PLACENTAL HANDLING OF FATTY ACID ETHYL ESTERS: PERFUSION AND SUBCELLULAR STUDIES

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(a)   Running title: The Metabolic fate of FAEE in the human placenta

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ABSTRACT

Background: The measurement of fatty acid ethyl esters (FAEE) in neonatal meconium is a novel test to confirm prenatal ethanol exposure. The origin of FAEE in the maternal-placental-fetal unit is not known. The objectives of this study were to investigate whether FAEE is transferred and metabolized by the human placenta. **Methods:** Isolated placental cotyledons were perfused with a mixture of 4 FAEE (palmitic, stearic, oleic, and linoleic EE) commonly detected in the meconium of neonates exposed to ethanol *in utero* and the transfer of FAEE to the fetal unit was investigated in the absence and presence of albumin. The metabolic degradation of FAEE by human placental microsomes was subsequently determined. **RESULTS:** FAEE disappeared from the maternal circulation but remained undetectable in the fetal unit following perfusions. The addition of albumin had no effect on FAEE transfer. The unrecoverable fraction of individual FAEE in the perfusion system accounted for >50% of the initial amount used, suggesting significant metabolic degradation. Subcellular studies documented the enzymatic degradation of FAEE by placental microsomes (Mean K_m: 35-95 µM; V_{max}: 0.6-1.8 nmol/min/mg for individual FAEE). **CONCLUSIONS:** FAEE at levels found in alcoholics that are originated from the mother are not transferred to the fetus, as they are taken up and degraded extensively by the human placenta. Hence FAEE detected in neonatal matrices are likely produced by the fetus from ethanol that has been transferred to and metabolized by the fetus, rendering FAEE a powerful direct biomarker reflective of true fetal exposure to ethanol *in utero*.

INTRODUCTION

Consumption of alcohol during pregnancy is the cause of Fetal Alcohol Spectrum Disorder (FASD), which affects approximately 1% of all live births (Sampson et al., 1997). According to the current diagnostic guidelines (Institute of Medicine (U.S.), 1996), except when the pathognomonic craniofacial dysmorphology is present, FASD can only be diagnosed with confirmed maternal drinking history, which is often difficult to obtained. As a result, large numbers of affected children cannot be properly diagnosed and receive appropriate interventions in early childhood; while studies have shown that early intervention leads to better prognosis of FASD (Streissguth et al., 1996).

Fatty acid ethyl esters (FAEE) are non-oxidative metabolites of ethanol. The production of FAEE from ethanol and its substrates, fatty acyl Co-A or free fatty acids, is facilitated by cytosolic and microsomal fatty acid ethyl ester synthases (Best and Laposata, 2003). FAEE have been proposed as biological markers of acute and chronic alcohol consumption (Laposata, 1999) as they have been reported to accumulate at high levels in the blood of adult drinkers (Doyle et al., 1996) and in critical organs most commonly affected by chronic alcoholism (Laposata and Lange, 1986). Recently, significantly elevated levels of FAEE have been documented in the meconium of neonates of mothers who had used alcohol heavily in pregnancy (Bearer et al., 1999;Bearer et al., 2003;Chan et al., 2003;Klein et al., 1999;Moore et al., 2003;Moore and Lewis, 2001). Hence, during the last 5 years the measurement of FAEE in meconium

has become a promising objective and non-invasive method to confirm prenatal exposure to ethanol.

Presently, the source of FAEE found in meconium is unknown. FAEE can be produced in the mother and crossing the placenta to the fetus, or produced by the placenta itself. Alternatively, if meconium FAEE are produced in the fetus only, then they would be a biomarker of the amount of ethanol that has circulated in the fetus and has been metabolized by the unborn baby. In such case, meconium FAEE would be superior to other measures such as maternal self-reporting, and maternal biochemical markers.

The objectives of the present study were to elucidate whether maternal FAEE cross the human placenta into the fetus, and whether the placenta is capable of metabolizing FAEE.

MATERIALS AND METHODS

Reagents

All standards and solvents were obtained from Sigma (Toronto, ON). Acetone and *n*-hexane were HPLC grade or better. Bovine albumin (BSA) was essentially fatty acid free (0.005%). FAEE, including palmitic (E16:0), heptadecanoic (E17:0), stearic (E18:0), oleic (E18:1), and linoleic (E18:2) acid ethyl esters were diluted in methanol and stored at -20°C. Capillary GC column (ZB-WAX) was obtained from Phenomenex (Torrance, CA). Enzyme linked immunosorbent assay (ELISA) kits for the analysis of human chorionic gonadotrophin (hCG) were obtained from Alpha Diagnostic International (San Antonio, Texas).

In vitro perfusion of isolated placental cotyledons

Placentas (n = 10) were obtained from caesarean sections or vaginal deliveries of uncomplicated, term pregnancies and transported to the laboratory on ice in heparinized phosphate buffered saline. Perfusion of a peripheral placental lobule was established within 30 minutes of delivery following a method previously described by our laboratory (Derewlany et al., 1991). Briefly, a chorionic artery and vein supplying a peripheral placental lobule were selected and cannulated. The fetal circulation was perfused at a rate of approximately 3 mL/min and recirculated in a 150 mL reservoir while the maternal circuit was perfused at a rate of approximately 15 mL/min and recirculated in a 250 mL reservoir.

The perfusion medium was maintained at 37°C and consisted of antipyrine (Maternal 1 mM), culture medium M199 (10.9 g/L), dextran (Maternal 7.5 g/L; Fetal 30.0 g/L), glucose (Maternal 2.77 mM), heparin (2000 units/L), kanamycin (100 mg/L), and sodium bicarbonate (Maternal 30 mM; Fetal 25 mM). Sodium bicarbonate was added to maintain the pH of the perfusates within physiologic range (Maternal, pH 7.4; Fetal, pH 7.35). The maternal circuit was equilibrated with 95% O_2 and 5% CO_2 whereas the fetal circuit was equilibrated with 95% N_2 and 5% CO_2.

(1) Perfusion studies without albumin (n = 4)

In 4 of the placentae, after an initial 10-minute "wash-out" period, the pre-experimental control period proceeded. Both circuits were closed and recirculated with fresh perfusate for 60 minutes. At the end of the 1-hour control, the fetal reservoir was replaced with 150 mL of fresh perfusate and the maternal reservoir was replaced with 250 mL of fresh perfusate containing four FAEE (palmitic, stearic, oleic, linoleic EE) at concentrations reported in the circulation of drinkers (final concentrations of 5 μM (n = 2) and 20 μM (n = 2) for each FAEE). In healthy human volunteers who had been given sufficient alcohol to raise their blood alcohol concentration (BAC) to the legal limit of 100 mg/dL, a mean FAEE concentration of 2 μM was measured (Doyle, Cluette-Brown, Dube, Bernhardt, Morse, and Laposata, 1996). Samples from the maternal and fetal reservoirs were collected every 15 minutes during the first 1.5-hour and then every 30 minutes until 240 minutes for the analysis of FAEE. At the end of the experiment, both circuits were switched to fresh perfusate for a 1-hour post-

experimental control. FAEE were extracted from the perfused tissue for the determination of the fraction bound to the placenta. The net disappearance of FAEE from the perfusion system was calculated from the following mass balance equation:

Net disappearance (%) = (Initial $[FAEE]_{maternal}$ − Terminal $[FAEE]_{maternal}$ − Terminal $[FAEE]_{fetal}$ − Terminal $[FAEE]_{placental}$) ÷ Initial $[FAEE]_{maternal}$ x 100%

(2) Perfusion studies with albumin (n = 6)

In 6 of the placentae, the same conditions were kept plus fatty acid free bovine albumin at 0.5% w/v was added. The addition of albumin to the perfusate aimed at elucidating whether it facilitates the transfer of FAEE across the placenta by acting as a carrier for FAEE in solution. Albumin has been a proposed carrier for FAEE in the circulation of drinkers (Bird et al., 1996;Bird et al., 1997;Doyle et al., 1994) and for *in vitro* assay systems involving FAEE (Chang and Borensztajn, 1999;Hasaba and Laposata, 2001;Kabakibi et al., 1998). The physiologic albumin to non-esterified (i.e. free) fatty acid ratio in third trimester is 0.78 as reported by Haggarty and colleagues (Haggarty et al., 1997), however, the ratio of albumin to FAEE *in utero* is not known. Therefore, albumin (0.5% w/v) was provided in sufficient excess to allow binding of all FAEE in both the maternal and fetal circulation.

Four individual FAEE commonly detected in meconium [palmitic (E16), stearic (E18), oleic (E18:1), and linoleic (E18:2) EE, diluted in methanol] were added to the maternal perfusion medium in concentrations reported in the circulation of drinkers (final concentrations of 2 μM and 20 μM for each FAEE) at

least 24 hours prior to the experiments to allow maximal equilibration between FAEE and albumin. The final concentration of methanol in the solution was less than 0.1%. At this stage, the lower concentration of FAEE was reduced from 5μM to 2 μM because of improved sensitivity in the chromatographic analysis of FAEE.

After an initial 10-minute "wash-out" period, the pre-experimental control period proceeded. Both circuits were closed and recirculated with fresh perfusate containing no albumin for 30 minutes, after which the circuits were switched to albumin-containing perfusate (0.5%, w/v) for another 30 minutes. At the end of the 1-hour control, the fetal reservoir was replaced with 150 mL of fresh albumin-containing perfusate and the maternal reservoir was replaced with 250 mL of fresh perfusate containing albumin (0.5%, w/v) and the four FAEE at equimolar concentrations [2 μM (n = 3) and 20 μM (n = 3)]. Samples from the maternal and fetal reservoirs were collected every 10 minutes during the first half-hour and then every 30 minutes until 180 minutes for the analysis of FAEE. At this stage, the experimental period was shortened from 4 hours to 3 hours based on the data obtained from the studies conducted in the absence of albumin showing that changes in FAEE disposition plateau after 2 hours. At the end of the experiment, both circuits were switched to fresh albumin-containing perfusate for a 1-hour post-experimental control. FAEE were extracted from the perfused tissue for the determination of the fraction bound to the placenta as described previously.

Sample Analysis

Arterial and venous samples were taken from the maternal and fetal circuits throughout the control and experimental periods for pH and pO_2 measurements. The physical integrity of the placental preparation was assessed by frequent monitoring of the fetal perfusion pressure (reject if > 10 mmHg) and excessive volume loss (reject if > 3 mL/ hr). Samples were taken from both reservoirs (every 15 minutes during control, and every 30 minutes during experiment) for the analysis of oxygen and glucose consumption, and the production of lactate and human chorinic gonadotrophin (hCG). Tissue hCG content was measured in both fresh and perfused tissue.

The perfusate concentrations of pH and pO_2 were measured using a blood gas analyzer (ABL 330; Radiometer, Copenhagen, Denmark). The concentrations of glucose and lactate in perfusate samples were measured as described previously (Derewlany, Leeder, Kumar, Radde, Knie, and Koren, 1991). For the analysis of hCG, samples of fresh and perfused placental tissue were homogenized with 5 mL of ice-cold phosphate buffered saline (0.1M, pH 7.4) per gram of tissue using a Polytron (Brinkmann Instruments). The homogenate was centrifuged at 8000 rpm, 4°C for 15 minutes and the supernatant aliquoted and stored at -20°C until analysis. The perfusate and tissue concentrations of hCG were measured by ELISA (Alpha Diagnostic International; San Antonio, Texas).

FAEE were extracted from perfusate samples, fresh and perfused placental tissue with 5 mL of hexane:acetone (5:2, v:v) and analyzed by gas chromatography with flame ionization detection (GC-FID) using a previously established method (Chan, Bar-Oz, Pellerin, Paciorek, Klein, Kapur, Farine, and Koren, 2003). The limit of detection was 0.5 pmol. Tissue samples were homogenized with 5 mL of ice-cold phosphate buffered saline (0.1M, pH 7.4) per gram of tissue using a polytron (Brinkmann Instruments, Switzerland), aliquoted and stored at -20°C until analysis. The recovery of FAEE from placental perfusate and tissue were determined by extrapolation from standard curves prepared by spiking known concentrations of FAEE in the respective blank matrices (i.e. blank perfusate and tissues), in order to account for matrix effects.

Degradation of FAEE by Placental Microsomes

Isolation of Placental Cytosol and Microsomes

Subcellular fractions of the fresh human placenta (n = 4) were isolated using standard differential centrifugation techniques. Pieces of villous tissue (about 70 g) were dissected and rinsed thoroughly in ice-cold saline to remove excess blood, and then blotted dry on gauze. The tissues were then homogenized in a blender (Waring Products Co.) with 5 mL ice-cold HEGM-D buffer (25 mM NaHepes, 1 mM Na_2EDTA, 10% glycerol, 20 mM $NaMolybdate.2H_2O$, 1mM dithiothreitol) per gram of tissue. The homogenate was aliquoted and centrifuged at 8000 rpm, 4°C for 15 minutes in a Sigma SK-30 Centrifuge (Sigma Laboratories, West Germany). The resulting pellet was discarded and the supernatant was centrifuged at 15000 rpm, 4°C for 15

minutes. The supernatant from this second spin was pooled and centrifuged at 35000 rpm, 4°C for 1 hour in a Beckman Optima LE-80K Ultracentrifuge (Beckman Coulter Inc., USA). The supernatant (cytosol) was aliquoted into cryovials, frozen in liquid nitrogen, and stored at -80°C until analysis. The pellet (microsomes) was resuspended in HEGM-D buffer using a Teflon pestle homogenizer (Canadian Laboratory Supplies Ltd.) and centrifuged at 35000 rpm, 4°C for 1 hour. The final supernatant was discarded and the microsomal pellet was resuspended with a Teflon pestle homogenizer in HEPES-sucrose buffer (5 mM HEPES, 0.25M sucrose, pH 7.4), aliquoted into cryovials, frozen in liquid nitrogen and stored at -80°C until analysis. The amount of proteins in the subcellular fractions was determined by the Bio-Rad microtiter plate method (Bio-Rad Laboratories, USA). The subcellular preparations were assayed within 6 months.

Assay Conditions

Initial experiments showed negligible FAEE degradation in cytosolic preparations, and thus only microsomal preparations were investigated in remaining experiments. Two experiments (each in triplicate) were conducted for each of the 4 placentas studied. Microsomal protein (100 µg) was pre-incubated in 0.1 M potassium phosphate buffer (0.01 M EDTA, 2 mg/ mL BSA, total volume 200 µl) for 20 minutes at 37°C. A mixture containing equimolar concentrations of palmitic, stearic, oleic, and linoleic EE (dissolved in methanol) was added (final concentrations of 0, 5, 25, 50, 100, 200 µM for each FAEE), and samples were incubated for 120 minutes at 37°C. For 0 µM, 10 µL of methanol was added, and

heat-inactivated microsomes were used in control experiments. The reaction was stopped by chilling on ice followed by the addition of 100 µl of ice-cold heptadecanoic EE (internal standard in hexane) and 5 mL of ice-cold extraction solvent (hexane:acetone, 5:2, v:v). FAEE were extracted and analyzed as described previously.

Kinetic Analysis

The velocity (V) versus substrate (FAEE) data were linearized by plotting [FAEE]/ velocity vs. [FAEE] (i.e. Hanes-Woolf Plot). The kinetic parameters (K_m, V_{max}) of FAEE degradation were derived from the line of best fit as determined by linear regression analysis. The maximum velocity (V_{max}) of FAEE degradation was calculated from the reciprocal of the slope and the apparent affinity (K_m) from the product of the y-intercept and V_{max}.

Statistical Analysis

The data was analyzed using the Student's T-test or the Mann Whitney Rank Sum Test (for non-parametric data) using the SigmaStat software (Version 2.03, SPSS Inc.)

RESULTS

In vitro perfusion of isolated placental cotyledons

(1) *Perfusion studies without albumin (n = 4)*

The mean mass of the perfused cotyledons was 15.27 ± 6.59 g (mean ± SD). There were no significant differences in the fetal arterial pressures between the control and experimental periods (mean ± SD): Pre-experimental control, 38.9 ± 1.4 mmHg; Experiment, 39.5 ± 0.9 mmHg; Post-experimental control, 43.1 ± 2.1 mmHg. The fetal flow rate was 2.48 ± 0.37 mL/min and maternal flow rate was 13.11 ± 0.74 mL/min. The rates of oxygen and glucose consumption, and the production of lactate and hCG did not differ in the control and experimental periods (data not shown).

The disposition of FAEE in the maternal and fetal reservoirs in the absence of albumin are shown in (Figure 1a). The levels of each ester decreased steadily during the 4-hour perfusion but none of the 4 FAEE was detectable in the fetal matrix throughout the experiment at both low (5 μM) and high maternal concentrations (20 μM). At the end of the low concentration (5 μM) perfusions, only 2 of the 4 FAEE species (stearic EE and oleic EE) remained in the maternal reservoir, at less than 20% of the initial concentration. Between 18% to 88% of the initial concentration of the 4 esters remained in the maternal matrix at the end of the high concentration perfusions (20 μM). A linear decay was documented for all 4 FAEE when the maternal concentration versus time data were log transformed (i.e. log [FAEE] vs. time), and the respective rates are shown in (Table 1). At high concentration, the rates of FAEE elimination from the

maternal reservoir were at least 3-fold slower than they were for low concentration perfusions ($p > 0.05$). The fate of each FAEE post perfusion is shown in (Figure 2a) as a mass balance. The amount of FAEE retained in the placenta was less than 6% at the end of low concentration perfusions and less than 17 % after perfusing at high concentration. Linoleic EE was not retained by the perfused placenta. Stearic EE was consistently the FAEE the least eliminated from the maternal reservoir and most retained by the perfused placenta at both low and high concentration. The net disappearance of individual FAEE decreased from greater than 85% at low concentration experiments to approximately 24% to 89% when high initial concentrations (20µM) were used.

(2) *Perfusion studies with albumin (n = 6)*

The mean mass of the perfused cotyledons was 9.85 ± 4.80 g (mean ± SD). There were no significant differences in the fetal arterial pressures between the control and experimental periods (mean ± SD): Pre-experimental control, 46.2 ± 12.7 mmHg; Pre-experimental control (with albumin), 49.3 ± 16.3 mmHg; Experiment, 54.7 ± 10.4 mmHg; Post-experimental control, 56.2 ± 8.1 mmHg. The fetal flow rate was 3.45 ± 0.67 mL/min and maternal flow rate was 14.51 ± 0.40 mL/min. The rates of oxygen and glucose consumption, and the production of lactate and hCG did not differ in the control and experimental periods (data not shown).

The disposition of FAEE in the maternal and fetal reservoirs in the presence of albumin are shown in (Figure 1b). The levels of each ester decreased steadily during the 3-hour perfusion but none of the 4 FAEE was

detectable in the fetal matrix throughout the experiment at both low (2 μM) and high maternal perfusate concentrations (20 μM). Less than 15% of the initial concentration of each FAEE remained in the maternal reservoir at the end of the low concentration (2 μM) perfusions whereas 14% to 49% of the initial concentration remained in the maternal matrix at the end of the high concentration perfusions (20 μM). A linear decay was documented for all four FAEE when the maternal concentration versus time data were log transformed (i.e. log [FAEE] vs. time), and the respective rates are shown in (Table 1). At high concentration, the rates of FAEE elimination from the maternal reservoir were approximately 50% slower than they were for low concentration perfusions ($p < 0.05$ for stearic EE). In addition, the rate of elimination of stearic EE was significantly slower than that of oleic and linoleic EE ($p < 0.05$) at 20μM. The rates of FAEE elimination in the presence of albumin were not significantly different from the values obtained during experiments without albumin. The fate of each FAEE post perfusion is shown in (Figure 2b) as a mass balance. The amount of FAEE retained in the placenta was less than 4% at the end of low concentration perfusions and less than 12% after perfusing at high concentration. Stearic EE was consistently the FAEE the least eliminated from the maternal reservoir and most retained by the perfused placenta at both low and high concentration. The net disappearance of individual FAEE decreased from greater than 85% at low concentration experiments to 49% to 86% when high initial concentrations (20μM) were used.

Degradation of FAEE by Placental Microsomes

Negligible metabolic activity was observed with cytosolic preparations during initial investigation (data not shown); therefore only microsomal proteins were assayed in subsequent experiments. The activity of placental microsomes in FAEE degradation is shown in (Figure 3) and the resulting kinetic values are summarized in (Table 2). No degradation was observed when heat-inactivated microsomes were used. Among the 4 placentas studied, there was up to 4-fold variability in the apparent affinities (K_m) and 2-fold variability in maximum velocities (V_{max}) for individual FAEE. In the same placenta, the variability in these kinetic values was between 2 to 5-fold for individual FAEE. Linoleic and palmitic EE were associated with the highest apparent affinities whereas the K_m of stearic EE was consistently the lowest among all placentas studied ($p < 0.05$). The V_{max} of stearic EE was significantly lower than the other 3 FAEE ($p < 0.05$) and it was the FAEE the least degraded by placental microsomes.

DISCUSSION

To the best of our knowledge, this is the first study to investigate the fate of FAEE within the maternal-placental-fetal unit and the role of the human placenta in FAEE metabolism. Understanding the origin of meconium FAEE is of critical importance in the development of FAEE as biological markers of fetal exposure to ethanol *in utero*. It has been reported previously that certain FAEE accumulate in various tissues and organs following maternal ethanol administration in mice (Bearer et al., 1992).

With the use of an *in vitro* perfusion model, our study demonstrates that FAEE originated from the mother do not cross the human placenta to reach the fetus. The addition of albumin in the perfusion system to serve as a carrier had no effect and did not facilitate the transfer of FAEE. A mixture containing equimolar concentrations of several FAEE that are commonly detected in the meconium of neonates born to drinking mothers was initially introduced to the maternal compartment. The levels of all FAEE were found to decrease steadily in the maternal reservoir over the course of the perfusions but this did not result in a parallel accumulation in fetal concentration, suggesting potential uptake and metabolism by the placenta. FAEE remained undetectable in the fetal circulation throughout the experiment both at low (2μM and 5μM) and high (20μM) initial concentrations.

The physiologic levels of FAEE in the circulation of drinking pregnant women have not been documented. The low concentrations (2μM and 5 μM) employed in our studies were extrapolated from previous investigations in

healthy human volunteers who had been given sufficient alcohol to raise their blood alcohol concentration (BAC) to the legal limit of 100 mg/dL (Doyle, Cluette-Brown, Dube, Bernhardt, Morse, and Laposata, 1996). This concentration was chosen to reflect the level that may be potentially seen in a socially drinking mother, and the 10-fold higher concentration was intended to represent a level that would be reached by a heavily drinking mother. The lack of transfer of FAEE across the human placenta even at such high concentrations suggests that FAEE detected in fetal meconium does not originate from the mother.

The linear relationships that resulted from the log-transformed analysis of the concentration-time data suggest that FAEE are eliminated by the placenta following first order kinetics. The rates of FAEE elimination from the maternal circulation were very similar in the presence or absence of albumin. When perfused at an initial concentration of 20μM, linoleic EE was cleared the fastest by the mother and stearic EE was the species eliminated most slowly from the maternal circulation and also most highly retained by the perfused placenta. This is in agreement with a previous report that showed preferential accumulation of stearic EE by the mouse placenta after maternal ethanol administration (Bearer, Gould, Emerson, Kinnunen, and Cook, 1992). This selective retention of individual species and the large unrecoverable fraction of FAEE from the perfusion system suggest differential rates of metabolism (i.e. degradation) of individual FAEE species by placental enzymes. The net disappearance of FAEE from the "closed" system decreased from greater than 90% at the end of low concentration perfusions to between 50% and 85% after perfusing at high

concentration, which suggests saturable uptake and metabolism of FAEE by placental tissue.

It has been proposed that FAEE are degraded via enzymatic hydrolysis into their parent fatty acids and ethanol (Laposata, 1999). The hypothesis that FAEE are degraded enzymatically by placental enzymes was investigated further using subcellular placental preparations and *in vitro* assays. The apparent rates of elimination and kinetic parameters obtained from our study may not represent the actual values associated with individual species of FAEE if each ester was assayed separately. However, it is physiologically impossible that the developing fetus would be exposed to each ester individually *in utero* and therefore, the use of a mixture of 4 FAEE that would be commonly detected in a fetal compartment is conceivable. The lack of metabolic activity demonstrated by heat-inactivated placental subcellular fractions suggests that the degradation of FAEE in the placenta is enzymatic. Negligible metabolic activity was observed with cytosolic preparations during initial investigation; therefore only microsomal proteins were assayed in subsequent experiments. This is concordant with a previous finding that only membrane bound enzymes localized to the microsomal and mitochondrial-lysosomal fractions of rat hepatoma cells displayed significant FAEE hydrolytic activity (Laposata et al., 1990). The variability associated with the kinetic parameters of individual FAEE corroborated the trends observed during perfusion experiments. Linoleic EE was consistently associated with the highest K_m and V_{max} values, and it was the ester that was cleared the fastest and least retained by the placenta. As important, the apparent affinities and

maximum velocities of stearic EE was the lowest among all placentas studied, which was consistent with the perfusion data that it was the ester associated with the slowest maternal elimination and the most retained by the placenta. The apparent affinity (K_m) of a substrate is inversely proportional to its affinity for the enzyme responsible for its metabolism, hence stearic EE has the highest affinity for the enzymatic activity observed in the human placenta and low concentrations are sufficient to saturate the enzymes.

The FAEE hydrolyzing capacities of various organs and tissues have been reported in humans and several animal species, and significant variability was observed. Rapid hydrolysis of FAEE has been observed in rat plasma (oleic EE) (Hungund et al., 1995), rat adipose tissue (linoleic EE) (DePergola et al., 1991), and human blood (oleic EE) (Saghir et al., 1999;Saghir et al., 1997) with elimination half-lives of less than 24 hours. The rates of FAEE hydrolysis were found to be significantly different in human and rat serum (0.34 ± 0.29 vs. 195 ± 31 nmol/ min/ mL, respectively) (Diczfalusy et al., 2001). Using tissue homogenates, Diczfalusy et al. investigated the capacity of various human organs and tissues to hydrolyze FAEE (palmitic EE) and concluded that the activity was highest in liver and pancreas (1100 –1600 nmol/ min/ g), and lowest in heart and adipose (10 – 15 nmol/ min/ g) (Diczfalusy, Bjorkhem, Einarsson, Hillebrant, and Alexson, 2001). There are very limited data on placental metabolism of FAEE in the literature. Using tissue homogenates, Bearer and colleagues have determined previously the FAEE synthetic activity of the human and mice placenta to be 0.2 and 1.2 nmol/ hr/ mg respectively, in the presence of

0.4 mM of oleic acid and 200 mM of ethanol (Bearer, Gould, Emerson, Kinnunen, and Cook, 1992). These rates of synthesis are very slow in comparison to the rates of FAEE degradation found in our study (V_{max} of various esters ranged between 0.6 and 1.8 nmol/ min/ mg), which supports our hypothesis that maternal FAEE are metabolized extensively by the human placenta and thus the net effect is to prevent their transfer to the fetus. The rate of FAEE degradation in our study is most comparable to that observed in purified human pancreatic triglyceride lipase (oleic EE, 0.5 nmol/ min/ mg) (Riley et al., 1990), but significantly slower than in liver microsomes of humans (palmitic EE, 40 nmol/ min/ mg) (Diczfalusy, Bjorkhem, Einarsson, Hillebrant, and Alexson, 2001) and rats (palmitic EE, 100 nmol/ min/ mg) (Diczfalusy et al., 1999), as well as purified porcine pancreatic carboxylester lipase (oleic EE, 13.8 μmol/ min/ mg) (Tsujita and Okuda, 1994).

The specific enzymes responsible for the degradation of FAEE in the human placenta are not known at this point. The linear relationships obtained from the kinetic studies may suggest several options: (1) the human placenta contains a single enzyme which is responsible for the degradation of FAEE; (2) multiple enzymes which are kinetically indistinguishable; or (3) one of these enzymes is dominant. The hydrolytic activities of FAEE in rat and human liver microsomes have been attributed to different species of carboxylesterases by the use of specific serine esterase inhibitors (Diczfalusy, Bjorkhem, Einarsson, Hillebrant, and Alexson, 2001;Diczfalusy, Bjorkhem, Einarsson, and Alexson, 1999). Significant carboxylesterase activity towards the hydrolysis of 1-

naphthylacetate and carboxylic esters of *p*-nitrophenol have been documented in the microsomal fraction of term human placenta and at least 3 isoforms have been identified (Yan et al., 1999). Whether the metabolic capacity observed in our study was associated with carboxylesterase or other enzymes requires further investigation.

Our studies show that FAEE originated in the maternal circulation are not transferred through the placenta to the fetus, and that the maternally circulated FAEE are taken up and degraded extensively by the human placenta. Hence FAEE detected in neonatal meconium or hair are likely produced by the fetus from the ethanol transferred to, and metabolized by the fetus, rendering FAEE as the first direct biomarkers of true fetal exposure to ethanol *in utero*.

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FOOTNOTES

LEGENDS FOR FIGURES

(Figure 1)

The disposition of FAEE in the maternal circulation (a) in the absence of albumin

(5 μM, n = 2; 20μM, n = 2) and (b) in the presence of 0.5% (w/v) albumin (2 μM,

n = 3; 20μM, n = 3). In the absence or presence of albumin, the concentrations

of individual FAEE species decreased steadily in the maternal reservoir while

they remained undetectable in the fetal matrix throughout the experiment.

(Figure 2)

The fate of FAEE post perfusion (a) in the absence of albumin and (b) in the

presence of albumin. The addition of albumin had no effects on the metabolic

fate of FAEE. Stearic EE was consistently the FAEE the least eliminated from

the maternal reservoir and most retained by the perfused placenta at both low

and high concentration perfusions.

(Figure 3)

Metabolism of FAEE by human placental microsomes (n = 4).

Table 1: The rates of elimination (min^{-1}) (mean ± SD) of individual FAEE from the maternal reservoir.

Log-transformed concentration vs. time plots (i.e. log [FAEE] vs. time) yielded linear relationships for all 4 FAEE, which suggest elimination following first order kinetics. Similar rates were found in experiments with and without the addition of albumin. At an initial concentration of 20 μM and in the presence of albumin, the rate of stearic EE elimination from the mother was significantly slower than oleic and linoleic EE ($p < 0.05$); and significantly slower than its rate at 2 μM ($p < 0.05$).

| FAEE	Palmitic EE	Stearic EE	Oleic EE	Linoleic EE
Initial 5μM (no albumin, n = 2)	0.013 ± 0.011	0.007 ± 0.006	0.012 ± 0.009	0.026 ± 0.024
Initial 20μM (no albumin, n = 2)	0.004 ± 0.003	0.002 ± 0.002	0.003 ± 0.002	0.008 ± 0.007
Initial 2μM (n = 3)	0.012 ± 0.005	0.011 ± 0.004	0.016 ± 0.008	0.019 ± 0.010
Initial 20μM (n = 3)	0.006 ± 0.002	0.004 ± 0.001	0.008 ± 0.002	0.010 ± 0.001

Table 2: Kinetic parameters of FAEE degradation by placental microsomes.

FAEE	K_m (µM)	V_{max} (nmol/min/mg)
Palmitic EE	95.42 ± 17.18	1.71 ± 0.22
Stearic EE	35.51 ± 12.53	0.57 ± 0.13
Oleic EE	66.53 ± 16.47	1.22 ± 0.20
Linoleic EE	94.47 ± 19.10	1.78 ± 0.27

Data shown are mean ± SEM; n = 4.

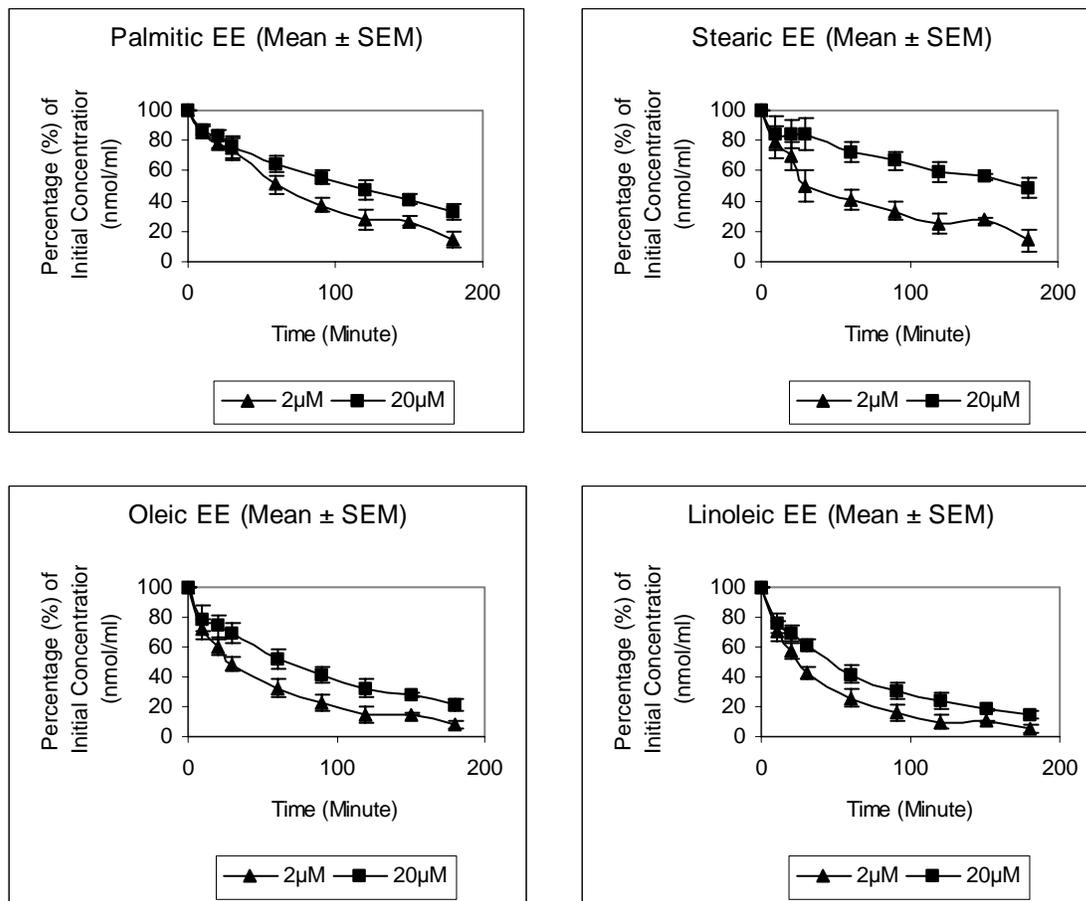**Figure 1: The disposition of FAEE in the maternal circulation.**
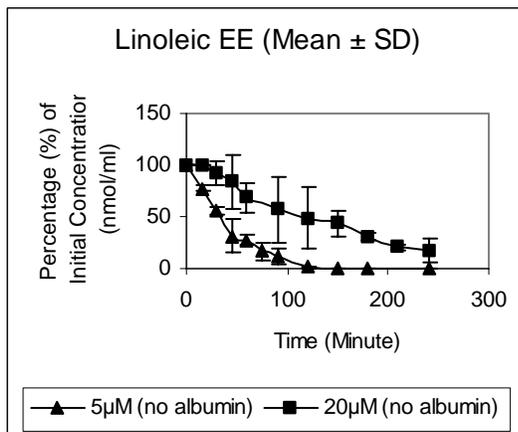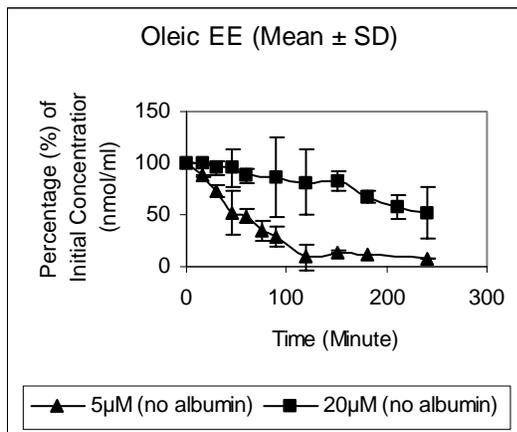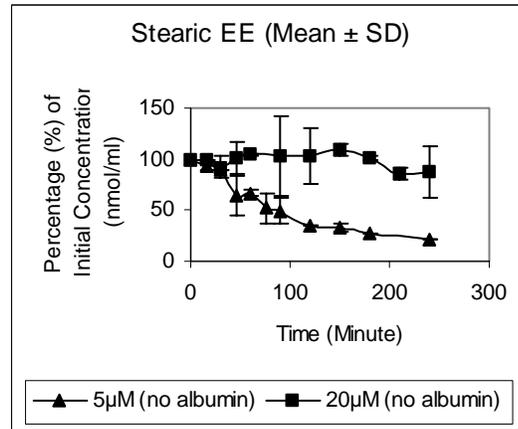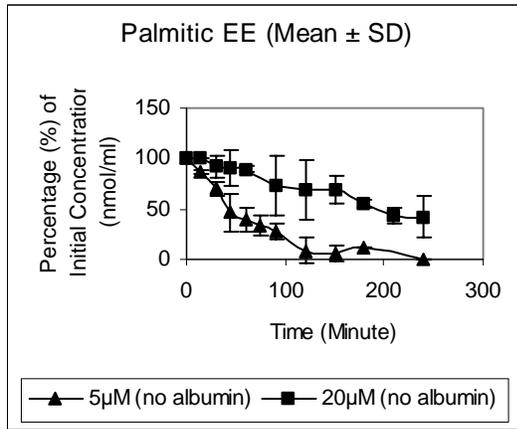
(a) In the absence of albumin

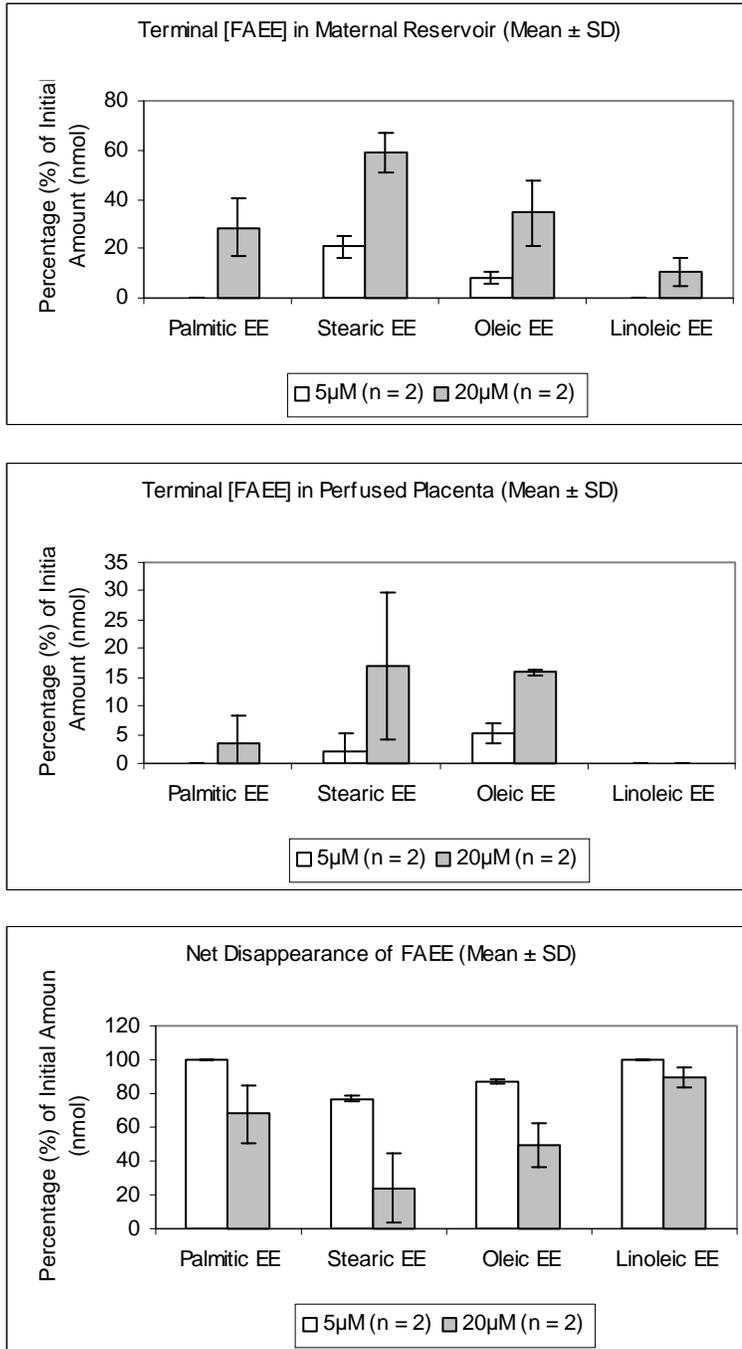## Figure 1: The disposition of FAEE in the maternal circulation.

(b) In the presence of albumin (0.5 %, w/v)

Figure 2: The fate of FAEE post perfusion.

(a) In the absence of albumin

Terminal [FAEE] in Maternal Reservoir (Mean ± SD)

Terminal [FAEE] in Perfused Placenta (Mean ± SD)

Net Disappearance of FAEE (Mean ± SD)

Figure 2: The fate of FAEE post perfusion.
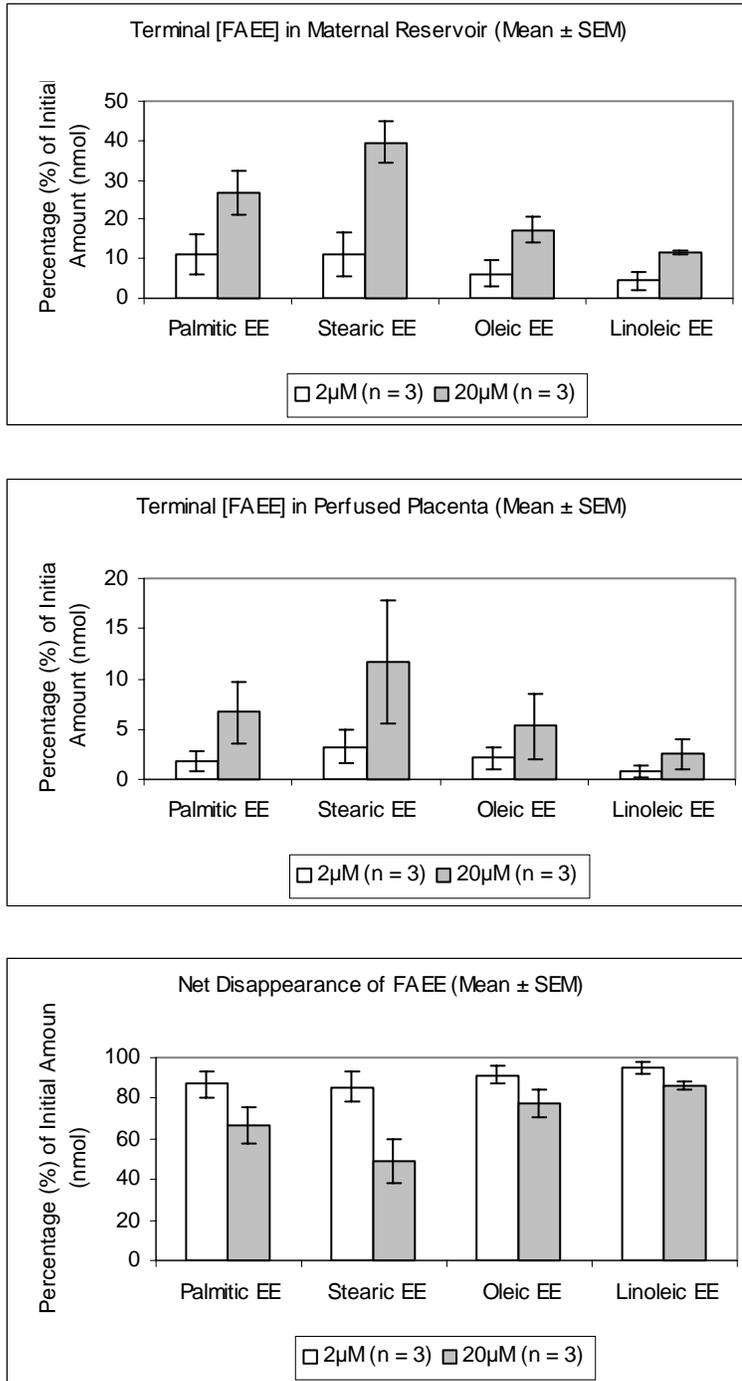
(b)   In the presence of albumin

Figure 3: Metabolism of FAEE by human placental microsomes (n = 4).