>Admission to using some amount of alcohol</td>
<td>29</td>
</tr>
<tr>
<td>Substantiated history of drinking</td>
<td>26</td>
</tr>
<tr>
<td>Suspicion of potential alcohol abuse because of proven abuse of other drugs</td>
<td>25</td>
</tr>
<tr>
<td>Social worker requested all available drug hair tests in order to &quot;cover their bases&quot;</td>
<td>20</td>
</tr>
<tr>
<td>Recorded criminal behaviour associated with alcohol use</td>
<td>13</td>
</tr>
<tr>
<td>Recorded ethanol intoxication or testing positive for alcohol</td>
<td>10</td>
</tr>
<tr>
<td>Admission to heavy amounts of alcohol use</td>
<td>8</td>
</tr>
<tr>
<td>To confirm subject's abstinence from alcohol</td>
<td>8</td>
</tr>
<tr>
<td>Observed drinking paraphernalia at the home</td>
<td>6</td>
</tr>
<tr>
<td>Previous treatment for alcohol abuse</td>
<td>5</td>
</tr>
<tr>
<td>Suspected partner/spouse of alcohol abuse</td>
<td>3</td>
</tr>
<tr>
<td>Previous child affected by fetal alcohol syndrome</td>
<td>1</td>
</tr>
<tr>
<td>Serious incident involving intoxication that had resulted in hospitalization</td>
<td>1</td>
</tr>
</tbody>
</table>
4.3.2 FAEE Analysis

All samples were processed and analyzed in either Toronto, Canada (n = 186) or in Berlin, Germany (n = 138), using the same method. The reason for the use of two laboratories was because earlier samples were processed in Berlin prior to the test being clinically available in Toronto. Method reliability between laboratories was assessed by comparing 35 samples tested concurrently by both laboratories. The results confirmed the test to be reproducible with 94% agreement between both laboratories, therefore all available data were included in the analysis.

The sample preparation, and HS-SPME/GCMS conditions for FAEE hair analysis have been previously described (Pragst et al., 2001; Auwarter et al., 2001). Briefly, samples of the proximal 6 cm hair, representing the last 6 months of growth, were obtained and rinsed with water, followed by a 30-minute wash in heptane. The hair was then finely chopped into fragments between 1-3 mm, and approximately 20 mg was used for each sample. The samples then underwent a 15-hour liquid-liquid extraction using dimethyl sulfoxide and n-heptane. The extracts were then isolated by phase separation and evaporation, and re-suspended in 1 mL of 0.1 M phosphate buffer (pH 7.6). The samples then underwent head-space solid phase microextraction (HS-SPME) and were analyzed by gas chromatography-mass spectrometry (GC-MS) using deuterated FAEE as internal standards. FAEE analyzed include: ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate. Instrumental co-efficient of variation (cv) values for same-day (intraday) results were below 3%, whereas between-day (interday) cv values were below 5%. The lowest limit of detection (LOD) and lowest limit of quantification (LOQ) were 0.02 ng/mg and 0.05 ng/mg for ethyl myristate, 0.02 ng/mg and 0.07 ng/mg for ethyl palmitate,
0.04 ng/mg and 0.12 ng/mg for ethyl oleate, and 0.01 ng/mg and 0.04 ng/mg for ethyl stearate, respectively. Positive levels for excessive drinking were defined as those equal to, or above, 0.50 ng of cumulative FAEE per mg of hair, a cut-off value which has previously been documented to be 90% sensitive and specific in detecting chronic heavy alcohol use (Pragst and Balikova, 2006).

### 4.4 Results

#### 4.4.1 Prevalence of Excessive Drinking among Parents

The cohort consisted of 324 subjects, 225 (69 %) women, 96 (30 % men), and for 3 (1%) individuals the gender was not recorded. Mean age of the cohort was 37 (range 18-66). The rate of positive samples (≥ 0.5 ng/mg) was 33.3 % (32.4 % among women, and 35.4 % among men) (n = 324), with similar proportions testing positive between both laboratories for both genders, confirming inter-lab reliability (Figure 4.1). The majority of samples had cumulative FAEE levels above 0.2 ng/mg (62 %), and one fifth was above 1.0 ng/mg (19 %). The clinical distribution of the measured hair FAEE concentrations is presented in Figure 4.2. Ethyl oleate, followed by ethyl palmitate, were the most prevalent esters when comparing average ester values for all tests, and for samples that tested positive.
Figure 4.1 Percent of positive (≥ 0.5 ng/mg) FAEE tests among parents
Figure 4.2 Levels of hair FAEE in a cohort of parents (n = 324); alcohol abuse almost certain above 1.0 ng/mg, most probable above 0.5 ng/mg, and moderate drinking likely above 0.2 ng/mg
4.4.2 Prevalence of Excessive Drinking among Pregnant Participants

Pregnancy status was available for a subset of 119 cases (mean age 28, range 19 - 50). Of these 119 cases, 26 (23 %) were pregnant either during hair sampling or within the last 6 months prior to sampling. Of these 26, 10 (38 %) had FAEE hair concentrations above a level that typically excludes strict abstinence (0.2 ng/mg) and 5 women (19 %) tested positive for excessive drinking, with levels above 0.5 ng/mg (Figure 4.3). Three of these 5 women had levels above 1.0 ng/mg. In 24 of the pregnant cases, social workers provided approximate pregnancy status during the time of hair sampling, or birth/due date of the baby (Table 4.2). In cases where social workers gave the date of birth or due date of the child, pregnancy status at the time of sampling was estimated by assuming a full term pregnancy (unless otherwise stated by the social worker) and using the due/birth date and the date of collection (or requisition if collection date was not available, as was the case in 2 cases).
Figure 4.3 Levels of hair FAEE in a cohort of pregnant women (n = 26); alcohol abuse almost certain above 1.0 ng/mg, most probable above 0.5 ng/mg, and moderate drinking likely above 0.2 ng/mg
Table 4.2 Hair levels of FAEE and approximate pregnancy status at the time of hair sampling for women reported to have been pregnant within the 6 months prior to hair sampling

<table>
<thead>
<tr>
<th>FAEE (ng/mg)</th>
<th>Approximate pregnancy status at sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.20</td>
<td>1 month postpartum</td>
</tr>
<tr>
<td>1.84</td>
<td>1 month postpartum*</td>
</tr>
<tr>
<td>1.24</td>
<td>2 months postpartum</td>
</tr>
<tr>
<td>0.67</td>
<td>3 months pregnant</td>
</tr>
<tr>
<td>0.64</td>
<td>Aborted a pregnancy within the last 6 months</td>
</tr>
<tr>
<td>0.46</td>
<td>&lt;1 month postpartum</td>
</tr>
<tr>
<td>0.45</td>
<td>Miscarried within the last 6 months</td>
</tr>
<tr>
<td>0.39</td>
<td>8 months pregnant</td>
</tr>
<tr>
<td>0.24</td>
<td>Unknown</td>
</tr>
<tr>
<td>0.23</td>
<td>7 months pregnant</td>
</tr>
<tr>
<td>0.19</td>
<td>2 months pregnant</td>
</tr>
<tr>
<td>0.18</td>
<td>7 months pregnant</td>
</tr>
<tr>
<td>0.18</td>
<td>3.5 months postpartum</td>
</tr>
<tr>
<td>0.17</td>
<td>3 months pregnant</td>
</tr>
<tr>
<td>0.16</td>
<td>7 months pregnant</td>
</tr>
<tr>
<td>0.13</td>
<td>Miscarried within the last 6 months</td>
</tr>
<tr>
<td>0.11</td>
<td>8 months pregnant</td>
</tr>
<tr>
<td>0.09</td>
<td>2 months pregnant*</td>
</tr>
<tr>
<td>0.06</td>
<td>6 months pregnant</td>
</tr>
<tr>
<td>0.05</td>
<td>20-25 weeks pregnant</td>
</tr>
<tr>
<td>0.04</td>
<td>2 months postpartum</td>
</tr>
<tr>
<td>0.01</td>
<td>9 months pregnant</td>
</tr>
<tr>
<td>0.00</td>
<td>Unknown</td>
</tr>
<tr>
<td>0.00</td>
<td>4 months pregnant</td>
</tr>
<tr>
<td>0.00</td>
<td>&lt;1 month postpartum</td>
</tr>
<tr>
<td>0.00</td>
<td>4 months pregnant</td>
</tr>
</tbody>
</table>
*Exact date of sample collection was unavailable, therefore dating was estimated based on sample requisition data

Infant was recorded as being born 2 months premature

4.5 Discussion

The current study revealed a high rate of excessive alcohol use among parents investigated by children’s aid authorities (33 %), with the majority of samples having FAEE levels above a level which typically excludes strict abstinence. We also report one of the highest rates of excessive drinking (19 %) ever reported among pregnant women (Ebrahim et al., 1999; Ebrahim et al., 1998; Stewart and Streiner, 1994; Ethen et al., 2008; Alvik et al., 2006; CDC, 2004; CDC, 2002; CDC, 1997; Sokol et al., 1980; Chambers et al., 2005; Chambers et al., 2006; Colvin et al., 2007; O'Connor and Whaley, 2003; Houet et al., 2006; Crome and Kumar, 2007; Caetano et al., 2006; Magnusson et al., 2005; Alvik et al., 2006). In accordance with our hypothesis, the rate of excessive alcohol use in this cohort greatly exceeded that which has been found in the general obstetric population, by hair analysis (7 %) (Wurst et al., 2008a), and which has been reported for the general adult Canadian population. According to the Canadian Addiction Survey of 2004,
79.3% of Canadians (78.7% of Ontarians, 79% of British Columbians) reported they consumed alcohol in the past 12 months, however only 7.9% (10.2% of Ontarians, 8.2% of British Columbians) consumed alcohol more than 4 times a week, and only 12.7% (12.4% of Ontarians, 13.3% of British Columbians) said their typical usage pattern of alcohol was more than 5 drinks on occasion (Adlaf et al., 2005). The present cohort was selected for FAEE testing by social workers, therefore a higher prevalence of alcohol use was expected; nevertheless, the high level of alcohol use confirms this population is at high risk for having children with FASD and in need of intervention.

Positive FAEE samples are defined as those equal to or above 0.5 ng/mg of FAEE in hair, and represent “heavy” or “excessive” chronic alcohol users. The studies upon which the cut-off level has been established, define heavy drinkers as alcoholic patients in treatment programs and report the amounts of alcohol they consumed (Auwarter et al., 2004; Wurst et al., 2004; Auwarter et al., 2001). The reported amounts are between 50 - 400 g ethanol/day (Auwarter et al., 2004; Auwarter et al., 2001), and 960 - 7600 g ethanol in the last month, mean 4440 g (representative of the last 6 months) (Wurst et al., 2004). Therefore, if one converts these amounts into North American standard drinks (SD) (Bondy et al., 1999; National Institute on Alcohol Abuse and Alcoholism, 2005), heavy drinkers, or excessive alcohol users, according to the 0.5 ng/mg cut-off are defined as people who consume between approximately 4 - 29 drinks a day or 70 - 558 drinks (mean 326 drinks) a month.

It is clear from this definition of heavy drinking that testing positive for hair FAEE is indicative of severe chronic alcohol use, and represents alcohol consumption in great excess of the Canadian low-risk drinking guidelines (Bondy et al., 1999). The high FAEE cut-off level is
extremely important, as it identifies individuals well within the alcohol exposure known to cause FASD and generally avoids lower levels, for which the association with FASD is still controversial (Jacobson and Jacobson, 1994). While the cut off of 0.5 ng/mg may exclude some women who drink excessively (10%), for the most part (90%), it avoids the serious issue of misclassifying social drinkers as chronic serious drinkers, and thus, minimizes the complexities of wrong assignment of pregnant women as alcohol abusers.

In addition, examining alcohol use in the family, specifically drinking by male partners of women, as a contributing factor in the occurrence of FASD is an issue that has not received much attention. Clearly maternal alcohol use during pregnancy is a necessary factor in the etiology of FASD, however, several studies have discussed the role of paternal alcohol use both as a direct teratogenic factor, and as a contributing factor towards maternal alcohol use before and during pregnancy. Several animal studies have demonstrated direct effects of paternal alcohol use resulting in a number of genetic conditions, birth defects, and negative impacts on cognitive ability and intelligence in the offspring, however this field remains largely understudied (Gearing et al., 2005; Abel and Tan, 1988; Abel, 1995b; Abel et al., 1990; Abel, 1993; Bielawski and Abel, 1997; Abel, 1991). Most importantly, paternal alcohol use has been shown to be a strong determinant of maternal alcohol use during pregnancy (Gearing et al., 2008). For example, having a stable relationship with strong emotional and social support, and a planned pregnancy can help to reduce alcohol use during pregnancy, whereas having an alcoholic partner who is not supportive of alcohol abstinence efforts or perhaps abusive can have the opposite effect (Gearing et al., 2005; Gearing et al., 2008). Alcohol use in the family has also been linked to increased risk of child abuse and future alcohol abuse by children, predisposing children to behaviors perpetuating the occurrence of FASD as they grow into child bearing years.
(Gearing et al., 2005; Jaudes et al., 1995; Smith et al., 2007; Fluke et al., 2008a; Barr et al., 2006; Baer et al., 2003; Baer et al., 1998; Seljamo et al., 2006). Therefore, a familial approach in the detection of alcohol use is warranted for advances in FASD research and intervention programs.

It is important to acknowledge the limitations of the current study. The present study was based on the evaluation of clinical records and corresponding test results of requested samples. Therefore, data regarding subjects such as sex, pregnancy status, demographic information, and reported alcohol intake, was limited, and if available was provided by the social worker. Also, the possibility of false positives has been documented in individuals using hair products containing over 62.5% ethanol on a regular basis (Hartwig et al., 2003), therefore, clinical data should be evaluated in conjunction with FAEE hair test results in order to accurately assess alcohol exposure. It would also have been interesting to compare maternal FAEE levels in relation to paternal FAEE, however due to the limitations of the study design this was not possible.

Nevertheless, the results of the current study demonstrate that the FAEE hair test is effective in corroborating clinical suspicion of heavy alcohol use in families suspected of abusing alcohol based on clinical and social data, and that these families are indeed at greater risk of having children with FASD. The present study is also the first to describe the use of FAEE hair analysis in establishing heavy drinking in pregnancy, a necessary criteria for the diagnosis of FASD, in a population at-risk for producing children with FASD. The FAEE hair test provides a unique advantage of being able to confirm or refute clinical suspicions of chronic alcohol abuse in an objective manner. Our results suggest that FAEE hair analysis may be a powerful tool in detecting heavy alcohol use in the perinatal context to help target resources to reduce the
incidence and severity of FASD. Further research using the FAEE hair test in pregnant women and their offspring is recommended.
5 Agreement between FAEE Hair Test for Alcohol and Social Workers’ Reports

5.1 Abstract

Aims: To examine the relationship between social worker reports and the novel FAEE biological marker. Design, Setting, Methods: In 2005 in Toronto, Canada, we established a new diagnostic program measuring hair FAEE to detect excessive parental drinking in a high-risk population for having children with FASD. A cross-sectional study of all cases submitted by CPS between May-December of 2007 (n = 172) was performed comparing social worker reports to FAEE test outcome using odds ratio analysis. A sub-analysis of mothers (n = 119), excluding fathers, was also performed. Results: Factors associated with testing positive for hair FAEE in parents, and mothers alone, were: the social worker having knowledge of a specific instance of problem drinking within the past 6 months (OR = 5.11, 2.57-10.16), (OR = 8.51, 3.59-20.18), respectively, and having received a third party report in regards to parental drinking (OR = 3.31, 1.69-6.46), (OR = 3.30, 1.45-7.50), respectively. Also, having mothers admit to heavy drinking (OR = 6.74, 1.50-30.38) was associated with testing positive for hair FAEE. Factors negatively associated with testing positive for hair FAEE were: social workers testing for FAEE without the suspicion of alcohol use but rather as a measure to “cover all bases”, or because of a history/suspicion of illicit drug use (OR= 0.09, 0.02 – 0.40), (OR = 0.2, 0.07-0.55), respectively,
and in mothers alone, (OR = 0.13, 0.03-0.58), (OR = 0.26, 0.08-0.80), respectively.

Furthermore, clinical agreement of 11 out of 15 social worker reports was observed between the amount of alcohol consumed by parents and FAEE test outcome. Conclusion: This is the first time the FAEE hair test is being used in the context of assessing parental alcohol use in a high-risk population and our results show the FAEE hair test agrees well with social worker reports. Reported factors directly related to alcohol use were significantly associated with testing positive for excessive alcohol use, whereas factors not directly related to alcohol use were negatively associated with testing positive.
5.2 Introduction

The consumption of alcohol during pregnancy can result in birth defects, neurodevelopmental delay, learning disabilities, and maladaptive behaviour (Sokol et al., 2003; Streissguth, 1992; Streissguth et al., 1994; Baer et al., 2003; Streissguth et al., 2004). FASD is one of the most prevalent causes of cognitive handicaps among children in North America (Abel and Sokol, 1987; Abel and Sokol, 1986; Abel, 1995a; Sampson et al., 1997; Abel, 1995a). Diagnosis requires the establishment of maternal alcohol consumption during pregnancy (Chudley et al., 2005); however, maternal self-reports of are often unreliable (Russell et al., 1996; Ernhart et al., 1988; Wurst et al., 2008a; Alvik et al., 2006; Alvik et al., 2006; Alvik et al., 2006), therefore the majority of cases are not diagnosed (Streissguth et al., 2004; Olson et al., 2007). Identification and diagnosis are key for intervention, however families at risk for having children with FASD are equally hard to identify because parents are reluctant to divulge heavy drinking habits. This has led us to propose using the FAEE hair test as an objective biological marker to assess parental alcohol use in the context of FASD research and diagnosis.

FAEE are a group of non-oxidative metabolites produced when ethanol is esterified to fatty acids in the body by one of several enzymes, primarily FAEE synthase (Laposata, 1998b). First detected in rabbit myocardial tissue (Lange et al., 1981) and suspected of being involved in ethanol induced toxicity (Lange, 1982; Laposata and Lange, 1986; Laposata, 1998a), FAEE have since been detected in human pancreas, liver, heart, brain, and white blood cells (Laposata and Lange, 1986; Laposata et al., 1987; Ben-Eliyahu et al., 1996). FAEE have also been shown to be
useful intermediate markers of alcohol exposure in blood (Doyle et al., 1994; Doyle et al., 1996), and retrospective markers of alcohol use post-mortem in adipose and liver tissue (Refaai et al., 2002). Also, FAEE have been used as markers of fetal alcohol exposure in meconium with success (Chan et al., 2004a; Bearer et al., 2003), however meconium is only available for the first 2-3 days of infant life. Hair on the other hand, is available for 1-3 months or more after birth in the neonate, and longer in the mother.

Cumulative levels of FAEE, specifically, ethyl myristate, palmitate, oleate, and stearate, have been measured in the hair of alcoholics and have successfully distinguished them from social and non-drinkers (Pragst and Balikova, 2006; Wurst et al., 2004). Low baseline levels of FAEE exist in non-drinkers, therefore a cut-off level needs to be used in order to differentiate heavy drinkers from social and non-drinkers. A cut-off level of equal to or above 0.5 ng of cumulative FAEE per mg hair has been established to differentiate excessive alcohol use (a positive result) for social use or abstinence. This cut-off level offers 90% sensitivity and specificity in detecting excessive drinking (Pragst and Balikova, 2006). Levels between 0.2-0.5 ng/mg are typically indicative of social use and generally exclude strict abstainers, whereas levels exceeding 1.0 ng/mg are considered highly positive, and are nearly 100% specific to heavy alcohol use, but offer lower sensitivity. Consequently, the cut-off level of 0.5 ng/mg is used clinically to distinguish a positive result (Pragst et al., 2001; Auwarter et al., 2001; Pragst and Balikova, 2006; Pragst and Yegles, 2008).

Until recently the FAEE hair test was primarily used in the clinical context of diagnosing heavy alcohol use in alcohol abusers, and in the medico-legal context of assessing cases involved with driving under the influence of alcohol (Wurst et al., 2008b; Kintz, 2007). However, a recent pilot
study using the FAEE hair test in pregnant women with low alcohol consumption was performed (Wurst et al., 2008a). The study employed the FAEE hair test among other biomarkers including the ethyl glucoronide hair test, to evaluate the additional benefit of using alcohol biomarkers over maternal self-report for assessing alcohol use in pregnancy (Wurst et al., 2008a). The study demonstrated a substantial benefit of biomarkers over maternal self-report. The FAEE hair test found 3% of women positive for excessive alcohol use, and 7% positive by combined FAEE and EtG hair tests (Wurst et al., 2008a). EtG is another minor metabolite of alcohol metabolism formed when alcohol is conjugated with activated glucoronic acid. Studies investigating the measurement of EtG in hair have demonstrated the test to be highly specific in the detection of heavy alcohol users, but lacking sensitivity with respect to the detection of moderate alcohol users, and some heavy alcohol users (Skopp et al., 2000; Alt et al., 2000; Janda et al., 2002; Yegles et al., 2004; Jurado et al., 2004; Klys et al., 2005; Politi et al., 2006; Morini et al., 2006; Politi et al., 2007; Appenzeller et al., 2007a; Appenzeller et al., 2007b; Politi et al., 2008; Pragst and Yegles, 2008). The FAEE hair test appears to be more sensitive, detecting even most social drinkers and therefore may be better suited for the detection of alcohol use during pregnancy, when women may curb their typical alcohol intake.

In October 2005, our laboratory established a new diagnostic program using FAEE hair analysis in order to diagnose excessive parental drinking. Since that time period we have observed an overall 33% level of positivity for heavy drinking (Kulaga et al., 2008), indicating that the cohort of parents we test is at high-risk for having children with FASD. The majority of cases referred for FAEE testing are referred by CPS for suspicion of alcohol abuse, however some are referred for other reasons such as to confirm abstinence, for example. Given this is the first time to apply the FAEE hair test in the context of assessing parental alcohol use in an at-risk
population, we wanted to know if agreement between social worker reports and FAEE test outcome exists. Therefore, the objective of the present study was to examine the relationship between social worker reports and the novel FAEE biological marker.

5.3 Materials and Methods

5.3.1 Study Sample

All samples ordered for FAEE testing at the Motherisk Laboratory by any CPS between May and December of 2007, were included in the analysis (n =172). Social worker reports consisted of clinical intake questionnaire filled out over the telephone upon requisition of the test. All CPS organizations were based in Canada with the majority residing in Ontario and British Columbia.

5.3.2 FAEE Analysis

All samples were processed and analyzed for FAEE using methodology adapted from a previously published method (Pragst et al., 2001). Sample preparation, HS-SPME, and GCMS conditions for FAEE hair analysis are described in detail in published works (Pragst et al., 2001; Auwarter et al., 2001). Briefly, 6 cm hair samples, representing the last 6 months of growth, were extracted for 15 hours using a liquid-liquid extraction involving dimethyl sulfoxide and n-heptane, after which the extracts were isolated by phase separation and evaporation, then re-
suspended in 0.1 M phosphate buffer (pH 7.6). The samples then underwent HS-SPME and were analyzed by GCMS using deuterated FAEE as internal standards. Instrumental cv values for same-day (intraday) results were below 3%, whereas between-day (interday) cv values were below 5%. LOD and LOQ were 0.015 ng/mg and 0.05 ng/mg for ethyl myristate, 0.02 ng/mg and 0.07 ng/mg for ethyl palmitate, 0.04 ng/mg and 0.12 ng/mg for ethyl oleate, and 0.01 ng/mg and 0.04 ng/mg for ethyl stearate, respectively.

5.3.3 FAEE Hair Test Intake Questionnaire Filled by Social Workers

The questionnaire consisted of 4 questions (3 yes/no, and 1 open-ended) that were administered to social workers over the telephone upon requisition of the FAEE hair tests. The questions were:

1. Do you know if the client was pregnant at the time of sampling or in the 6 months prior to sampling?

2. Are you aware of specific instances of problem drinking in this individual over the six months prior to hair sampling?

3. Do you know how much alcohol the person consumed on average either per day or per week, during the 6 months prior to sampling?

4. What led you to suspect alcohol abuse in this patient or what was the reason for requesting the alcohol hair test?
Answers for open-ended question number four were later grouped into 16 categories for analysis: 1) third party reports alleging alcohol abuse, 2) admission to some use of alcohol including heavy amounts, 3) substantiated history of drinking, 4) history or suspicion of illicit drug use, 5) all drug hair tests requested to "cover their bases", 6) recorded criminal behaviour associated with alcohol use, 7) recorded ethanol intoxication or testing positive for alcohol, 8) admission to heavy amounts of alcohol use, 9) to confirm subject's abstinence, 10) observed drinking paraphernalia at the home, 11) previous treatment for alcohol abuse, 12) partner/spouse abuse or suspected abuse of alcohol, 13) by accident, 14) don’t know, 15) previous child affected by fetal alcohol syndrome, 16) serious incident involving intoxication that had resulted in hospitalization.

5.4 Statistics

Odds ratio analysis was performed to evaluate if FAEE levels above 0.5 ng/mg, or 1.0 ng/mg, in parents and in mothers alone, were associated with any risk factors identified by social workers in clinical intake questionnaires. Question 1 was evaluated only in females as it pertained to pregnancy. Question 3 and the following categories for question 4: 13) by accident, 14) don’t know, 15) previous child affected by fetal alcohol syndrome, and 16) serious incident involving intoxication that had resulted in hospitalization, were excluded from odds ratio analysis as data was either insufficient or inconsequential such as “by accident”, for example. Furthermore, data categories could not be grouped for analysis nor could multivariate analysis be performed as the
categories were not mutually exclusive (i.e. many social workers reported several reasons for requesting FAEE test).

5.5 Results

Thirty three percent of parents tested positive for FAEE above 0.5 ng/mg (56/172 parents, and 40/119 women). The social worker having knowledge of a specific instance of problem drinking within the 6 months prior to hair sampling strongly predicted testing positive for heavy alcohol use in parents (OR = 5.11, 2.57 – 10.16), and in mothers alone (OR = 8.51, 3.59-20.18).

Fifteen social workers recorded the amounts of alcohol parents may have consumed on average. Strong agreement between the reported amount of alcohol intake and the FAEE hair test result (positive or negative) was observed; 9 out of 13 subjects that were reported to use heavy amounts of alcohol tested positive, and the two subjects that either claimed abstinence or were reported to use low amounts of alcohol tested negative (Table 5.1).
<table>
<thead>
<tr>
<th>Result (ng/mg)</th>
<th>Gender</th>
<th>Pregnant</th>
<th>Social Worker Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.55</td>
<td>Male</td>
<td>n/a</td>
<td>Self-reported 6 drinks per day</td>
</tr>
<tr>
<td>2.50</td>
<td>Female</td>
<td>No</td>
<td>2-6 drinks, 4-7 times a week</td>
</tr>
<tr>
<td>2.20</td>
<td>Female</td>
<td>Yes</td>
<td>6 drinks every other day</td>
</tr>
<tr>
<td>1.89</td>
<td>Male</td>
<td>n/a</td>
<td>12 drinks per day</td>
</tr>
<tr>
<td>1.79</td>
<td>Female</td>
<td>No</td>
<td>Drank daily, unknown amount</td>
</tr>
<tr>
<td>1.22</td>
<td>Male</td>
<td>n/a</td>
<td>Self-reported 3-4 drinks, 2-4 times a month, sometime 6 or more drinks</td>
</tr>
<tr>
<td>1.02</td>
<td>Female</td>
<td>No</td>
<td>Binge on weekends, unknown amount</td>
</tr>
<tr>
<td>0.74</td>
<td>Female</td>
<td>No</td>
<td>3-4 drinks per day</td>
</tr>
<tr>
<td>0.65</td>
<td>Male</td>
<td>n/a</td>
<td>A few drinks per day</td>
</tr>
<tr>
<td>0.39</td>
<td>Female</td>
<td>Yes</td>
<td>Drank on daily basis</td>
</tr>
<tr>
<td>0.21</td>
<td>Female</td>
<td>No</td>
<td>Self-reported abstinent except on one occasion when she had 2-3 drinks</td>
</tr>
<tr>
<td>0.15</td>
<td>Female</td>
<td>No</td>
<td>15-20 drinks per occasion every weekend or every other weekend</td>
</tr>
<tr>
<td>0.13</td>
<td>Female</td>
<td>No</td>
<td>Self-reported 1-2 beers, 1-2 times a month</td>
</tr>
<tr>
<td>0.02</td>
<td>Female</td>
<td>No</td>
<td>3-5 drinks per day</td>
</tr>
<tr>
<td>0.00</td>
<td>Female</td>
<td>No</td>
<td>A lot, unknown frequency or amount</td>
</tr>
</tbody>
</table>

The three main reasons for requesting an alcohol hair test were 1) receiving a third party report, 2) the parent admitting to using alcohol, and 3) the parent having had a substantiated history of drinking (Table 5.2). (Note the reasons were not mutually exclusive therefore the percent of social workers reporting different reasons will not sum to one hundred percent). Receiving a third party report was significantly associated with testing positive for heavy alcohol exposure in parents, and in women alone (Table 5.2 and 5.3). The fourth and fifth most common reasons for testing positive for hair FAEE were: having a history or suspicion of illicit drug use, and requesting all drug hair tests available in order to “cover the social worker’s bases” and these were negatively associated with testing positive for heavy alcohol use (Tables 5.2 and 5.3).
When women were analyzed separately, self-admission of heavy alcohol use was significantly associated with testing highly positive for hair FAEE (Table 5.3). Twenty-six out of 119 women were reported as being pregnant within the last six months prior to hair sampling, and pregnancy was not associated (positively or negatively) with testing positive for hair FAEE. Five out of 26 (19 %) pregnant women were found positive for heavy alcohol exposure.

**Table 5.2 Reasons why social workers suspected alcohol abuse/ordered FAEE hair test for parents (n = 172)**

<table>
<thead>
<tr>
<th>Reasons</th>
<th>Number</th>
<th>Percent</th>
<th>FAEE above 0.5 ng/mg OR</th>
<th>CI</th>
<th>FAEE above 1.0 ng/mg OR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third party reports alleging alcohol abuse</td>
<td>60</td>
<td>35</td>
<td><strong>3.31</strong></td>
<td>1.69-6.46</td>
<td><strong>2.30</strong></td>
<td>1.18-4.74</td>
</tr>
<tr>
<td>Admission to some use of alcohol including heavy amounts</td>
<td>50</td>
<td>29</td>
<td>1.41</td>
<td>0.71-2.81</td>
<td>1.06</td>
<td>0.49-2.30</td>
</tr>
<tr>
<td>Substantiated history of drinking</td>
<td>44</td>
<td>26</td>
<td>1.87</td>
<td>0.92-3.80</td>
<td>1.83</td>
<td>0.85-3.94</td>
</tr>
<tr>
<td>History or suspicion of illicit drug use</td>
<td>43</td>
<td>25</td>
<td><strong>0.20</strong></td>
<td>0.07-0.55</td>
<td><strong>0.27</strong></td>
<td>0.09-0.80</td>
</tr>
<tr>
<td>All drug hair tests requested to &quot;cover their bases&quot;</td>
<td>35</td>
<td>20</td>
<td><strong>0.09</strong></td>
<td>0.02-0.40</td>
<td><strong>0.74</strong></td>
<td>0.06-6.81</td>
</tr>
<tr>
<td>Recorded criminal behaviour associated with alcohol use</td>
<td>23</td>
<td>13</td>
<td>1.40</td>
<td>0.56-3.45</td>
<td>0.91</td>
<td>0.31-2.01</td>
</tr>
<tr>
<td>Recorded ethanol intoxication or testing positive for alcohol</td>
<td>17</td>
<td>10</td>
<td>1.98</td>
<td>0.72-5.45</td>
<td>1.94</td>
<td>0.67-5.63</td>
</tr>
<tr>
<td>Admission to heavy amounts of alcohol use</td>
<td>14</td>
<td>8</td>
<td>2.22</td>
<td>0.74-6.69</td>
<td>2.74</td>
<td>0.89-8.42</td>
</tr>
<tr>
<td>To confirm subject's abstinence</td>
<td>13</td>
<td>8</td>
<td>0.60</td>
<td>0.16-2.23</td>
<td>0.99</td>
<td>0.26-3.79</td>
</tr>
<tr>
<td>Observed drinking paraphernalia at the home</td>
<td>11</td>
<td>6</td>
<td>1.80</td>
<td>0.58-5.16</td>
<td>1.98</td>
<td>0.55-7.16</td>
</tr>
<tr>
<td>Previous treatment for alcohol abuse</td>
<td>8</td>
<td>5</td>
<td>0.68</td>
<td>0.13-3.48</td>
<td>1.10</td>
<td>0.21-5.70</td>
</tr>
<tr>
<td>Partner/spouse abuse or suspected abuse of alcohol</td>
<td>5</td>
<td>3</td>
<td>0.99</td>
<td>0.22-4.00</td>
<td>0.99</td>
<td>0.33-2.90</td>
</tr>
<tr>
<td>By accident</td>
<td>5</td>
<td>3</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
</tr>
<tr>
<td>Don’t know, social worker unaware of why tested for FAEE</td>
<td>3</td>
<td>2</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
</tr>
<tr>
<td>Previous child affected by fetal alcohol syndrome</td>
<td>2</td>
<td>1</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
</tr>
<tr>
<td>Serious incident involving intoxication that had resulted in hospitalization</td>
<td>1</td>
<td>1</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
<td>p/a</td>
</tr>
</tbody>
</table>

* Denotes statistical significance.
Table 5.3 Reasons why social workers suspected alcohol abuse/ordered FAEE hair test for mothers (n = 119)

<table>
<thead>
<tr>
<th>Reasons</th>
<th>Number</th>
<th>Percent</th>
<th>FAEE above 0.5 ng/mg OR CI</th>
<th>FAEE above 1.0 ng/mg OR CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third party reports alleging alcohol abuse</td>
<td>36</td>
<td>30</td>
<td>3.30* 1.45-7.50 2.27 0.93-5.52</td>
<td></td>
</tr>
<tr>
<td>Admission to some use of alcohol including heavy amounts</td>
<td>36</td>
<td>30</td>
<td>1.40 0.62-3.15 1.20 0.48-3.01</td>
<td></td>
</tr>
<tr>
<td>Substantiated history of drinking</td>
<td>39</td>
<td>25</td>
<td>1.76 0.76-4.12 1.69 0.66-4.31</td>
<td></td>
</tr>
<tr>
<td>History or suspicion of illicit drug use</td>
<td>28</td>
<td>24</td>
<td>0.26* 0.06-0.86 0.34 0.09-1.21</td>
<td></td>
</tr>
<tr>
<td>All drug hair tests requested to “cover their bases”</td>
<td>25</td>
<td>21</td>
<td>0.13 0.03-0.50 0.73 0.64-0.83</td>
<td></td>
</tr>
<tr>
<td>Recorded ethanol intoxication or testing positive for alcohol</td>
<td>13</td>
<td>11</td>
<td>1.27 0.39-4.17 1.60 0.45-5.68</td>
<td></td>
</tr>
<tr>
<td>To confirm subject’s abstinence</td>
<td>9</td>
<td>8</td>
<td>0.99 0.23-4.17 1.79 0.42-7.70</td>
<td></td>
</tr>
<tr>
<td>Recorded criminal behaviour associated with alcohol use</td>
<td>8</td>
<td>7</td>
<td>2.06 0.49-9.81 0.47 0.05-3.97</td>
<td></td>
</tr>
<tr>
<td>Admission to heavy amounts of alcohol use</td>
<td>8</td>
<td>7</td>
<td>3.62 0.82-16.00 6.74* 1.50-30.38</td>
<td></td>
</tr>
<tr>
<td>Observed drinking paraphernalia at the home</td>
<td>6</td>
<td>5</td>
<td>2.05 0.49-10.68 1.76 0.39-10.17</td>
<td></td>
</tr>
<tr>
<td>Previous treatment for alcohol abuse</td>
<td>4</td>
<td>3</td>
<td>2.03 0.29-14.94 3.60 0.49-26.05</td>
<td></td>
</tr>
<tr>
<td>Partner/spouse abuse or suspected abuse of alcohol</td>
<td>3</td>
<td>3</td>
<td>0.96 0.92-1.01 0.97 0.93-1.00</td>
<td></td>
</tr>
<tr>
<td>By accident</td>
<td>4</td>
<td>3</td>
<td>n/a  n/a      n/a  n/a</td>
<td></td>
</tr>
<tr>
<td>Previous child affected by fatal alcohol syndrome</td>
<td>2</td>
<td>2</td>
<td>n/a  n/a      n/a  n/a</td>
<td></td>
</tr>
<tr>
<td>Serious incident involving intoxication that had resulted in hospitalization</td>
<td>1</td>
<td>1</td>
<td>n/a  n/a      n/a  n/a</td>
<td></td>
</tr>
<tr>
<td>Don’t know, social worker unaware of why tested for FAEE</td>
<td>0</td>
<td>0</td>
<td>n/a  n/a      n/a  n/a</td>
<td></td>
</tr>
</tbody>
</table>

* Denotes statistical significance.
5.6 Discussion

Our laboratory is the first to apply the FAEE hair test in the context of assessing parental alcohol use in a population at high-risk of having children with FASD. Our results show the FAEE hair test is a useful in corroborating and confirming social workers’ suspicion of heavy alcohol use by parents when it is present. Reported factors directly related to alcohol use were significantly associated with testing positive for excessive alcohol use, whereas factors not directly related to alcohol use were negatively associated with testing positive. In addition, there was strong agreement between the reported amount of alcohol intake and the FAEE hair test result (positive or negative), with 11 out of 15 results agreeing with social worker reports.

Factors associated with testing positive for FAEE can also be viewed by social workers as risk factors or predictive factors for heavy alcohol use by parents. Specifically, social workers having knowledge of a specific instance of problem drinking within the past 6 months resulted in a five-fold increased risk for parents testing positive for excessive alcohol use. Similarly, social workers receiving third party reports alleging that the parent was drinking resulted in a 3-fold increased risk for testing positive. These risk factors were also significant when mothers were analyzed separately, except that mothers were 8 times more likely to be positive for FAEE if the social worker was aware of instances of problem drinking within the past 6 months.

Furthermore, when mothers self-reported using heavy amounts of alcohol they were 7 times more likely to test highly positive (above 1.0 ng/mg) for hair FAEE. This association was not observed when mothers and fathers were analyzed together, and was only significant for FAEE
levels above 1.0 ng/mg, and not when applying the 0.5 ng/mg cut-off level. This suggests that the added specificity of the higher cut-off level was required to reach statistical significance. These results suggest that hair analysis of FAEE may be a more sensitive marker of alcohol exposure in women as compared to men, and therefore specificity may be lower in women at comparable levels. Previous investigations have mainly focused on men (Auwarter et al., 2001; Pragst et al., 2001; Wurst et al., 2004), and further investigation using this marker in women is clearly warranted. It does however remain a possibility that this observation may have resulted from women being more truthful about their alcohol intake relative to men but this remains speculative at this point.

Pregnancy status was not associated with likelihood to test positive or negative for FAEE, suggesting that being pregnant did not affect the drinking habits of this cohort. Alternatively, the present study may have lacked the statistical power to detect such an effect. Regardless of the fact weather or not women curtail their drinking habits during pregnancy, five out of 26 (19 %) pregnant women were found positive for heavy alcohol exposure. Although this figure is less than that found for the entire cohort (33 %), it is still staggeringly high, confirming this cohort is indeed at risk for having children with FASD. In addition, the social workers reported that one of these women consumed 6 alcoholic drinks every other day. To the best of our knowledge this is the first study to report data on alcohol consumption for a women who has tested positive for hair FAEE during pregnancy.

Also important is the fact that requesting an FAEE hair test without the suspicion of alcohol use, but solely as a measure to “cover all bases” by the social worker, or because illicit drug use was documented or suspected, was negatively associated with testing positive for hair FAEE in
parents. Our results therefore strongly suggest that if there are no indications for social workers to suspect alcohol abuse, parents are less likely to test positive for alcohol, most likely because alcohol abuse is too obvious to go unsuspected in families involved with CPS.

Parental alcohol abuse is a major concern for child welfare because it puts families at high risk of having children with FASD, and providing poor rearing environments that can be equally harmful (Curtis and McCullough, 1993; Freisthler et al., 2007; Freisthler and Weiss, 2008). A recent study by Sarkola et al. found that daily alcohol consumption before and/or during pregnancy, among other factors, was significantly associated with out-of-home placement of children (Sarkola et al., 2007). Several studies have demonstrated that rearing environment can have an even larger negative impact on children than prenatal exposure to drugs and alcohol itself (Arendt et al., 2004; Lewis et al., 2004; Thyssen Van et al., 2000). Furthermore, studies involving fetal alcohol affected children have shown a child’s rearing environment to be critical in determining a child’s progress and positive/negative outcomes (Cone-Wesson, 2005).

A recent Canadian study confirmed suspicions of a high prevalence of FASD among children’s aid client families (Gough and Fuchs, 2006). The study reported that out of children with disabilities in the care of child welfare services in Manitoba, 34 % were diagnosed with FASD, representing 11 % of all children in care (Gough and Fuchs, 2006). Furthermore, parental alcoholism and drug use have been strongly associated to child neglect and abuse, both detrimental to the child’s development and well-being (Jaudes et al., 1995; Kelleher et al., 1994; Walsh et al., 2003; Nair et al., 2003; Fluke et al., 2008b). Consequently, identifying parental alcohol abuse and risk factors associated with that use is of critical importance for social workers to be able to identify families in need of intervention.
In conclusion, the present study shows that social worker reports are in agreement with the novel FAEE biomarker test results, confirming that the FAEE hair test is useful in corroborating and confirming social workers’ suspicion of heavy alcohol use by parents. Furthermore, we identified predictive factors associated with heavy alcohol use in parents, and mothers specifically. This is also the first study to report alcohol consumption data among pregnant women testing positive for hair FAEE. We suggest the FAEE hair test has potential for use in the context of FASD research and diagnosis.
6 Correlation between Alcohol and Other Drugs of Abuse by Hair Analysis: Parents At-Risk for Having Children with FASD and Polydrug Exposures

6.1 Abstract

The FAEE hair test, a biomarker of excessive alcohol exposure, has demonstrated potential for use in FASD diagnosis. FASD may be compounded by polydrug exposure. Our objective was to determine the risk of other drug abuse among parents testing positive for excessive alcohol use by FAEE hair analysis. Samples submitted for FAEE hair analysis by CAS between October 2005 and May 2007, also concurrently tested for cocaine, cannabinoids, opiates, methamphetamine, amphetamine, barbiturates, benzodiazepines, methadone, and oxycodone, were included in our analysis. Subjects consisted of parents suspected of using excessive amounts of alcohol. Parents with heavy chronic alcohol use were found to have a 5-fold increased risk of testing positive for methamphetamine (OR = 4.7, 1.1 – 19.1), and a 2-fold increased risk for testing positive for cocaine (OR = 2.3, 1.0 – 5.3). Parents with heavy chronic alcohol use also were found less likely to test positive for methadone (OR = 0.79, 0.63 – 0.99). Mothers with heavy chronic alcohol use were found to have a 3-fold increased risk of testing positive for cocaine (OR = 3.26, 1.1 – 9.7). The positive predictive values (PPV) of most hair drug tests in predicting positive FAEE tests were upwards of 30 %, with the highest being 100 %
for benzodiazepines, followed by methamphetamine, which had a PPV of 56%. Our results suggest that parents abusing alcohol are at greater risk of using stimulants, which may put their unborn children at further risk. When assessing babies exposed in-utero to alcohol, it is also critical to address the possibility of exposure to other drugs of abuse. Furthermore, because testing positive for stimulants was found to be a positive predictor for excessive alcohol use, a full-scale evaluation of any relevant children for FASD should be considered.
6.2 Introduction

Exposure to alcohol during pregnancy can have devastating consequences for the fetus, producing life-long impairments including birth defects, mental retardation, learning disabilities, and maladaptive behaviour (Sokol et al., 2003; Streissguth, 1992; Streissguth et al., 1994; Baer et al., 2003; Streissguth et al., 2004). FASD, is one of the most prevalent causes of neurocognitive handicap among children in North America, with a frequency estimated to be as high as 1 in 100 births (Abel and Sokol, 1987; Abel and Sokol, 1986; Abel, 1995a; Sampson et al., 1997; Abel, 1995a). Presently the majority of these cases remain undiagnosed early in life when interventions can be relatively effective (Streissguth et al., 2004; Olson et al., 2007). A serious challenge in diagnosis is the need to establish evidence of excessive maternal consumption of alcohol during pregnancy (Chudley et al., 2005), as maternal self-reports of are often unreliable (Russell et al., 1996; Ernhart et al., 1988; Wurst et al., 2008a; Alvik et al., 2006; Alvik et al., 2006; Alvik et al., 2006). Therefore, a biological marker that can determine past or chronic heavy alcohol use in the mother would greatly facilitate the detection and diagnosis of children with FASD.

Recently, there has been a growing body of evidence that the measurement of FAEE in hair could be used as biomarker for excessive alcohol use, and aid in FASD diagnosis and intervention (Wurst et al., 2008a; Pragst and Yegles, 2008; Kulaga et al., 2008). Although the majority of alcohol is metabolized oxidatively to acetaldehyde and then to acetic acid, a portion undergoes non-oxidative metabolism, resulting in the formation of FAEE. FAEE are produced
when ethanol is conjugated with fatty acids in the body, a reaction that is most often catalysed by FAEE synthase, or AEAT (Laposata, 1998b). Cumulative levels of four FAEE, ethyl myristate, palmitate, oleate, and stearate, measured in hair have been shown to be highly sensitive and specific in the detection of excessive drinking in adults (Pragst and Balikova, 2006; Wurst et al., 2004).

Since the FAEE hair test’s inception, the test has been validated and used in the medical context of alcohol abuse and the medicolegal context of assessing alcohol exposure in cases of driving under the influence of alcohol (Wurst et al., 2008a; Pragst and Yegles, 2008). Since then there has been a growing interest in using the test to assess alcohol use in the perinatal context. A recent pilot study conducted in Sweden (n = 103) investigated whether biomarkers of alcohol consumption would provide additional information to the use of a validated alcohol questionnaire in pregnant women. The study reported that the investigators were able to detect alcohol consumers by FAEE hair analysis that were previously undetected by questionnaire or other markers alone (Wurst et al., 2008a). In our laboratory we have recently described for the first time ever the use of the FAEE hair test in a cohort of parents at high-risk for having children with FASD and reported a prevalence of 33% for excessive alcohol use (Kulaga et al., 2008).

Women who consume excessive amounts of alcohol also tend to have many other reproductive risk factors that need be considered. Among poor prenatal care and nutrition, poverty and neglect, epidemiological studies have suggested women who drink alcohol are also at higher risk to consume other recreational drugs concomitantly (Alpert et al., 1981; Project CHOICES Research Group, 2002; Weiner et al., 1983; Sokol et al., 1980; Abel and Hannigan, 1995;
Snodgrass, 1994). Therefore, the objective of the current study was to determine the risk of other drug abuse among parents testing positive for heavy chronic alcohol use by FAEE hair analysis.

6.3 Materials and Methods

6.3.1 Study Sample

Starting in October 2005, we established a diagnostic program for hair analysis of FAEE to detect excessive parental drinking. Samples ordered for FAEE testing at the Motherisk Laboratory by CAS between October 2005 and May 2007 (n = 224) that were also concurrently tested for any of the following drugs: cocaine (n = 205), cannabinoids (n = 196), opiates (n = 136), methamphetamine (n = 94), amphetamine (n = 83), benzodiazepines (n = 65), barbiturates (n = 61), methadone (n = 21), and oxycodone (n = 9), were evaluated. All CAS organizations were based in Canada with the majority residing in Ontario and British Columbia. CAS clients that underwent hair testing consisted of parents at risk of having children with FASD, based on clinical suspicion of chronic heavy alcohol use.
6.3.2 FAEE Analysis

All samples were processed and analyzed for FAEE in either Toronto, Canada or in Berlin, Germany, using similar methodology. The reason for the use of two laboratories was because earlier samples were processed in Berlin prior to the test being clinically available in Toronto. Quality assurance testing was performed where numerous samples were concurrently analyzed in both laboratories and compared. The results confirmed the tests to be reproducible between both laboratories, therefore all available data was included in the analysis.

The sample preparation, and HS-SPME/GCMS conditions for FAEE hair analysis have previously been described (Pragst et al., 2001; Auwarter et al., 2001). Briefly, 6 cm hair samples, representing the last 6 months of growth, underwent a 15-hour liquid-liquid extraction using dimethyl sulfoxide and n-heptane, after which extracts were isolated by phase separation and evaporation, then re-suspended in 0.1 M phosphate buffer (pH 7.6). The samples then underwent HS-SPME and were analyzed by GCMS using deuterated FAEE as internal standards. Instrumental cv values for same-day (intraday) results were below 3%, whereas between-day (interday) cv values were below 5%. The LOD and LOQ were 0.015 ng/mg and 0.05 ng/mg for ethyl myristate, 0.02 ng/mg and 0.07 ng/mg for ethyl palmitate, 0.04 ng/mg and 0.12 ng/mg for ethyl oleate, and 0.01 ng/mg and 0.04 ng/mg for ethyl stearate, respectively.

6.3.3 Drug Analysis

Samples were analyzed for amphetamine, methamphetamine, barbiturates, benzodiazepines, cannabinoids, cocaine, opiates, methadone, or oxycodone using ELISA by standard methods of
the Motherisk laboratory. Briefly, approximately 5-10 mg of hair was weighed and then thoroughly mulched using scissors. Then 1 mL of methanol was added to each sample. The vials were then capped and incubated at 56°C for 15-18 hours. Supernatent was then transferred, evaporated, and reconstituted in 400 µL phosphate buffer solution (PBS) (pH 7.4). The samples were analyzed twice by ELISA using kits from Immunoanalysis and Neogen. An 8-point calibration curve of fresh standards with concentrations ranging from 0.1 ng/mL to 100 ng/mL was prepared from stock solutions obtained from Cerilliant, Texas, USA. Stock solutions were spiked into reconstituted PBS solution after evaporation of drug-free hair and then diluted as appropriate for each drug. LOD for all drugs except methadone was 0.1 ng/mL, and LOQ was 0.1 ng/mL for cannabinoids, cocaine, opiates, and oxycodone, and 0.2 ng/mL for amphetamine, methamphetamine, and barbiturates, and 0.15 ng/mL for benzodiazepines. LOD and LOQ was 0.2 ng/mL for methadone. Results were confirmed by GCMS analysis.

6.3.4 Statistics

Logistic regression was performed to evaluate if an association was present between having FAEE hair levels above 0.5 ng/mg, or 1.0 ng/mg, and testing positive for any drugs in hair. We also calculated sensitivity, specificity, and predictive values of other drugs in predicting excessive alcohol use.
6.4 Results

Of samples tested for FAEE, 57% had levels of FAEE above 0.2 ng/mg, 29% above 0.5 ng/mg, and 16% above 1.0 ng/mg. Also, there was up to a 53% positivity rate for concomitantly tested drugs, with the highest being for cocaine (Table 6.1). The sensitivity, specificity, positive predictive values, negative predictive values, and efficiency of each drug in predicting excessive alcohol use are given in Table 6.2. Methamphetamine showed the highest efficiency in predicting FAEE results, followed by benzodiazepines, while testing positive for cannabinoids had the greatest sensitivity in detecting alcohol users. Significant odds ratios were found between testing positive for FAEE above 0.5 ng/mg and methamphetamine (OR = 4.7, 1.1 – 19.1), and between testing positive for FAEE above 1.0 ng/mg and cocaine (OR = 2.3, 1.0 – 5.3) (Table 6.3). A significant negative association was found between testing positive for FAEE above 1.0 ng/mg and methadone (OR = 0.79, 0.63 – 0.99) (Table 6.3). Also, after excluding samples from fathers and examining mothers, a significant association was found between testing positive for FAEE above 1.0 ng/mg and cocaine (OR = 3.26, 1.10 – 9.71) (Table 6.4).
Table 6.1 Number of drug tests that returned positive results and the number of drug tests that were positive for both drugs and FAEE

<table>
<thead>
<tr>
<th>Drug</th>
<th>N</th>
<th>Positive (No.)</th>
<th>Positive (%)</th>
<th>Drug and FAEE Positive (No.)</th>
<th>Drug and FAEE Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>205</td>
<td>109</td>
<td>53.2</td>
<td>35</td>
<td>17.1</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>196</td>
<td>68</td>
<td>34.7</td>
<td>23</td>
<td>11.7</td>
</tr>
<tr>
<td>Opiates</td>
<td>136</td>
<td>32</td>
<td>23.5</td>
<td>10</td>
<td>7.4</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>94</td>
<td>9</td>
<td>9.6</td>
<td>5</td>
<td>5.3</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>83</td>
<td>3</td>
<td>3.6</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>65</td>
<td>2</td>
<td>3.1</td>
<td>2</td>
<td>3.1</td>
</tr>
<tr>
<td>Barbituates</td>
<td>61</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Methadone</td>
<td>21</td>
<td>4</td>
<td>19.0</td>
<td>1</td>
<td>14.8</td>
</tr>
<tr>
<td>Oxycodon</td>
<td>9</td>
<td>2</td>
<td>22.2</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 6.2 Predictive value of drug tests in predicting FAEE results

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>27.8%</td>
<td>77.1%</td>
<td>31.3%</td>
<td>74.0%</td>
<td>63.6%</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>41.1%</td>
<td>67.9%</td>
<td>33.8%</td>
<td>74.2%</td>
<td>60.2%</td>
</tr>
<tr>
<td>Opiates</td>
<td>27.8%</td>
<td>78.4%</td>
<td>31.3%</td>
<td>75.5%</td>
<td>65.2%</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>21.7%</td>
<td>94.4%</td>
<td>55.6%</td>
<td>78.8%</td>
<td>76.6%</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>4.2%</td>
<td>96.6%</td>
<td>33.3%</td>
<td>71.3%</td>
<td>69.9%</td>
</tr>
<tr>
<td>Barbituates</td>
<td>0.0%</td>
<td>100.0%</td>
<td>N/A</td>
<td>72.1%</td>
<td>72.1%</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>11.1%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>75.0%</td>
<td>75.8%</td>
</tr>
<tr>
<td>Methadone</td>
<td>27.8%</td>
<td>40.5%</td>
<td>31.3%</td>
<td>36.6%</td>
<td>34.2%</td>
</tr>
<tr>
<td>Oxycodon</td>
<td>0.0%</td>
<td>71.4%</td>
<td>0.0%</td>
<td>71.4%</td>
<td>55.6%</td>
</tr>
<tr>
<td></td>
<td>FAEE above 0.5 ng/mg</td>
<td>FAEE above 1.0 ng/mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------</td>
<td>---------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OR</td>
<td>CI</td>
<td>OR</td>
<td>CI</td>
<td></td>
</tr>
<tr>
<td>Cocaine</td>
<td>1.59</td>
<td>(0.85-2.97)</td>
<td>2.31*</td>
<td>(1.00-5.32)</td>
<td></td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>1.47</td>
<td>(0.78-1.47)</td>
<td>1.23</td>
<td>(0.56-2.71)</td>
<td></td>
</tr>
<tr>
<td>Opiates</td>
<td>1.36</td>
<td>(0.57-3.25)</td>
<td>0.92</td>
<td>(0.28-3.02)</td>
<td></td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>4.65*</td>
<td>(1.13-19.13)</td>
<td>1.92</td>
<td>(0.35-10.46)</td>
<td></td>
</tr>
<tr>
<td>Amphetamine</td>
<td>1.24</td>
<td>(0.11-14.34)</td>
<td>0.96</td>
<td>(0.91-1.01)</td>
<td></td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>1.13</td>
<td>(0.96-1.33)</td>
<td>6.00</td>
<td>(0.34-104.79)</td>
<td></td>
</tr>
<tr>
<td>Methadone</td>
<td>1.56</td>
<td>(0.12-20.61)</td>
<td>0.79*</td>
<td>(0.626-1.00)</td>
<td></td>
</tr>
<tr>
<td>Oxycodon</td>
<td>0.71</td>
<td>(0.45-1.14)</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates statistical significance
Table 6.4 Odds ratio and confidence intervals for associations between mothers testing positive for various drugs and testing positive for hair FAEE above 0.5 ng/mg, and 1.0 ng/mg

<table>
<thead>
<tr>
<th>Drug</th>
<th>FAEE above 0.5 ng/mg</th>
<th>FAEE above 1.0 ng/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>CI</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2.01</td>
<td>(0.94-4.29)</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>1.02</td>
<td>(0.45-2.33)</td>
</tr>
<tr>
<td>Opiates</td>
<td>1.93</td>
<td>(0.72-5.20)</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>4.08</td>
<td>(0.89-18.72)</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>1.06</td>
<td>(0.94-1.18)</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>1.17</td>
<td>(0.94-1.45)</td>
</tr>
<tr>
<td>Methadone</td>
<td>2.17</td>
<td>(0.14-32.53)</td>
</tr>
<tr>
<td>Oxycodon</td>
<td>0.83</td>
<td>(0.58-1.19)</td>
</tr>
</tbody>
</table>

* Indicates statistical significance

6.5 Discussion

The current study revealed a significant association between alcohol and other drug use. More specifically, testing positive for hair FAEE was significantly associated with testing positive for stimulants, methamphetamine and cocaine, in hair. Parents who were found to chronically use heavy amounts of alcohol were 5 times more likely to use methamphetamine and twice as likely to use cocaine. Mothers who were found to chronically use heavy amounts of alcohol were 3
times more likely to use cocaine, suggesting that future pregnancies may be at risk for polydrug exposure. Other studies have confirmed that alcohol use in pregnancy is often associated with other drug use. In a multi-site survey of women aged 18-44 (n = 2672), 333 met a priori definition of “at-risk” for an alcohol-exposed pregnancy (women who were fertile, sexually active, and consuming more than 7 drinks per week or more than 5 drinks per occasion within the last 6 months). Recent drug use (OR = 3.1, 1.3 – 2.7) and a history of inpatient treatment for drugs or alcohol (OR = 1.8, 1.3 – 2.4) were significantly associated with being at risk for having alcohol-exposed pregnancy (Project CHOICES Research Group, 2002). Furthermore, data from the largest clinical database of children prenatally exposed to alcohol, Washington state’s FAS DPN, showed that 84.5% of children referred to clinic for FASD diagnosis have been exposed to other drugs of abuse concomittantly (Olson et al., 2007). Therefore, this is not the first report of an association between parental alcohol use and illicit drug use, but it is the first study to confirm such an association using an independent biomarker by means of hair analysis.

Clinically in our laboratory we use a cut-off level of 0.5 ng of cumulative FAEE per mg hair, to identify heavy drinkers with 90% sensitivity and specificity (Pragst and Balikova, 2006). Levels between 0.2-0.5 ng/mg are indicative of social use and typically exclude strict abstainers, whereas levels in excess of 1.0 ng/mg are nearly 100% specific to heavy alcohol use, but offer lower sensitivity so that many cases may be missed (Pragst et al., 2001; Auwarter et al., 2001; Pragst and Balikova, 2006; Pragst and Yegles, 2008). Although it has not yet been determined, it is possible that higher levels of hair FAEE reflect higher levels of alcohol use, for example chronic daily binging versus periodic binging, or simply consuming greater amounts of alcohol. Therefore, the two cut-off levels 0.5 and 1.0 ng/mg may reflect slightly different populations of drinkers as well, which of course overlap. Consequently, the fact that in the present study we
were able to see an association between cocaine and alcohol use only with the more specific cut-off of 1.0 ng/mg, may reflect a preferential use of cocaine in this “heavily using” population; whereas the association between methamphetamine and alcohol use, which was apparent using the lower (more sensitive) FAEE cut-off level of 0.5 ng/mg, may reflect the growing popularity of methamphetamine use over cocaine in general, with the higher 1.0 ng/mg cut-off lacking the sensitivity required to reveal it. Irrespective of cut-off levels, a significant association between alcohol and stimulant use exists and poses an added risk of polydrug exposure to unborn children.

Polydrug use may pose a more severe threat to unborn children than just alcohol exposure or exposure to any other single drug. Stimulant use during pregnancy, such as cocaine or methamphetamine have been shown to increase the risk for growth retardation and placental abruption (Covington et al., 2002; Doris et al., 2006; Hulse et al., 1997; Oyelese and Ananth, 2006; Shankaran et al., 2007; Addis et al., 2001; Loebstein and Koren, 1997; Zimmerman, 1991). Also, cognitive deficits from prenatal exposure to cocaine have been supported by many studies, however some debate still exists as to whether these deficits are attributable to confounding variables such as concomitant alcohol or other drug use, and or other co-occurring reproductive risk factors (Addis et al., 2001; Arendt et al., 2004; Bandstra et al., 2002; Church et al., 1998; Cone-Wesson, 2005; Covington et al., 2002; Frank et al., 2002; Frank et al., 2001; Harvey et al., 2001; Lewis et al., 2004; Lumeng et al., 2007; Morrow et al., 2001; Noland et al., 2003; Schuetze et al., 2007; Thyssen Van et al., 2000). Biochemically, ethanol and cocaine are esterified hepatically to cocaethylene, a potent neurotoxin. However, the exact effects of combining prenatal alcohol exposure with other drugs are unknown. Studies suggest that women who use multiple drugs are however more likely to suffer other reproductive risk factors such as
malnutrition and having multiple sex partners (Snodgrass, 1994; Shankaran et al., 2007; Day et al., 1993; Frank et al., 1988). An early study by Qazi et al., reported that of 18 pregnant women identified as heavy drinkers who gave birth to 5 infants affected by FAS, 7 with PFAS, and 6 non-affected infants, that lower socioeconomic status, higher parity, and increased use of tobacco and other drugs appeared to influence the outcome of the pregnancies (Qazi et al., 1982). Animal studies have also suggested a possible synergistic effect in teratogenicity between drugs of abuse and alcohol (Abel, 1985). Therefore, fetal exposure to alcohol and other drugs poses a serious threat to offspring, the consequences of which are still not yet fully understood.

Another aspect to consider in identifying heavy drinking and alcohol use in parents is the harmful environmental risk factors associated with drug and alcohol use for child rearing. A recent study investigating risks for out-of-home custody found that among other factors, drug in urine during pregnancy, and daily alcohol consumption before and/or during pregnancy were significantly associated with out-of-home placement of children (Sarkola et al., 2007). Children’s environment significantly affects their development, Cone-Wesson reviews evidence that suggests prenatal effects of alcohol and other drug exposure may be ameliorated by stimulating and sensitive care-giving environments (Cone-Wesson, 2005). Similarly, several studies have demonstrated that although prenatal exposure to stimulants can lead to impairments independently, poor rearing environment can have an even larger negative impact on the child (Arendt et al., 2004; Lewis et al., 2004; Thyssen Van et al., 2000). As important, parental alcoholism and drug use have been linked to child neglect and abuse, both detrimental to the child’s development and well-being (Jaudes et al., 1995; Kelleher et al., 1994; Walsh et al., 2003; Nair et al., 2003; Fluke et al., 2008b). Therefore, identifying parental abuse of drugs and alcohol is of critical importance.
The current study demonstrated that most drug hair tests had a PPV in predicting positive FAEE tests upwards of 30%. The two exceptions were barbiturates and oxicodon, the first of which no tests returned positive for, the latter of which only 2 tests returned positive for (Table 6.2); therefore because of the low prevalence of use coupled with the relatively small sample size it is difficult to assess the true PPV of these two drugs. The highest PPV was seen for benzodiazepines, which had a PPV of 100%, followed by methamphetamine, which had a PPV of 56%. However, similarly for benzodiazepines, only 2 tests in total returned positive, therefore caution should be exercised when interpreting this result.

Methamphetamine, followed by opiates, was the most efficient in predicting chronic heavy alcohol use (Table 6.2). Cannabinoids, had the highest sensitivity in detecting alcohol abuse with a modest specificity of 67%. With the exception of methadone, the majority of drug tests showed very high specificity (Table 6.2) for detecting alcohol use, indicating that if a subject tested positive for illicit drug use it is likely they would test positive for alcohol use as well. Therefore, our results indicate that testing positive for illicit drugs is predictive of testing positive for heavy chronic alcohol use. This is of clinical importance in identifying risk factors for heavy alcohol use and FASD.

However, it should be mentioned that PPV values of the present study may be over inflated because samples selected for FAEE and drug testing, in the current sample population, were selected by social workers based on clinical suspicion of heavy alcohol use. Consequently, higher PPV of drug hair tests in predicting positive hair FAEE are expected, confirming social workers’ suspicion of heavy alcohol use in these subjects.
Interestingly, a negative association between alcohol and methadone use was found in parents. This association likely reflects the fact that methadone patients are in intensive treatment programs for drug abuse, and possibly for alcohol use as well, therefore they receive support in abstaining from their addictions. The fact that we see a negative association between alcohol and methadone use suggests that these treatment programs offer a protective effect in abstaining from alcohol use, as might be expected.

In conclusion, the present study revealed a significant association between stimulants use and parental heavy alcohol use. Therefore, because testing positive for stimulants can serve as a positive predictor for excessive alcohol use, a full-scale examination of any relevant children for FASD is warranted. Likewise, when assessing babies exposed in-utero to alcohol, it is also critical to address the possibility of exposure to other drugs of abuse.
7 Discussion

7.1 Bridging the Gap

The FAEE hair test is a powerful biomarker for excessive alcohol use, and for confirmation of alcohol use during pregnancy, a necessary criterion in FASD diagnosis. Yet a major gap exists between previous FAEE research efforts, primarily involving the development and validation of the FAEE hair test, and research applying the FAEE hair test in the context of FASD diagnosis. This thesis strives to address this gap. Translational research, bridging laboratory findings with clinical use is a critical part of the development of any new diagnostic means.

In 2001, Pragst and colleagues developed the assay that measures FAEE in hair. Since that time, the group has conducted numerous studies assessing the hair test’s sensitivity and specificity, segmental analysis of FAEE, the test’s resilience to cosmetic applications and grooming habits, and its applicability for use in forensic cases (Pragst et al., 2001; Auwarter et al., 2001; Hartwig et al., 2003a; Hartwig et al., 2003b; Auwarter et al., 2004; Wurst et al., 2004; Yegles et al., 2004; Pragst and Balikova, 2006; Pragst and Yegles, 2008; Wurst et al., 2008b). Pragst and Yegles, also recently published a review of these studies, and proposed that the hair test be used for detection of alcohol abuse during pregnancy (Pragst and Yegles, 2008).
There has been progress in this direction. Pragst and colleagues have made a valuable contribution to the field by using the hair test, along with other biomarkers, to assess alcohol use in a sample of pregnant women obtained from the general population in Sweden (Wurst et al., 2008a). In addition, our laboratory has been involved in animal research using the guinea pig as a model to investigate FAEE in neonatal and maternal hair as a biomarker for fetal alcohol exposure, with promising results (Caprara et al., 2005a; Kulaga et al., 2006).

There is a significant challenge, however, towards implementing the use of the FAEE hair test in the field of FASD diagnosis. The ideal study to bridge this gap would be one that contains the following elements: a large sample of pregnant women with and without heavy alcohol exposure, an accurate history of alcohol use in pregnancy, a large sample of corresponding children with and without FASD diagnosis, neonatal hair samples of those children, and maternal hair samples from in and around the time of pregnancy.

However, the challenge of such a study is that it has several intrinsic flaws that are, if not theoretically insurmountable, are at least difficult to implement practically. First and foremost are the obvious ethical considerations when studying pregnant women consuming alcohol. Alcohol is a known and potent teratogen, therefore it cannot be deliberately administered during pregnancy, and women already consuming it should be offered treatment and encouraged to discontinue its use. Furthermore, because of the stigmatization associated with alcohol use in pregnancy many women are reluctant to report their drinking, let alone participate in a scientific study. Oftentimes there are also legal ramifications associated with alcohol abuse, such as potentially losing custody of the infant once it is born, or of any existing children. Consequently, it is notoriously difficult to recruit and retain pregnant women who use heavy amounts of
alcohol. It is also very difficult to obtain accurate reporting histories. Other important considerations are that once babies are born they are too young to be diagnosed with fetal alcohol related disorders until they reach an age where neurocognitive tests can be applied. Furthermore, most infants that are exposed to significant amounts of alcohol will not be affected.

Although the true number is not known, experts in the field estimate that only 40% of infants exposed heavily to alcohol in-utero will be affected by FASD. This poses a logistical nightmare in terms of recruiting a sufficient number of mothers and exposed infants, and for retaining them for enough years for the necessary follow-up. Conversely, if children already known to have FASD are recruited, the problem becomes obtaining neonatal hair samples from their infancy, and or maternal hair samples from the time of pregnancy, which are not likely to be available.

To overcome these challenges my approach in the current thesis has been to use existing data, animal models, and high-risk parents as a model for alcohol exposure in pregnancy and rearing environment, to help bridge the gap between previous FAEE research as a biomarker for alcohol exposure, and the application of the FAEE hair test in FASD diagnosis and research. All studies contained in this thesis contribute towards bridging this gap and also support the main hypothesis that the FAEE hair test holds significant potential for use in the FASD context.
7.2 Major Findings

1) Alcohol dose is difficult to assess accurately among problem-drinking mothers. Therefore, the use of an animal model, where accurate amounts of alcohol can be delivered and FAEE incorporated and measured, is invaluable. Therefore, our laboratory has employed the use of the guinea pig. As a result, it was critical to validate its use in the context of what is known about FAEE in humans. An order of magnitude difference in the rate of FAEE incorporation, into hair, was found between guinea pigs and humans, with humans incorporating more than 10-fold greater amounts of FAEE per equivalent alcohol exposure. A significant correlation between hair FAEE (ethyl oleate) and systemic exposure to alcohol was also found in both species, suggesting that ethyl oleate may be the most sensitive indicator of alcohol exposure. These findings support my hypothesis that a positive dose-concentration relationship between alcohol exposure and FAEE exists, and that the FAEE response to alcohol, as measured in hair, is more pronounced in one species over the other, in this case the human. Therefore, FAEE in hair appear to be a more clinically sensitive marker of alcohol exposure in the human versus the guinea pig.

Alternative hypotheses exist that can explain the results. For example, the guinea pig hair was white, whereas human hair was multicoloured, and for the most part pigmented. If the presence of melanin in hair affects FAEE incorporation by increasing it, this could explain the greater amount of incorporation seen in humans. However, based on the chemical nature of FAEE molecules and melanin granules, and on evidence from my second study, this is an unlikely hypothesis.
Another hypothesis, is that because the present study compared human and guinea pig FAEE data resulting from unequivalent alcohol exposures, and used mathematical modeling to correct for these differences, it is possible that despite attempts to correct for this, differences in the calculated incorporation rates may have resulted from the unequal length of exposure. Specifically, full length analysis of guinea pig fur, representative of approximately 2 months of regular exposure, was used for FAEE analysis in the guinea pig, whereas, 6 cm samples of hair obtained from humans, representative of 6 months of varying exposure, was used in human analysis, but the final results from both sample types were calculated as nanograms of FAEE per milligrams of hair. Therefore, the difference in cumulative exposure represented in hair between species is not accounted for. However, this is a necessary limitation, and regardless of this fact, under normal experimental conditions the results and outcome would likely be the same, therefore, it may be irrelevant whether or not this difference contributed to the difference observed in ICR.

As a consequence, it appears that guinea pigs incorporate an order of magnitude less FAEE for equivalent exposure. A previous study conducted by our laboratory investigated the measurement of hair FAEE in maternal, and neonatal hair, of guinea pigs exposed to alcohol throughout gestation (Caprara et al., 2005a). It was discovered that exposed dams and offspring had significantly higher levels of FAEE in their hair relative to controls, demonstrating the test as an effective tool in assessing fetal alcohol exposure. If I apply the findings of the current study to this previous one, I can surmise that the FAEE hair test will be an even more effective clinical tool in assessing fetal alcohol exposure in the human, because the human response of hair FAEE to alcohol appears to be much more pronounced.
Therefore, in light of previous research the present study, the first in my thesis, contributes towards bridging the gap between previous FAEE research and FAEE research in FASD by providing evidence that the test will be clinically sensitive enough for application in fetal alcohol exposure assessment. Furthermore, these results support the main hypothesis of my thesis that the FAEE hair test holds significant potential for use in the context of FASD diagnosis and research.

2) No significant difference was found between FAEE levels in black and white hair of the same rodents. These findings support my hypothesis that FAEE incorporation is not influenced by hair pigment, and therefore that the FAEE hair test is not vulnerable to hair colour bias.

Alternative hypotheses do exist and should be considered. For example, during data analysis it was discovered that the initial sample weight influenced extraction efficiency, with lower initial sample weights leading to higher extraction yields. Twenty milligrams of hair was typically used in analysis, however, this amount was not always available, as was the case for several white hair samples. Consequently, a significant linear relationship ($r^2 = 0.90$) was observed between initial sample weight and extraction efficiency, and it was used to standardize all samples to 20 mg for the purpose of comparison. It could be speculated that this procedure may have somehow obscured a true difference in FAEE incorporation between black and white hair. However, this procedure in effect actually reduced the originally measured FAEE values in white hair, because initial weights in these samples were lower (and the relationship between initial sample weight and extraction efficiency is inverse), and if an interaction between melanin and FAEE existed, lower levels in white fur would be expected. Therefore, lowering them further would simply
exacerbate any significant difference between black and white hair values, rather than obscure it. Conversely, it can be argued that this procedure may have obscured a significantly greater rate of incorporation into white hair, over that in black hair, however, there is no biological basis for this. Apart from melanin, other possible binding sites within the hair cell membrane complex and keratin fibers do not differ between black and white hair (Kintz, 2007).

Alternatively, it is possible that treatment with ethanol was not sufficiently long to produce enough FAEE to detect a statistically significant difference between black and white hair. This remains a possible hypothesis, however it is unlikely as FAEE levels were high enough to be measurable in both pigmented and non-pigmented hair. Previous research where significantly higher levels of drug have been detected in pigmented hair, relative to non-pigmented hair, have reported substantial differences of greater than 20-fold and often with no drug detected in non-pigmented hair (Hubbard et al., 2000; Slawson et al., 1998; Slawson et al., 1996; Borges et al., 2001b; Wilkins et al., 1998; Potsch et al., 1997a; Gygi et al., 1997; Slawson et al., 1996; Gygi et al., 1996; Green and Wilson, 1996; Boyd et al., 1991; Uematsu et al., 1992; Nakahara et al., 1998).

Therefore, my hypothesis that FAEE incorporation is not influenced by hair pigment is the most plausible explanation for the results. This hypothesis is also supported by what is known about drug-melanin interactions in hair; FAEE are not likely to interact with melanin to any significant extent. Previous investigators have demonstrated that melanins typically interact preferentially with basic drugs through their cation exchange properties (Borges et al., 2001b; Borges et al., 2003; Borges et al., 2002; Nakahara et al., 1998; DeLauder and Kidwell, 2000; Nakahara and
Kikura, 1996; Nakahara et al., 1995; Gygi et al., 1997). FAEE are lipophillic and are not expected to interact in this manner.

This study, the second study in my thesis, helps bridge the gap between previous FAEE research and its application in FASD diagnosis and research, because the pediatric arena where the test will be used, involves issues that are highly sensitive, dealing with child custody matters, stigmatization issues, and consequences that can have profound psychosocial repercussions. Therefore, the avoidance of bias is an imperative.

3) Over a third of parents submitted for FAEE testing by CPS, and one fifth of pregnant women within the cohort, were positive for excessive alcohol exposure. These results support the hypothesis that this cohort is at significantly higher risk for using heavy amounts of alcohol then the general population, and therefore also at higher risk for having children with FASD.

A thirty percent positivity rate in parents, and twenty percent in pregnant women is exceptionally high. The Canadian Addiction Survey reports that only 7.9% of Canadians consume alcohol more than 4 times a week, and only 12.7% say their typical usage pattern is more than 5 drinks per occasion (Adlaf et al., 2005). Furthermore, 20% is one of the highest heavy alcohol use rates ever reported amongst pregnant women (Ebrahim et al., 1999; Ebrahim et al., 1998; Stewart and Streiner, 1994; Ethen et al., 2008; Alvik et al., 2006; CDC, 2004; CDC, 2002; CDC, 1997; Sokol et al., 1980; Chambers et al., 2005; Chambers et al., 2006; Colvin et al., 2007; O'Connor and Whaley, 2003; Houet et al., 2006; Crome and Kumar, 2007; Caetano et al., 2006; Magnusson et al., 2005; Alvik et al., 2006).
Among pregnant women in North America, the prevalence of binge drinking (5 or more drinks per occasion) has been reported to range between 0.7 % and 2.9 %, and for frequent drinking (average consumption of seven drinks or more per week, or one binge episode in the last month) between 0.8 % to 3.9 % (CDC, 1997; CDC, 2002; CDC, 2004; Ebrahim et al., 1999; Ebrahim et al., 1998). However, these reported rates are likely underestimates. Wurst et al. report a rate of 7 % for excessive alcohol use, in pregnant women recruited from the general obstetric population, as assessed by biomarker (hair) analysis (Wurst et al., 2008a). Nevertheless, the rates of heavy alcohol use in the present cohort are much higher than those in the general population.

It should be noted that the majority of the present cohort was selected for alcohol testing by CPS based on clinical and social data that suggested alcohol abuse or misuse. However, not all parents were tested for FAEE because of a suspicion of alcohol abuse specifically, some were tested for other reasons such as to confirm abstinence for example. In fact, that is why the fourth study was performed to determine if social worker reports were in agreement with FAEE test outcome. Nevertheless, parents were selected for alcohol testing, therefore, a higher prevalence of heavy drinking is expected, and this rate is not necessarily reflective of the rate among parents involved with CPS in general.

Most importantly, the present study demonstrates that the FAEE hair test is a useful test in assessing parental alcohol use. A 33 % positive rate indicates that the test is good at corroborating social workers suspicion of alcohol use. If the rate was very low, for example between 1 and 5 %, it might put into question the test’s sensitivity in detecting heavy alcohol use or suggest that social workers are unnecessarily testing parents without basis, which would not likely be the case. If on the other hand, a very high rate of positivity was observed, for example
between 90 and 100%, it would suggest that either the test lacked specificity or that social workers are potentially missing a lot of additional heavy-alcohol using parents. A thirty percent positivity rate is a comfortable rate that is similar to the positivity rate for other drugs of abuse tested for in hair by our laboratory, and it suggests the FAEE hair test is performing well in its capacity.

The present study helps bridge the gap between previous FAEE research and the use of the FAEE biomarker assay in FASD because it is the first study to assess alcohol exposure in a cohort of parents at high risk of having children with FASD. The study supports the main hypothesis that the FAEE hair test holds significant potential for use in the FASD context because it demonstrates the test’s clinical utility in identifying alcohol abusing parents.

4) Based on social worker reports, social workers having knowledge of a specific instance of problem drinking within the last 6 months and or a third party report alleging alcohol abuse, were significantly associated with testing positive for heavy alcohol use. Similarly, mothers admitting to heavy alcohol use were at significantly higher risk of testing positive for FAEE as well. However, requesting an alcohol test without suspicion of alcohol use, but rather as a means to “cover all bases”, or because of a history/suspicion of illicit drug use, was negatively associated with testing positive for FAEE.

Therefore, the results support the hypothesis that the FAEE hair test agrees well with social worker reports; reports that were congruent with heavy alcohol use were associated with testing positive, whereas reports that were not necessarily congruent with alcohol use were in fact
negatively associated with testing positive. Furthermore, the results also suggest that alcohol abuse when present is so pervasive, and obvious, that it is hard to miss.

It is true that many of the factors reported by social workers were not significantly associated with FAEE test results. For example, confirming abstinence was not negatively (or positively) associated with testing positive for FAEE. This is likely because some reasons/factors for testing for FAEE occurred much less frequently than others, consequently the study may have been underpowered to assess associations for all factors identified by social workers. Nonetheless, the associations that were found support agreement between hair FAEE and social worker reports.

Furthermore, factors associated with testing positive can be considered predictive factors for social workers to consider when assessing parents for alcohol use. The current sample represents all parents tested over a 6-month period. Any larger sample size may detect additional statistically significant effects, but not likely ones of clinical significance. Social workers need to be aware of what are the strongest indicators of alcohol use that will maximize the efficiency of limited resources.

The present study contributes in bridging the gap between previous FAEE research and the application of the FAEE hair test in FASD research/diagnosis by confirming that the FAEE hair test is good at corroborating social workers suspicions for testing parents. Likewise, the study supports the main hypothesis that the FAEE hair test holds significant potential for use in the FASD context because it demonstrates the test is functioning well in its capacity of assessing alcohol misuse by parents.
5) A significant association between heavy alcohol use and stimulant use was found in parents, with parents that tested positive for FAEE being five times more likely to test positive for methamphetamine, and twice as likely to test positive for cocaine. These results support the hypothesis that parents who use alcohol heavily are at greater risk of using other drugs concomitantly.

Testing positive for illicit drugs may indicate experimental or social use, however the fact that the use of methamphetamine and cocaine were significantly associated with alcohol use, suggests that these were likely repeated patterns consistent with abuse. The difference between occasional use and abuse is important as the latter will more severely affect the potential for fetal exposure and poor rearing environment.

Noteworthy, the present study had different sample sizes for different drugs because of limitations within the study design. Therefore, it is possible that associations between alcohol and other drugs exist, but may have been underpowered to reach statistical significance. This is still true even though cases exist where drugs such as methamphetamine, that contained less samples (n = 94) and still produced a statistically significant association compared to drugs such as opiates, that contained more samples (n = 136) and did not. This is because each drug has its own unique chemical properties that affect its metabolism, elimination, and incorporation into hair, leading to different clinical sensitivities and specificities for each biomarker assays. Consequently, it remains possible that other drugs may still be associated with alcohol use in addition to stimulants.

The hypothesis that parents who use alcohol heavily are at greater risk of abusing other drugs concomitantly is also supported by previous research. Specifically in pregnant women, our own
laboratory found a significant association between heavy alcohol use and amphetamine use, as well as opiate use, as measured by meconium analysis (unpublished results). Also, in a large survey (n = 2672), recent drug use and a history of inpatient treatment for drugs/alcohol were found to be significantly associated with having an alcohol-exposed pregnancy (Project CHOICES Research Group, 2002). Furthermore, 84.5% of children referred to clinic for FASD diagnosis have been exposed to drugs of abuse, based on findings from the largest clinical database of children prenatally exposed to alcohol (Olson et al., 2007).

The current study contributes to bridging the gap between previous FAEE research and FAEE research in FASD in demonstrating how this unique tool can be applied in investigating FASD related issues. In the same regard, the study supports the main hypothesis that the FAEE hair test holds significant potential for use in the context of FASD. Concomitant drug exposure is of great concern to clinicians as it may have direct effects on the fetus compounding FASD, and compromising rearing environment, which has shown to be crucial in FASD management and outcome (Sarkola et al., 2007; Cone-Wesson, 2005; Arendt et al., 2004; Lewis et al., 2004; Thyssen Van et al., 2000; Streissguth et al., 2004).
7.3 Limitations

Many of the study-specific limitations have been discussed in the previous section, and in the discussions within the study chapters; therefore they will not be revisited.

The clinical studies conducted by Pragst and colleagues have determined the sensitivity and specificity of the FAEE hair test, using a cut-off level of 0.5 ng/mg, to be both 90% with respect to identifying heavy drinking (Pragst and Balikova, 2006; Pragst and Yegles, 2008). Therefore, 10% of heavy drinkers may be missed, and 10% may be incorrectly identified as heavy drinkers when they are not. False positives above levels of 1.0 ng/mg have also been known to occur as a result of using hair products heavily concentrated with ethanol, however these appear to be rare exceptions. Furthermore, sensitivity and specificity have never been assessed in the pregnant population, consequently interpretation may vary in this population and needs to be further studied. Therefore, FAEE hair test results need to be considered within the context of accompanying clinical data supporting heavy alcohol use.

Furthermore, FAEE are primarily incorporated through sebum, therefore they get deposited most heavily in the proximal 5-10 cm of hair close to the scalp, regardless of the timing of exposure (Yegles et al., 2004). Consequently, the test has been standardized to the use of 6 cm fragments, obtained from the proximal 6 cm of hair from the scalp, in order to be able to assess and compare results from different samples. However, because hair grows approximately a 1 cm a month, the FAEE hair test cannot assess exposure more than 6 months prior to sample collection. Furthermore, it is not presently possible to resolve when the alcohol was consumed within those
6 months. However, it is generally the case that heavy alcohol consumers use alcohol on an ongoing basis, unless they have decided to stop and or enter treatment. If a subject claims abstinence has begun within the 6 month prior to testing, the FAEE test cannot confirm or deny that claim, but rather the test must be performed at least 6 months after abstinence has begun in order to confirm or refute such a claim. Therefore, the FAEE hair test is limited in its ability to analyze alcohol consumption in a time-wise manner, however this is rarely the investigator or clinician’s goal.

Also, many subjects may have hair shorter than 6 cm, especially in the case of males. Consequently, values obtained from these subjects may differ slightly in their interpretation, but previous literature does not suggest this deviation to be substantial.

7.4 Implications, Significance, and Future Applications

The challenge in the mind of FASD researchers, clinicians, and advocates is how can we begin to impact and change the course of this silent tragedy that has reached epidemic proportions (1 in 100 births)?

The answer, I suggest through the work of my thesis, is through objective identification using the FAEE hair test. Alcohol abuse during pregnancy is difficult to identify because of maternal reluctance to divulge this information, and effects from fetal alcohol are not always obvious, sharing characteristics with many other disorders such as attention deficit disorder, and various
other learning disabilities. Identifying children and families for the purpose of diagnosis and intervention is the first step in changing the course of this epidemic.

My thesis has provided evidence that the FAEE hair test, a biomarker that can assess chronic, heavy alcohol exposure is:

- Sensitive to assess fetal alcohol exposure using maternal and neonatal hair
- Not prone to being biased by hair colour
- Able to assess alcohol exposure in parents, agreeing with social worker reports
- Able to shed light on important concomitant drug exposures

Therefore, my work supports wide use of the FAEE hair test in the field of FASD. The test holds potential for widespread screening of suspected mothers and or babies for fetal alcohol exposure. The test can also be used to identify families at risk for having children with FASD, as was done in my thesis. Further work is required for the validation of the method in pregnancy as well as in neonates, and as mentioned, significant obstacles exist with this challenge.

The FAEE hair test has the potential to revolutionize the field of FASD. Identification and diagnosis will not only reduce the tremendous economic burden that fetal alcohol has on society, but also the immeasurable burden of human suffering caused by this disorder. Early intervention can also help to stabilize child and family life so that affected kids can lead healthier, happier, and more productive lives.
8 Conclusion

The body of work in this thesis has shown the FAEE hair test holds significant potential for use in FASD diagnosis and research. Despite significant challenges in bridging the gap between previous FAEE research and the use of FAEE in the context of FASD, all the studies contained in this thesis have contributed towards bridging this gap.

The first two studies demonstrated how animal models can be applied to further our knowledge regarding FAEE/FASD, and that the FAEE hair test is indeed an effective clinical tool in measuring alcohol exposure because of its high sensitivity in humans and invulnerability towards hair colour bias. The last three studies took advantage of using parents as a model for exposures in pregnancy, and child rearing environment. The studies examined the prevalence of alcohol exposure, the extent of polydrug use, and clinical agreement between the FAEE hair test and social worker reports. These studies demonstrated how the FAEE hair test can be widely applied in context of FASD diagnosis and research.

In conclusion this thesis has added hope for resolving the silent epidemic of fetal alcohol affected children.


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The following works, located in chapters 2, 3, and 4, of this thesis, have been previously published or accepted for publication:

