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ANNI LEHIKOINEN

Fetal alcohol exposure

Biochemical findings and insights into clinical outcome

FETAL ALCOHOL EXPOSURE

BIOCHEMICAL FINDINGS AND INSIGHTS INTO CLINICAL OUTCOME

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ABSTRACT

Fetal alcohol exposure causes a spectrum of adverse effects on the unborn fetus. Fetal alcohol spectrum disorder (FASD) is an umbrella term that describes all fetal alcohol effects. The FASD includes fetal alcohol syndrome (FAS), fetal alcohol effects (FAE), fetal alcohol-related neurodevelopmental disorder (ARND) and alcohol-related birth defects (ARBD). Despite the known adverse effects of alcohol use during pregnancy, we lack a reliable way to detect fetal alcohol exposure.

The purpose of this study was to investigate the effects and long-term sequelae of alcohol use during pregnancy. This work focuses on three timeframes: Firstly, we studied metabolomics and trisomy screening parameters of alcohol- and drug-abusing pregnant mothers during the first trimester. Secondly, we investigated alcohol- and drug- exposed fetuses during the second trimester of pregnancy by ultrasonography and followed their outcome at the age of 2.5 years. Thirdly, we performed MRI and SPECT imaging in children with FAS/FAE and ophthalmological examination in young adults diagnosed with FAS/FAE as children.

The metabolite profile of alcohol- and drug-abusing mothers seems to differ from that of non-abusing mothers during the first trimester of pregnancy. Alcohol- and drug- abusing mothers had increased glutamate and decreased glutamine levels, and alcohol use was associated with

decreased serotonin levels. However, the first trimester screening parameters (free β-hCG, PAPP-A and NTT) for trisomy 21 were not affected by alcohol and drug abuse. Nonetheless, smoking increased free β-hCG levels and decreased PAPP-A levels. Mothers giving birth to an SGA child showed decreased PAPP-A levels.

Alcohol- and drug- abusing mothers (n=23) and their controls (n=22) were followed during pregnancy and fetal ultrasonography measures were analysed during mid-pregnancy. We found that smaller head size was associated with alcohol and drug exposure. Head circumference and height of exposed children remained reduced compared to the population reference at 2.5 years of age (mean -0.82 and -0.75 SD-scores respectively, $P<0.01$for both).

MRI showed smaller absolute volumes of the amygdala, caudatus, putamen and hippocampus in FAS/FAE children than in controls. SPECT imaging using a specific radioligand showed reduced serotonin transporter (SERT) and increased dopamine transporter (DAT) binding in striatal nuclei of FAS/FAE children (n=12) indicating similarities with alcohol dependency characterized by antisocial, impulsive, and aggressive personality and early-onset alcohol dependence (type 2 alcoholism). Additionally, when examined as young adults (n=10), reduced retinal nerve fibre layer thickness on optical coherence tomography (OCT) was found, which is in line with the previous animal and human studies.

In conclusion, this study increases understanding of the structural and biochemical changes in alcohol-using mothers and their children. The results of this study indicate that alcohol use during the pregnancy causes a spectrum of harmful effects to the pregnanct mother and exposed children. However, further research is needed to find a reliable way to detect alcohol use and to control for confounding factors.

Keywords: Fetal Alcohol Spectrum Disorders; Tomography, Optical Coherence; Ultrasonography, Prenatal; Magnetic Resonance Imaging; Tandem Mass Spectrometry; Fetus; Chromatography, Liquid; Adolescent; Ethanol

Lehikoinen, Anni
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TIIVISTELMÄ

FASD, fetal alcohol spectrum disorder on termi, joka kuvaa sikiöaikaisen alkoholialtistuksen aiheuttamaa oirekirjoa. Sikiöaikaisen alkoholialtistuksen oirekirjoa kuvataan neljällä diagnoosilla: 1. fetaalialkoholioireyhtymä (FAS, fetal alcohol syndrome), 2. osittainen fetaalialkoholioireyhtymä (FAE, fetal alcohol effects), 3. alkoholin aiheuttamat keskushermoston vauriot (ARND, alcohol-related neurodevelopmental disorder) ja 4. alkoholin aiheuttama epämuodostuma (ARBD, alcohol-related birth defect). Sikiöaikaisen alkoholialtistuksen tiedetään olevan haitallista kehittyvälle sikiölle, mutta kliinisessä työssä ei ole luotettavaa keinoa tunnistaa raskauden aikainen alkoholin käyttö.

Tämän väitöskirjan tavoitteena oli tutkia raskauden aikaisen alkoholin käytön vaikutusta ja seurauksia eri vaiheissa raskautta ja altistuneen lapsen elämää. Tässä työssä tutkimme tarkemmin kolmea eri ajanjaksoa: 1. Ensimmäisen raskauskolmanneksen aikana tutkimme äitien metabolomiikkaa ja trisomiaseulan tuloksia. 2. Toisen raskauskolmanneksen aikana teimme alkoholille ja muille päihteille altistuneiden sikiöille ultraäänitutkimuksen ja seurasimme lasten kehitystä aina 2.5 vuoden ikään saakka. 3. Kouluikäisille FAS/FAE diagnoosin saaneille lapsille teimme pään MRI- ja SPECT- tutkimuksen ja teimme näille lapsille varhaisessa aikuisiässä silmätutkimuksen.

Ensimmäisen raskauskolmanneksen aikana alkoholia ja päihteitä käyttävien äitien glutamaattitasot olivat korkeammat ja glutamiinitasot matalammat kuin kontrolliäideillä. Alkoholia ja päihteitä käyttävien äitien serotoniinitasot olivat myös kontrolliäitien tasoja matalammat. Ensimmäisen raskauskolmanneksen trisomiaseulan tulokset eivät eronneet kliinisesti merkittävästi kontrolliaineistosta. Vapaa β-hCG oli alkoholia ja päihteitä käyttävillä äideillä korkeampi ja PAPP-A matalampi kuin kontrolliäideillä, mutta nämä löydökset selittynevät tupakoinnilla. Myös äideillä, joiden vastasyntynyt lapsi oli raskauden kestoon nähden pienipainoinen, oli verrokkiäitejä matalammat PAPP-A tasot.

Keskiraskaudessa teimme ultraäänitutkimuksen alkoholia ja muita päihteitä käyttävien äitien (n=23) ja kontrolliäitien (n=22) sikiöille (n=11 alkoholille ja muille päihteille altistuneet sikiöt; n=20 kontrollit). Sikiön pienempi pään koko näytti assosioituvan äidin alkoholin ja päihteiden käyttöön raskausaikana. Alkoholille ja muille päihteille altistuneiden lasten päänympärysten ja pituuksien keskiarvot olivat vielä 2,5 vuoden iässä 0,82 ja 0,75 SD-yksikköä alhaisemmat kuin samanikäisten suomalaisten lasten vastaavat keskimitat (P<0.01).

FAS/FAE-diagnoosin saaneilla lapsilla oli MRI-tutkimuksessa pienemmät amygdala, caudatus, putamen ja hippocampus kontrolleihin verrattuna. SPECT kuvauksessa havaitsimme, että FAS/FAE-diagnoosin saaneilla lapsilla (n=12) käytetyn spesifin radioligandin sitoutuminen serotoniinitransporttereihin oli vähäisempää ja dopamiinitransporttereihin voimakkaampaa verrokkeihin verrattuna. Nämä löydökset ovat samansuuntaisia kuin aggressiivisuuteen, impulsiivisuuteen ja epäsosiaaliseen käyttäytymiseen taipuvaisilla alkoholisteilla (tyypin 2 alkoholistit). Lisäksi FAS/FAE-diagnoosin saaneilla lapsilla oli MRI-tutkimuksessa pienemmät amygdala, caudatus, putamen ja hippocampus sekä aivojen kokonaistilavuus kontrolleihin verrattuna.

Yhteenvetona voidaan todeta, että tämä tutkimus tuo lisätietoa sikiöaikaisesta alkoholialtistuksesta. Tutkimuksen tulokset tukevat käsitystä siitä, että raskauden aikaisen alkoholin käytön vaikutusten kirjo on laaja ja että raskauden aikainen alkoholin käyttö vaikuttaa sekä äidin että lapsen terveyteen. Lisätutkimukset ovat tarpeellisia, jotta pystyisimme

luotettavasti tunnistamaan raskauden aikaisen alkoholinkäytön ja huomioimaan paremmin sekoittavat tekijät. Vaikka tutkimus toi lisätietoa sikiöaikaisesta alkoholialtistuksesta, meillä ei edelleenkään ole luotettavaa keinoa tunnistaa raskaudenaikaista alkoholin käyttöä.

Avainsanat: sikiön alkoholioireyhtymä; alkoholi (päihteet); ultraäänitutkimus; magneettikuvaus; yksifotoniemissiotomografia; optinen koherenssitomografia; nestekromatografia; massaspektrometria; sikiö; leikki-ikäiset; kouluikäiset; nuoret aikuiset

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Lehikoinen A, Kärkkäinen O, Lehtonen M, Auriola S, Hanhineva K, Heinonen S. Alcohol and substance use are associated with altered metabolome in the first trimester serum samples of pregnant mothers. *Eur J Obstet Gynecol Reprod* Biol 223:79-84, 2018

II Lehikoinen A, Voutilainen R, Romppanen J, Heinonen S. The effect of maternal alcohol and drug abuse on first trimester screening analytes: a retrospective cohort study. *BMC Pregnancy and Childbirth* 25;20(1):562, 2020

III Lehikoinen A, Ordén MR, Heinonen S, Voutilainen R. Maternal drug or alcohol abuse is associated with decreased head size from mid-pregnancy to childhood. *Acta Paediatr* 105:817-22, 2016

IV Riikonen R, Nokelainen P, Valkonen K, Kolehmainen A, Kumpulainen K, Könönen M, Vanninen R, Kuikka J. Deep serotonergic structures in fetal alcoholic syndrome: a study with nor-beta-CIT-single-photon emission computed tomography and magnetic resonance imaging volumetry. *Biol Psychiatry* 57:1565-72, 2005

V Lehikoinen A, Sorri I, Voutilainen R, Heinonen S. Optical coherence tomography shows decreased thickness of retinal nerve fibre layer among fetal alcohol exposed young adults in a case-control study. *Acta Ophthalmologica*, 99(7): e1243-e1244, 2021

CONTENTS

ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HTOL	5-Hydroxytryptophol
5-HIAL	5-hydroxyindole-3-acetaldehyde
ADH	Alcohol dehydrogenase
ADHD	Attention deficit hyperkinetic disorder
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APAs	Acetaldehyde-protein adducts
ARBD	Alcohol-related birth defect
ARND	Alcohol-related neuroldevelopmental disorder
AST	Aspartate aminotransferase
AUDIT	The Alcohol Use Disorder Identification Test
CBCL	Child Behavior Check List
CDI	Children´s Depression Inventory
CDT	Carbohydrate-deficient transferrine
CNS	Central nervous system
DAT	Dopamine transporter
EAAC-1	Excitatory amino acid carrier 1
EAAT	Excitatory amino acid transporter
E18:2	Ethyl linoleate
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
EtG	Ethyl glucuronide
EtS	Ethyl sulphate
FAE	Fetal alcohol effects
FAEE	Fatty acid ethyl ester
FAS	Fetal alchol syndrome
FASD	Fetal alcohol spectrum disorder
free β-hCG	Free β-human chorionic gonadotropin subunit
FTS	First trimester screening
GGT (γ-GT)	Gamma-glutamyl transferase

GMDS	Griffiths Mental Developmental Scales
GC	Gas chromatography
HC	Head circumference
HPLC	High performance liquid chromatography
iGlu	Ionotropic glutamate
KA	Kainate
LC	Liquid chromatography
LysoPC	Lysophosphatidylcholine
MAO	Monoamine oxidase
MCV	Mean corpuscular volume (mean blood red cell volume)
MD	Mean difference
mGlu	Metapotropic glutamate
MoM	Multiples of medians
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NA	Not available
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NTT	Nuchal translucency thickness
OCT	Optical coherence tomography
PAG	Phosphate–activated glutaminase
PAPP-A	Pregnancy-associated plasma protein A
PEth	Phosphatidylethanol
RF	Radio frequency
RNFL	Retinal nerve fibre layer
ROS	Reactive oxygen species
SD	Standard deviation
SERT	Serotonin transporter
SGA	Small for gestational age
SPE	Solid phase extraction
SPECT	Single photon emission computed tomography
VGLUT	Vesicular glutamate transporter

1 INTRODUCTION

It is assumed that public has had some awareness of the harmfulness of drinking during pregnancy for centuries. Already Old Testament in *Book of Judges 13:3-4* indicates harmful effects of alcohol to pregnant women, although it took time before scientists published their observations about the harmful fetal effects of maternal alcohol consumption: the Frenchman Paul Lemoine was the first to publish the pattern of symptoms and findings for fetal alcohol spectrum disorder (FASD) in 1968 (Lemoine, Harousseau, Borteyru, & Menuet, 1968).

The severity of symptoms depends on multiple factors such as exposure time and amount, socioeconomical status, maternal nutritional status, parity, gravidity, additional exposure agents (e.g. smoking and drugs), genetic and epigenetic factors (Hayes et al., 2021; Kaminen-Ahola, 2020; Sarman, 2018; Young, Giesbrecht, Eskin, Aliani, & Suh, 2014). Nevertheless, FASD is a preventable cause of cognitive impairment and even a low level of fetal alcohol exposure may cause adverse effects: there is no time or amount alcohol that is safe during pregnancy (Flak et al., 2014; Römer et al., 2020; Sarman, 2018). Therefore, preventive actions directed towards alcohol users and fetal alcohol exposure is crucial for allocating the necessary help.

The prevalence of alcohol and drug consumption during pregnancy has been shown to vary in different countries (U. S. Department of Health and Human Services, 2012). The exact prevalence of alcohol use during pregnancy in Finland is unknown. The prevalence estimations of fetal alcohol exposure in Finland is often based on the study by Pajulo (Pajulo, M., 2001), who reported that 6% of pregnant Finnish women were dependent on alcohol or drugs. Nonetheless, Pajulo´s study did not measure alcohol or drug dependency during the pregnancy; it measured risky alcohol and drug abuse before or during the pregnancy.

Alcohol use during pregnancy is a transgenerational problem. Alcohol not only has harmful effects on the fetus but also on maternal health. Alcohol use during pregnancy is influenced by a range of contextual and

structural factors, including poverty, histories of trauma and violence, physical and mental health concerns, sociocultural and economic vulnerabilities (Hayes et al., 2021; Lyall et al., 2021). Additionally, alcohol use during pregnancy impairs absorption of essential amino acids and nutrients (Madruga de Oliveira, Rondó, & Oliveira, 2009), and it increases the risk for high blood pressure and pre-eclampsia (Grum, Seifu, Abay, Angesom, & Tsegay, 2017).

Lack of reliable information about alcohol use is one of the major problems in diagnosing FASD and targeting preventive intervention. Asking about alcohol use is the easiest way to obtain information of alcohol consumption during pregnancy. However, people are likely to underestimate their drinking, and they are unable or unwilling to estimate the alcohol dose size properly (Schultz, Kohn, Schmerbauch, & Correia, 2017; Witbrodt, Kaskutas, Korcha, & Armstrong, 2008). Currently, we lack a laboratory-based screening tool for detection of alcohol use. So far, we do not have a reliable way to detect alcohol use during pregnancy (Bearer et al., 2003).

In this study, our general aim was to assess tools to recognize and detect fetal alcohol exposure. This study has three main time points in the timeline. Firstly, we evaluated first trimester metabolites and trisomy screening results of pregnant alcohol- and drug-abusing mothers. We wanted to assess whether metabolite profiling would reveal alcohol consumption biomarkers. Secondly, we investigated the effects of alcohol and drug exposure during the second trimester of pregnancy by fetal ultrasonography and followed the children´s development until 2.5 years of age. By doing so, we wanted to evaluate whether the harmful effects of alcohol are detectable already during mid-pregnancy. Thirdly, we performed single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) volumetry on FAS/FAE subjects to evaluate brain dopamine and serotonin metabolism in their childhood. Furthermore, we performed an ophthalmological examination and optical coherence tomography in the same FAS/FAE subjects during early adulhood to evaluate retinal nerve fibre layer thickness.

2 REVIEW OF THE LITERATURE

2.1 FETAL ALCOHOL SPECTRUM DISORDER

2.1.1 Background for the diagnostic criteria of FASD

FASD is an umbrella term that describes all fetal alcohol effects. The spectrum of the exposed children varies from non-detectable changes to severe cognitive impairment. Fetal alcohol syndrome (FAS) is the most severe form of fetal alcohol exposure. FASD also includes partial fetal alcohol syndrome (fetal alcohol effects, FAE), alcohol-related neurodevelopmental disorder (ARND) and alcohol-related birth defect (ARBD). The diagnostic criteria of FASD varies remarkably: the study of Coles et al. showed that selected diagnostic criteria are only moderately similar (Coles, C. D. et al., 2016). This study among the other shows that there is an urgent need for an international consensus on the diagnostic criteria of FASD (Astley, 2006; Coles, C. D. et al., 2016; Hemingway et al., 2019).

2.1.2 Diagnostic criteria of FASD in Finland

Even though diagnostic criteria differ remarkably, it is necessary to find and diagnose FASDs. Uncertainty of the FASD diagnostic criteria at least partly explains why FASD is thought to be underdiagnosed in Finland. Although FASD diagnosis is considered to be stigmatising, it is the interest of children that they get the right diagnose. Official national diagnostic criteria do not exist in Finland, but in the Finnish Physician´s Handbook (Lääkärin käsikirja) Autti-Rämö has recommended to use criteria for FASD that are based on IOM criteria and national research (Autti-Rämö, 2021). Confirmed maternal alcohol exposure is needed for the diagnosis of FASD. However, if alcohol exposure is uncertain (e.g. in case of adoption), additional information should be added to the diagnosis (“without confirmed alcohol exposure”). The following diagnostic criteria are suggested to be used:

FAS

Requires features A, B and C:

A. Typical facial features, 2≤ features needed
    a. short palbepral fissures (<10 percentile)
    b. thin upper lip (rank 4 or 5 on lip/philtrum guide)
    c. smooth philtrum (rank 4 or 5 on lip/philtrum guide)
B. Prenatal and/or postnatal growth deficiency
    - Prenatal growth deficiency can resolve when growing up. Growth is evaluated first at birth.
    - Length or weight < -2SD, relative weight <-10%

C. Deficient brain growth, structural brain abnormality, or cognitive impairment that is detected as a
    a. Brain imaging abnormality or
    b. Small (< -2SD) head circumpherence or
    c. Neurobehavioral impairment, which is not explained by genetical or environmental factors. These impairments can be difficulties in performing complex tasks (problem solving, planning and evaluating, mathematical tasks); demanding lingual tasks (understanding and production); specific behavioral features (e.g. problems in social interaction and mood regulation impairment)

FAE

Requires features A and B

A. Typical facial features, 2≤ features needed
    a. short palbepral fissures (<10 percentile)
    b. thin upper lip (rank 4 or 5 on lip/philtrum guide)
    c. smooth philtrum (rank 4 or 5 on lip/philtrum guide)
B. Requires one of the following
    a. Prenatal or postnatal growth deficiency
        i. Prenatal growth deficiency can resolve when growing up. Growth is evaluated first at birth.
        ii. Length or weight < -2SD, relative weight <-10%

b. Deficient brain growth or structural brain abnormality, as in the FAS criteria
c. Neurobehavioral impairment that is not explained by genetical or environmental factors. These impairments can be difficulties in performing complex tasks (problem solving, planning and evaluating, mathematical tasks), demanding lingual tasks (understanding and production), specific behavioural features (e.g., problems in social interaction and mood regulation impairment)

ARND

- Neurobehavioral impairment that is not explained by genetical or environmental factors. These impairments can be difficulties in performing complex tasks (problem solving, planning, and evaluating, mathematical tasks), demanding lingual tasks (understanding and production), specific behavioural features (e.g., problems in social interaction and mood regulation impairment)
- Growth deficiency is permitted, but typical facial features are not present.

ARBD

- Confirmed binge drinking during the first trimester of pregnancy
- Congenital malformation

2.1.3 Alcohol amounts during pregnancy

The amount and timing of fetal alcohol exposure varies. There are no internationally acknowledged standard definitions for mild, moderate, and heavy alcohol consumption during the pregnancy. Flak et al. (Flak et al., 2014) defined that mild drinking was defined as up to 3 drinks per week, mild to moderate as up to 6 drinks per week including individuals who consumed at least 3 drinks per week, moderate as up to 6 drinks per week, and heavy as more than 6 drinks per week. One drink was defined as 13.7 g of alcohol. Teratogenic consequence of the alcohol exposure depends on the timing of the exposure (Figure 1). However, even mild drinking can be harmful and therefore there is no safe amount or time to use alcohol during the pregnancy (Flak et al., 2014).

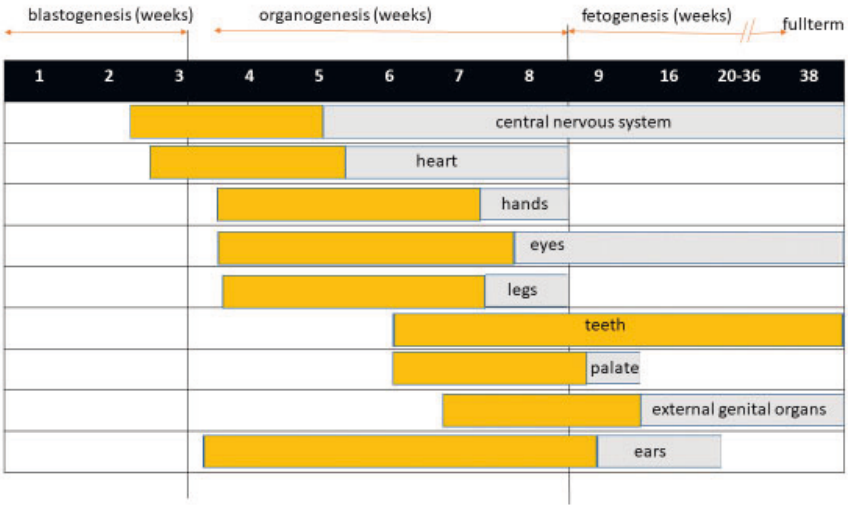

Figure 1. Teratogenic consequences at different developmental ages. Fetal organs are most vulnerable to malformation during the organogenesis (yellow lines). Modified from Sariola et al. (Sariola, 2015).

2.2 FEATURES OF FETAL ALCOHOL EXPOSURE

2.2.1 Overview

Fetal alcohol exposure causes a spectrum of neurocognitive and psychiatric problems ranging from mild learning difficulties to severe cognitive disabilty. Generally accepted features of fetal alcohol exposure are growth deficiency, microcephaly or other structural brain anomalies, neurobehavioral impairment (cognitive and behavioral) and typical facial features (smooth philtrum, thin vermilion border of the upper lip and short palpebral fissures).

2.2.2 Neurobehavioral outcome of FASD

The estimated prevalence of learning difficulties varies from 8-20 % in general population and approximately 5 % of the population has cognitive disability. The literature on cognitive impairments and behavioral features in FASD does not provide a consistent profile (Kodituwakku, P. W., 2009).

Although alcohol exposure during pregnancy is the main non-genetic cause of mental retardation, the majority of FASD subjects do not show cognitive delay, but an IQ score in the low normal or borderline range (Aragón et al., 2008; Kodituwakku, P. et al., 2006; Streissguth et al., 1991)

Executive functioning refers to the ability to develop and retain appropriate problem-solving strategies to attain objectives and goals (Welsh & Pennington, 1988). Good executive functioning depends on intact cognitive functions related to the ability for planning, response inhibition, working memory and the involvement of more basic cognitive processes like attention span, memory functions, perceptual and motor activities (Pennington, Bennetto, McAleer, & Roberts Jr., 1996). Individuals with FASD have difficulties to plan and solve problems, difficulties with abstract thinking and inhibition of their responses to stimuli (Kodituwakku et al., 2006; Mattson & Riley, 1999). These symptoms are associated with attention deficit disorder (ADHD). A meta-analysis by Kingdon et al. (Kingdon, Cardoso, & McGrath, 2016) showed that even though similarities in excecutive functions exist between the FASD groups and ADHD, it seemed that children with FASD showed greater deficits on measures of planning, set shifting, fluency, and working memory than non-alcohol exposed children with ADHD, although this difference was not statistically significant. Addition to neurocognitive problems, the social skills and interpersonal relationship, behavioral and emotional problems cause a remarkable burden in the lives of those with FASD (Fryer, McGee, Matt, Riley, & Mattson, 2007; Nash et al., 2006).

2.2.3 Alcohol exposure and brain

The brain is the most severely impacted organ by fetal alcohol exposure. Typical findings caused by fetal alcohol exposure are reduced total brain volume (microencephaly) (Archibald et al., 2001; Astley et al., 2009; Coles, Claire D. et al., 2011; Johnson, Swayze, Sato, & Andreasen, 1996; Lebel et al., 2008; Sowell et al., 2002; Swayze et al., 1997; Willoughby, Sheard, Nash, & Rovet, 2008), cerebral volume (Archibald et al., 2001; Mattson et al., 1996) and cerebellar volume (Archibald et al., 2001; Astley et al., 2009;

Mattson et al., 1996; O'Hare et al., 2005; Riikonen, R., Salonen, Partanen, & Verho, 1999; Sowell et al., 1996).

The corpus callosum is crucial for the interhemispheric communication and it has been frequently described to be damaged by fetal alcohol exposure. Different types of corpus callosum damage have been described including complete agenesis (Astley et al., 2009; Johnson et al., 1996; Riley, E. P. et al., 1995; Swayze et al., 1997), partial agenesis (Autti-Ramo et al., 2002; Johnson et al., 1996) and callosal thinning (Autti-Ramo et al., 2002; Clark, Li, Conry, Conry, & Loock, 2000). Abnormalities have been reported across the corpus callosum, but the splenium seems to be the most affected region (Autti-Ramo et al., 2002; Riley et al., 1995; Sowell, Mattson et al., 2001).

Ultimately no brain structure is safe from the harmful effects of alcohol (Archibald et al., 2001; Mattson et al., 1996; Olney, Ishimaru, Bittigau, & Ikonomidou, 2000). Despite the association between brain damage and alcohol exposure, the abnormalities are not specific for fetal alcohol exposure; e.g. prenatally detected corpus callosum agenesis is associated with chromosomal abnormalities in approximately 18% of cases (Santo et al., 2012).

2.2.4 Fetal alcohol exposure effects on heart

Although extensive research has been done on the effect of maternal alcohol consumption on congenital heart defects, conclusions are still inconsistent. The systematic review and meta-analysis by Sun et al. concluded that there is no association between maternal alcohol consumption and the risk of congenital heart defects (Sun, Chen, Chen, Ma, & Zhou, 2015).

2.2.5 Kidney, liver, and gastrointestial birth defects in FASD

Hofer et al. (Hofer & Burd, 2009) reviewed published studies on birth defects of renal, liver and gastrointestinal organ systems. The existing literature is limited and more research is needed to determine if a specific

pattern of organ specific abnormalities or functional deficits exists in subjects with FASD.

2.2.6 Fetal alcohol exposure and eye abnormalities

The eye is a sensitive indicator of prenatal adverse events and therefore it is a useful object in the investigation of teratogens. As the timeline of early stages of eye development is known, ocular birth defects can be studied in terms of critical time periods for the action of teratogenic agents. An especially vulnerable time to get structural defects is from the 3rd week of pregnancy to the 8th week of pregnancy, whereas functional defects are possible throughout pregnancy (Sadler & Langman, 1995).

Typical periocular facial features are short horizontal palpebral fissures, telecanthus, epicanthus and unilateral or bilateral blepharoptosis (Hoyme et al., 2016; Stromland, 2004). Strabismus, most often esotropia, is a frequent finding in children with FAS (Hinzpeter, Renz, & Loser, 1992; Hug, Fitzgerald, & Cibis, 2000; Miller et al., 1981; Miller et al., 1984), and it was reported in up to 43% of Swedish cases (Stromland, 1985).

Eye abnormalities detected by inspection are microphthalmia, buphthalmia and coloboma of the iris and uvea. Previously, microphthalmia has been used as a diagnostic criterion for FAS (Rosett, 1980). However, due to the lack of generally available objective methods to measure eye size, this criterion has been abandoned.

Abnormalities of the anterior segment and optical media, such as Peters and Axenfeld anomaly (defects of cornea, anterior chamber and iris) (Hinzpeter et al., 1992; Miller et al., 1984), microcornea, iris and uveal coloboma, small decentered non-reactive pupil (Stromland, 1985), corneal endothelial abnormalities (Carones, Brancato, Venturi, Bianchi, & Magni, 1992) and diffuse corneal clouding (Edward et al., 1993), are associated with fetal alcohol exposure. Some cases of glaucoma, cataract and persistent hyperplastic primary vitreous body have also been reported (Hinzpeter et al., 1992; Miller et al., 1981; Miller et al., 1984; Stromland, 1985).

The retinal fundus abnormalities in FAS range from mild, discrete lesions of the optic disc and retinal vessels to severe malformations of

both retina and the optic nerve. The most frequent finding is the optic nerve hypoplasia and tortuosity of retinal vessels (Hinzpeter et al., 1992; Miller et al., 1984; Pinazo-Duran, Renau-Piqueras, Guerri, & Stromland, 1997; Stromland, 1985).

Hypoplasia of the optic nerve head is characterized by subnormal vision and a subnormal number of optic nerve axons, showing morphological signs such as small size, pallor, irregular margins and an abnormal retinal vascular pattern of the optic disk. In a group of Swedish children with FAS 48% of the optic discs were hypoplastic (Stromland, 1985). During recent years, methods to evaluate optic nerve thickness and retinal nerve fiber layer (RNFL) have evolved. Optical coherence tomography (OCT) examination allows measurement of the size of the optic disc and the different layers of retina, including both the macular and peripapillary RNFL (Gyllencreutz, Aring, Landgren, Landgren, & Gronlund, 2020; Menezes, Ribeiro, Coelho, Mateus, & Teixeira, 2016).

2.3 FETAL ALCOHOL EFFECTS IN ADULTS

Few research reports exist concerning FASD in adulthood. It seems that some of the structural, cognitive and behavioral problems are permanent (Gyllencreutz et al., 2020; Landgren et al., 2019; Rasmussen, 2005). However, the key facial features that characterize FASD in childhood diminish or evolve while growing up (Jacobson et al., 2021). Previous studies indicate that prenatal alcohol exposure is associated with alcohol problems in early adulthood (Weeks et al., 2020) and mental health problems are highly prevalent in FASD (Pei, Denys, Hughes, & Rasmussen, 2011). In Canada, FASD was explored among adults involved with justice. The prevalence of FASD was as high as 17.5% of the study participants (McLachlan et al., 2019).

FASD also predisposes to metabolic abnormalities, including type 2 diabetes, low HDL, high triglycerides, and female-specific overweight and obesity. This might be due to behavioral and primary organ dysfunction (Weeks et al., 2020). FASD increases hospitalization and mortality (Jacobson, Chiodo, Sokol, & Jacobson, 2002).

Economical costs of FASD to society are assumed to be high. However, the research data on the economic burden of FASD are scarce. In a comprehensive review article of Greenmyer et al. (Greenmyer, Klug, Kambeitz, Popova, & Burd, 2018) the mean annual costs were estimated at $22810 for children and $24308 for adults. However, it is likely that these numbers underestimate the costs (McLachlan et al., 2019). To sum up, FASD causes a life-long personal and family burden, and it is a serious public health problem associated with a remarkable economic burden.

2.4 ALCOHOL METABOLISM

After ingestion, ethanol is rapidly absorbed by the gasterointestinal tract. Absorbtion rate is varied by the timing, dosage and drinking pattern, in addition to nutrition status (Norberg, Jones, Hahn, & Gabrielsson, 2003). Alcohol diffuses through the placenta where it distributes rapidly in the fetus (Idänpään-Heikkilä et al., 1972; Norberg et al., 2003).

Alcohol is metabolised at constant rate by several pathways. The majority of alcohol is removed by oxygenation (Figure 2) and less than 10% of alchol is excreted in breath, sweat and urine (Cederbaum, 2012; Gemma, Vichi, & Testai, 2007). Maternal alcohol consumption of pregnant ewes showed that the elimination of alcohol from the fetus is primarily regulated by maternal elimination (Brien, Clarke, Richardson, & Patrick, 1985). Fetal elimination rate of ethanol is slower than the maternal rate mainly due to reuptake of amniotic fluid containing alcohol by the fetus (Brien et al., 1985; Burd, Blair, & Dropps, 2012). Sex, age, race, food ingestion, weight, biological rhythms, alcoholism (advanced liver disease) and drugs used influence alcohol metabolism (Cederbaum, 2012).

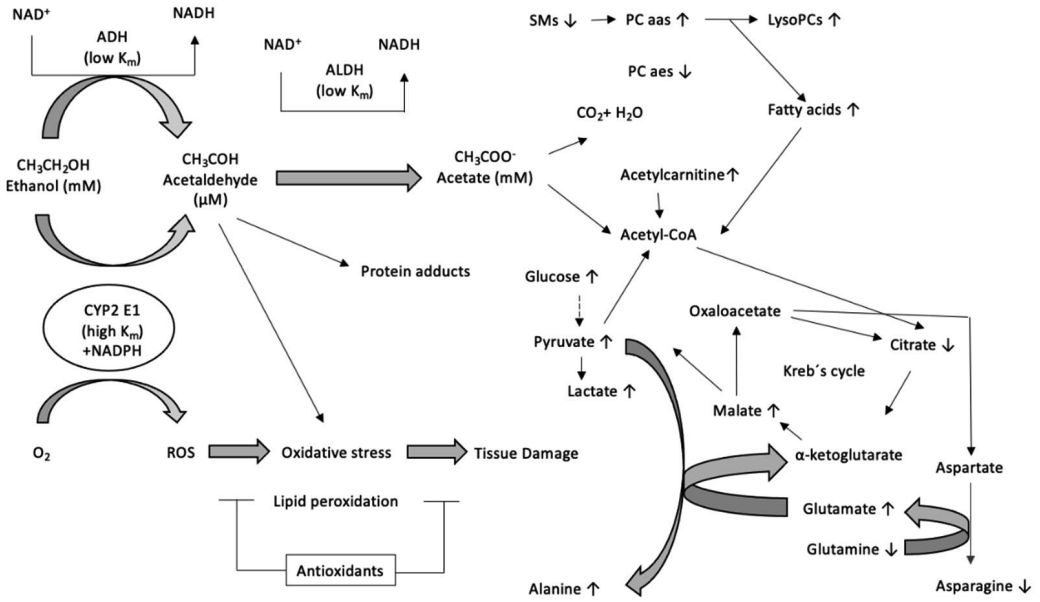

Figure 2. Schematic representation of hepatic ethanol oxidative metabolism, related effects and metabolites associated with significant changes after alcohol consumption (modified from Gemma 2007 and Voutilainen 2019 (Gemma et al., 2007; Voutilainen & Kärkkäinen, 2019). The left side of the figure presents ethanol oxidative metabolism and the right side of the figure the metabolites. An upward arrow indicates an increase and a downward arrow indicates a decrease of the metabolite after alcohol ingestion. Abbreviations: Acetyl-CoA: acetyl coenzyme A; ADH: alcohol dehydrogenase; ALDH aldehyde dehydrogenase; CYP2 E1: Cytochrome P450 2E1; LysoPC: lysophosphatidylcholine; PC aes: Phosphatidylcholine acyl-alkyls; PC aas, phosphatidylcholine diacyl; ROS: reactive oxygen species; SM, hydroxysphingomyelin

2.5 METABOLOMICS OF ALCOHOL USE

Metabolomics (metabonomics, metabolic profiling) is an omics approach applied to study metabolic changes (Bujak, Struck-Lewicka, Markuszewski, & Kaliszan, 2015; Gemma et al., 2007; Voutilainen & Kärkkäinen, 2019). Metabolome represents directly the functional changes in cellular metabolism, and it provides a view about the current physiological state (Voutilainen & Kärkkäinen, 2019). Therefore metabolomics can be used in biomarker research to identify predictive markers (Guijas, Montenegro-Burke, Warth, Spilker, & Siuzdak, 2018). The general methods used in metabolomics research are nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with either liquid or gas chromatography (LC or GC, respectively) (Ulaszewska et al., 2019). MS-based techniques are more sensitive than NMR-based methods.

Previous metabolomic studies have shown that fatty acid, phosphatidylcholine diacyls and steroid metabolites tend to increase and phosphatidylcholine acyl-alkyls and hydroxysphingomyelins decline among alcohol users (reviewed in Voutilainen & Kärkkäinen, 2019). In addition, several organic acids associated with alcohol use are important for energy metabolism (Figure 2). Glucose, alanine and lactate are commonly found to be increased whereas glutamine and asparagine are found to decline (Voutilainen & Kärkkäinen, 2019).

2.5.1 Serotonin

2.5.1.1.1 General aspects to serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a monamine derived from the amino acid tryptophan. In the central nervous system it acts as a neurotransmitter. However, most serotonin is found outside the central nervous system and all described 15 serotonin receptors are expressed outside as well as within the brain. Brain-derived serotonin counts only for around 5% of total serotonin (Berger, Gray, & Roth, 2009). The remaining 95% of serotonin is produced in the peripheral organs, including the cardiovascular, pulmonary, gastrointestinal and genitourinary systems (Andrews, Bharwani, Lee, Fox, & Thomson, 2015; Roth, 2006). In the

periphery the vast majority of serotonin is produced by enterochromaffin cells in the gut. 5-HT cannot cross the blood brain barrier, so peripheral measures do not reflect brain levels (El-Merahbi, Löffler, Mayer, & Sumara, 2015). Serotonin is synthesized through a multistep pathway from one of the essential aminoacids, tryptophan. Dietary tryptophan is provided e.g. from white and red meat, seeds and beans (Fadda, 2000).

There is abundant evidence suggesting that the placenta directly synthesizes 5-HT (Bonnin et al., 2011; Bonnin & Levitt, 2011; Huang, Zhang, Di, & Zhang, 1998; Laurent et al., 2017). Potentially, the placenta is the sole source of 5-HT during the early stage of fetal brain development (Bonnin & Levitt, 2011; Bonnin et al., 2011; Rosenfeld, 2021). While an elevated concentration of 5-HT from the placenta can disrupt early brain development, hyposerotonemia may also impair sensory, motor, and cognitive abilities, collectively leading to autism spectrum disorder or other neurobehavioral disorders (Rosenfeld, 2021; Sato, 2013; Yang, C. J., Tan, & Du, 2014).

Peripheral serotonin has several roles as a regulator of multiple physiological functions. It has a function in the regulation of glucose and lipid homeostasis (El-Merahbi, Löffler, Mayer, & Sumara, 2015). Serotonin produced in pancreatic β-cells promotes insulin secretion and during pregnancy also β-cell proliferation (Kim, H. et al., 2010; Ohara-Imaizumi et al., 2013; Paulmann et al., 2009). Intestinal serotonin acts on the liver by promoting gluconeogenesis and suppressing hepatic glucose uptake (Kim et al., 2010; Ohara-Imaizumi et al., 2013; Paulmann et al., 2009; Sumara, Sumara, Kim, & Karsenty, 2012). Besides these functions, serotonin has many other functions such as modulation of cardiac function. For example, high serotonin levels can cause atrial fibrillation (Langer et al., 2007). Serotonin signaling in the periphery is complex due to its multiple sites of production, its capacity to act as an auto-, para- and endocrine factor, and the existence of at least 14 serotonin receptors.

Even though the vast majority of total body serotonin is found outside CNS, serotonin has an important role in modulating all behavioral processes (Berger et al., 2009). All brain regions express multiple serotonin receptors in a receptor subtype-specific fashion (Mengod, Vilaro, & Cortes,

2007) and, in addition to that, individual neurons may express multiple serotonin receptors (Araneda & Andrade, 1991). The behavioral and neuropsychological processes modulated by serotonin include mood perception, reward, anger, aggression, appetite, memory, sexuality, and attention (Canli & Lesch, 2007; Roth, Hanizavareh, & Blum, 2004; Roth, 2006). In fact, it is difficult to find a human behavior that is not regulated by serotonin.

2.5.1.1.2 Serotonin metabolism

Circulating plasma serotonin is rapidly inactivated by Phase I enzymes in the liver and lungs. The major Phase I enzymes involved in the biotransformation of serotonin to inactive metabolites are monoamine oxidase (MAO) and aldehyde and alcohol dehydrogenases (ALDH and ADH, in order) (Figure 3). MAO exists as two isoforms, MAO-A and MAO-B. Serotonin is a selective substrate for MAO-A which is found in neurons, intestines, kidneys, liver and lungs (Shih & Chen, 2004). In non-alcohol drinking state, 5-hydroxyindoleacetic acid (5-HIAA) is the predominant metabolite compared to 5-hydroxytryptophol (5-HTOL) by a factor of about 1000:1 (Lin et al., 2020).

When ethanol is metabolized in the liver, there is a shift in 5-HT metabolism towards 5-HTOL, which results in increased urinary 5-HTOL/5-HIAA ratio (Lin et al., 2020). The detection window for alcohol consumption by an elevated 5-HTOL/5-HIAA ratio in urine is about 5–15 hours longer than for conventional ethanol testing, and it has been considered as a 24-hour alcohol use marker. The sensitivity of the 5-HTOL test in the detection of recent alcohol intake depends closely on the dose ingested and on the time passing between drinking and urine sampling. Accordingly, consumption of low, non-intoxicating, amounts (<10 g ethanol) in the evening may not result in an elevated 5-HTOL the following morning, while intake of doses >50 g are generally detectable (Beck & Helander, 2003).

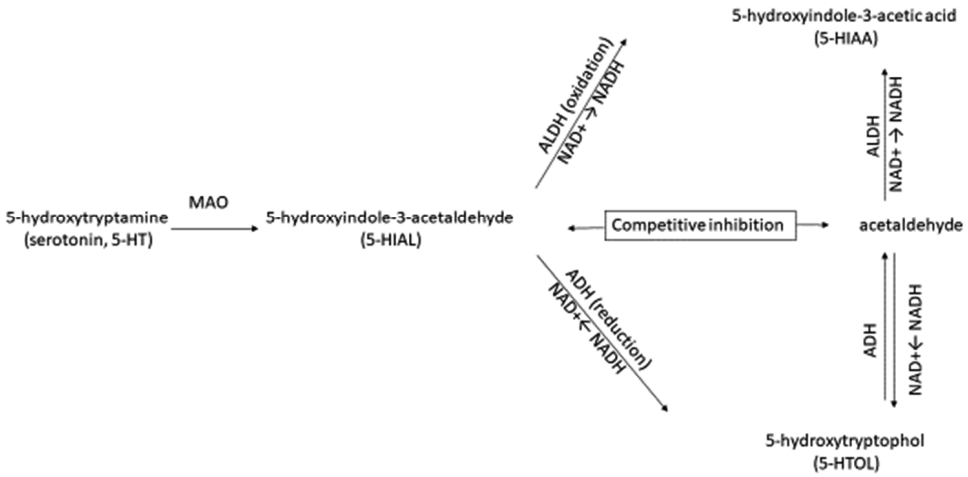

Figure 3. Scheme showing the metabolism of serotonin (5-HT) to the corresponding aldehyde (5-HIAL) by monoamine oxidase and conversion of the latter to 5-hydroxyindoleacetic acid (5-HIAA) or the reduced metabolite 5-hydroxytryptophol (5-HTOL). When ethanol is metabolized in the liver, there is a shift in 5-HT metabolism towards the alcohol metabolite (5-HTOL), which results in increased urinary 5-HTOL/5-HIAA ratios (Source: Lin et al., 2020).

2.5.1.1.3 Serotonin (5-hydroxytryptamine, 5-HT) measurement

Currently, liquid chromatography tandem mass spectrometry (LC-MS/MS) is the most sensitive and widely used method to measure serotonin (Szeitz & Bandiera, 2018). Measurement of serotonin levels in the living human brain is not easily accessible. Cerebrospinal fluid can be used for analysing circulating serotonin levels (Szeitz & Bandiera, 2018). Indirect methods are used to evaluate serotonin levels in the brain (more in paragraph Single photon emission computed tomography).

2.5.2 Glutamate and glutamine

Glutamate, a nonessential amino acid, is the most abundant free amino acid in the brain. It plays several critical roles in neural functioning: it is both the primary excitatory neurotransmitter and important in oxidative

metabolism. The primary supply for glutamate is the nonessential aminoacid glutamine. Glutamatergic neurotransmission is tightly coupled to cerebral oxidative metabolism (de Graaf, Mason, Patel, Behar, & Rothman, 2003).

Glutamate can be considered to be responsible for many neurological functions, including cognition, memory, behavior, movement, and sensation. It also plays significant roles in the brain development, including synapse induction and the relationship of synapses with astrocytes, cell migration, synaptic spatial organization in the cerebellum, cell differentiation, and cell death (Balakrishnan, Dobson, Jackson, & Bellamy, 2014; de Graaf et al., 2003; Kim, S. K., Nabekura, & Koizumi, 2017; Moriyama et al., 2000). Glutamatergic neurotransmission plays a role in many neurological diseases such as temporal lobe epilepsy, multiple sclerosis and amyotrophic lateral sclerosis (Sepkuty et al., 2002; Todd & Hardingham, 2020; Waxman, 2007).

Glutamate exerts its effects by binding to and activating cell surface receptors known as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate (KA) receptors, NMDA receptors, and ionotropic glutamate (iGlu) and metapotropic glutamate (mGlu) receptors (Kandel, 2014). Glutamate transporters are a family of neurotransmitter transporter proteins that move glutamate across the membrane. There are two general classes of glutamate transporters, the excitatory amino acid transporter (EAAT) family and vesicular glutamate transporter (VGLUT) family. Currently at least 14 amino acid transporters have been identified to transport glutamine (Rubio-Aliaga & Wagner, 2016). Cystine/glutamate transporter is an antiporter that acts in nonvesicular glutamate release. In addition to its role in the central nervous system, glutamate signalling has been implicated in peripheral non-neuronal tissues such as kidney, lung, liver, heart, stomach and immune system. Glutamate and its receptors have been reported to participate in the regulation of the inflammatory reaction and cell fibrosis in some non-neurological diseases (Du, Li, & Li, 2016) (Table 1).

Table 1. Expression of glutamate system and related disease in peripheral tissue

Organs	Glutamate receptors (relation to disease)	Glutamate transporters (relation to disease)
Kidney	Ka receptor subunit 2, NMDA1 receptor (chronic kidney disease), mGlu2/3 receptors (Cancer)	EAAC1 (dicarboxylic aminoaciduria)
Lung	NMDA1 receptor (acute lung injury, hyperreactivity of bronchial asthma),	EAAT1, EAAT5
	NMDA2B receptor (non-small cell carcinoma), Ka 2, mGlu2/3 receptors	Cystine/glutamate transporter (small-cell lung cancer)
Liver	mGlu receptor, NMDA1 receptor (inflammation, central obesity, type2diabetes, liver injury)	EAAT1, EAAT-2, EAAT5, Cystine/glutamate transporter (liver cancer)
Heart	AMPA receptor (cardiac arrhythmias), NMDA1 receptor (ischaemia), Ka 2, mGlu5 receptor, mGlu1/2/3 receptors	EAAT1, EAAT5, Cystine/glutamate transporter
Stomach	Ka 2 receptor, NMDA1 receptor, mGlu2/3 receptors	EAAC1, EAAT1, EAAT2, Cystine/glutamate transporter (gastric mucosa injury)
Immune system	iGlu receptors, mGlu receptors (T cell leukemia/lymphoma, HIV-1 infection, rheumatoid arthritis, systemic lupus erythematosus)	Cystine/glutamate transporter, EAAT-1

Abbreviations: Ka: Kainate; NMDA: N-methyl-D-aspartate; mGlu: metapotropic glutamate; iGlu: ionotropic glutamate; EAAT: excitatory amino acid transporter; EAAC: excitatory amino acid carrier. Adapted from Du et al. (Du et al., 2016).

The importance of glutamate receptor-mediated signaling in brain development is highlighted by the fact that deprivation of stimulation and inhibition of NMDA receptors cause apoptotic cell death in the developing brain (Ikonomidou et al., 1999; Olney, 2002). Many animal models have demonstrated that prenatal alcohol exposure interacts with glutamatergic neurotransmission (Baculis, Diaz, & Valenzuela, 2015; Valenzuela, Puglia, & Zucca, 2011). Embryonic ethanol exposure induces widespread neuronal apoptosis through N-Methyl-D-aspartate (NMDA) receptor blocking, which results in reduced brain mass and neurobehavioral disturbances in adulthood (Ikonomidou et al., 2000). Therefore, any action modulating developmental glutamate receptor signaling, like alcohol exposure, may modify brain development, with long-lasting consequences.

Acute alcohol exposure has been found to attenuate glutamate release from presynaptic neurons (Goodwani, Saternos, Alasmari, & Sari, 2017; Ikonomidou et al., 2000). This effect may be attributed to an ethanol-induced downregulation of brain vesicular glutamate transporters (VGLTs), as shown in adult rodents (Zhang, Ho, Vega, Burne, & Chong, 2015). Baggio et al. showed that adult zebrafishes previously exposed to alcohol during their embryonic development presented a dose-dependent reduction of brain glutamate uptake (Baggio et al., 2017). This reduction might be implicated in the increased anxiety-like behaviors and the disrupted social behavior in adulthood in the zebrafish FASD model (Baggio, Mussulini, de Oliveira, Gerlai, & Rico, 2018). In addition to parenchymal effects, fetal alcohol exposure has also been shown to alter fetal brain blood flow (Parnell et al., 2007). L-glutamine supplementation was able to mitigate the alcohol-induced acid–base imbalances and the alterations of fetal regional brain blood flow (Sawant, Ramadoss, Hankins, Wu, & Washburn, 2014).

The glutamate-glutamine cycle refers to the sequence of events by which an adequate supply of the neurotransmitter glutamate is maintained in the central nervous system (Purves et al., 2008). This is critical for the rapid and efficient clearance of glutamate from the synaptic cleft and extracellular space, the maintenance of neuronal mitochondrial metabolism, and the detoxification of the ammonia generated by neurotransmission (Purves et al., 2008; Todd & Hardingham, 2020).

During glutamatergic neurotransmission neurons release glutamate into the extracellular space; the glial glutamate transporters rapidly remove the released glutamate (Figure 4). To minimize the likelihood of glutamate transporter reversal during depolarization, the cell surface of glutamatergic neurons expresses low levels of glutamate transporters (Hertz, 2006). Studies of glutamatergic synapses have shown them to be closely surrounded by glial end processes possessing high densities of glutamate transporters. Reuptake of glutamate from the extracellular space primarily by glia uses the sodium-dependent, electrogenic glutamate transporters EAAT1 and EAAT2 (Danbolt, 2001). Under normal conditions, EAAT1 and EAAT2 are located on astrocytic membranes and terminate excitatory neurotransmission by first binding glutamate (buffering) then transporting glutamate into the astrocytic cytosol in an energy-consuming step (via the citric acid cycle, CAC) (Cavelier, Hamann, Rossi, Mobbs, & Attwell, 2005). In the presynaptic neuron, glutamine is converted by the phosphate-activated glutaminase (PAG) to glutamate and ammonia. The rate of glutamate synthesized by PAG is proportional to the rate of glutamate used by neurons (Waxman, 2007).

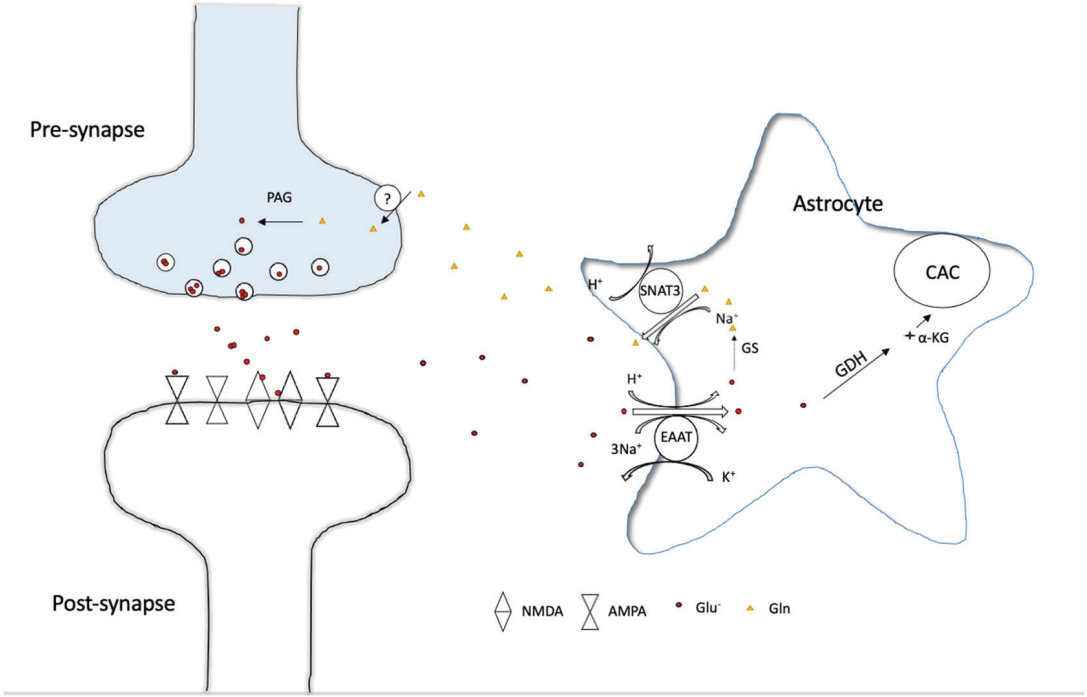

Figure 4. The glutamate-glutamine cycle. Glutamate (Glu) released after excitatory transmission is collected by astrocytic EAAT transporters 1 and 2. Glutamate is then either converted into α-ketoglutarate (α-KG) via glutamate dehydrogenase (GDH) or a transaminase reaction and enters the citric acid cycle (CAC), or is converted into glutamine (Gln) by glutamine synthetase (GS). Astrocytes excrete Gln back into the extracellular environment via the Na+driven SNAT3 transporter, which is then taken up by an as yet unconfirmed neuronal Gln transporter. Neurons then convert Gln back to Glu via a phosphate-activated glutaminase (PAG) reaction to replenish their vesicular Glu−stores. Adapted from Todd 2020 (Todd & Hardingham, 2020).

2.6 TRADITIONAL ALCOHOL BIOMARKERS, METABOLOMICS AND PREGNANCY

2.6.1 Introduction to alcohol biomarkers

A number of biomarkers have been proposed and evaluated for their ability to detect alcohol use during pregnancy. They can be divided into direct and indirect markers and further according to exposure time into short- and long-term use (Bearer et al., 2003; Joya et al., 2012; Witbrodt et al., 2008). Direct biomarkers are products of ethanol metabolism, whereas indirect biomarkers are those that reflect the toxic effects of ethanol on organs, tissues, or body biochemistry. Traditionally used testing matrices are blood, urine, breath and saliva, eventhough meconium and hair samples are becoming more common testing matrices. Newborn hair and meconium analyses show alcohol use post festum.

The use of available biomarkers of alcohol consumption is hampered by a number of problems: the time window for detection of alcohol use biomarkers is not suitable for clinical use, the use of the biomarker has not been validated, pregnancy itself affects the behavior of the biomarker or the biomarker may be insensitive or non-specific (Aertgeerts, Buntinx, Ansoms, & Fevery, 2002; Saunders, Aasland, Babor, de la Fuente, J R, & Grant, 1993).

2.6.2 Direct alcohol use markers

2.6.2.1.1 Alcohol

Direct alcohol detection from breath (Hlastala, 1998), blood (golden standard) (Kraut, 2015), and urine (Hadland & Levy, 2016) are indicators of acute alcohol use. Breath test and blood analysis reveal the current status of alcohol amount whereas urine test detects alcohol use up to 10-12 hours after ingestion (Hadland & Levy, 2016).

2.6.2.1.2 Ethyl glucuronide and ethyl sulphate

Ethyl glucuronide (EtG) is a metabolite of alcohol produced through a reaction with glucuronic acid in the liver. Direct human biotransformation products of ethanol, such as EtG, ethyl sulphate (EtS), and fatty acid ethyl esters, are considered specific for alcohol consumption (Pragst & Yegles, 2008; Wurst et al., 2006). EtG and EtS can be detected in urine for up to 5 days after a drinking episode, but great interindividual variability exists in the excretion (Mercurio et al., 2021). EtG accumulates in hair, where it remains detectable for several weeks to months, depending on the length of the hair. EtG in hair has proved to be a reliable biomarker for detection of chronic alcohol consumption (Pragst & Yegles, 2008). EtG and EtS have been mainly analysed in blood, meconium and urine, but also from hair (Cappelle et al., 2018). With meconium EtG ≥30 ng/g as the gold standard condition and maternal self-report at 19 weeks' gestation as the test condition, 82% clinical sensitivity (95% CI 71.6–92.0) and 75% specificity (95% CI 63.2–86.8) were observed for meconium EtG. A significant dose–concentration relationship between self-reported drinks per drinking day and meconium EtG ≥30 ng/g also was observed (P<0.01) (Himes et al., 2015).

2.6.2.1.3 Fatty acid ethyl esters

Fatty acid ethyl esters (FAEEs) are nonoxidative metabolites that are formed when alcohol conjugates to endogenous free fatty acids and fatty acyl-CoA (Laposata, 1998). Although at least 15 different FAEEs have been identified in the human body, particularly four FAEEs are used as ethanol consumption-related markers: ethyl myristate (14:0), ethyl palmitate (16:0), ethyl stearate (18:0), and ethyl oleate (18:1) (Luginbühl, Schröck, König, Schürch, & Weinmann, 2016). In the blood FAEEs are detectable up to 24 hours (Soderberg et al., 1999), but FAEEs accumulate in the meconium (Hutson, Magri, Gareri, & Koren, 2010). To our best knowledge, there are no data on FAEE levels in the blood or plasma from a population of pregnant women. FAEEs originating from the mother are not transferred into the fetus because they are taken up and degraded extensively by the human placenta. Hence, FAEEs detected in neonatal matrices are likely

produced by the fetus from ethanol that has been transferred to and metabolized by the fetus (Chan, Knie, Boskovic, & Koren, 2004). Therefore, FAEEs are considered good indicators of true alcohol exposure in utero (Riley, E. P., Infante, & Warren, 2011).

2.6.3 Indirect maternal biomarkers

2.6.3.1.1 Carbohydrate-deficient transferrin

Carbohydrate-deficient transferrin (CDT) is synthesized and secreted in the liver and it acts as a carrier for iron in the blood (Joya et al., 2012). It is elevated 1-3 weeks after heavy alcohol consumption and maesurements of CDT are made from serum samples. CDT is a group of minor isoforms of human transferrin with a lower degree of glycosylation than major isoforms of this glycoprotein (Allen & Litten, 2003). The mean half-time of CDT is approximately 14-17 days (Maenhout, Baten, De Buyzere, & Delanghe, 2012).

The most common CDT measurement technique is microcolumn anion-exchange chromatography followed by immunoassay for transferrin quantification. Additionally, high-performance liquid chromatography, capillary electrophoresis and isoelectric focusing methods are used to analyse CDT (Helander, Vabo, Levin, & Borg, 1998). The hormonal status of women influenced CDT levels: CDT was 9.9% higher in pregnant women and 7.5% lower among those who used oral contraceptives wheareas postmenopausal women had 10.3% lower levels of CDT. Women using oral contraceptives and hormone intrauterine device for contraception had lower CDT (Sillanaukee et al., 2000b).

There are some studies analysing CDT and alcohol use during pregnancy (Azurmendi-Funes et al., 2019; Bakhireva, Ludmila N. et al., 2012; Bianchi, Ivaldi, Raspagni, Arfini, & Vidali, 2011; Comasco, Hallberg, Helander, Oreland, & Sundelin-Wahlsten, 2012; Howlett, Abernethy, Brown, Rankin, & Gray, 2017; Kenan, Larsson, Axelsson, & Helander, 2011; Magnusson, Göransson, & Heilig, 2005; Niemela et al., 2016; Sarkola, Eriksson, Niemela, Sillanaukee, & Halmesmaki, 2000). The clinical utility of CDT in alcohol use identification, especially in pregnancy, seems to be substantial. Niemelä et al. (Niemela et al., 2016) showed 39.5% sensitivity

for FAS outcome and 54% sensitivity for the combination of GGT-CDT for FAS outcome with a %CDT cut-off 1.7%. Sarkola and collegues found low sensitivity and specificity for CDT during pregnancy (Sarkola et al., 2000). However, based on interpretation of the previous studies, it seems that accuracy to detect alcohol use increases when analysing CDT together with other biomarkers and questionnaires (Azurmendi-Funes et al., 2019; Howlett et al., 2017).

2.6.3.1.2 Gamma-glutamyl transferase, alanine aminotransferase and aspartate aminotransferase

Liver enzymes, including gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are elevated 1-3 weeks after the last exposure (Bakhireva, L. N. & Savage, 2011). ALT is aggregated primarily in the cytosol of hepatocytes and consists of 496 amino acids and has a half-life of approximately 47 hours. ALT is normally detectable in serum at low concentrations (typically <30 IU/L). However, any process that leads to loss of hepatocyte membrane integrity or necrosis results in the release of ALT in high concentrations into the plasma (Moriles & Azer, 2021). These markers can measure recent changes in alcohol consumption from blood, but they are not increased by binge drinking. Only 30-50% of excessive drinkers in the general community have elevated levels of GTT (Poikolainen & Vartiainen, 1997; Sillanaukee, P. et al., 2000a). Liver enzymes are elevated by other forms of liver damage (Bakhireva & Savage, 2011). A systematic review of Howlett et al. (2017) and the review of Cook (2003) concluded that none of the blood biomarkers GGT, ALT and AST had both high specificity and sensitivity.

2.6.3.1.3 Mean corpuscular volume

Mean corpuscular volume (MCV) is an index of red blood cell size. Alcohol and its metabolites have toxic effects on the production of hematologic precursor cells and on red cell morphology. MCV is naturally elevated in mid-to late stage pregnancy. Macrocytosis, enlarged erythrocytes, is a common finding in chronic alcoholics.

The sensitivity and specificity of MCV are low as a marker for recent excessive alcohol intake during the pregnancy, but its sensitivity and specificity are superior to CDT (Sarkola et al., 2000). Sillanaukee et al. (Sillanaukee, P., Aalto, & Seppä, 1998) found that in detecting excessive drinking in the early phase, MCV in women was more sensitive (40%) than CDT (29%) or GGT (34%) in a primary care sample. However, the best sensitivity was reached in using combination of three biomarkers (MCV, CDT and GGT). Other reports support the poor usefulness of MCV in women to detect heavy drinking, even though it seems to be a slighltly better indicator of alcohol use among women than among men (Allen, Litten, Fertig, & Sillanaukee, 2000; Mundle, Munkes, Ackermann, & Mann, 2000; Sillanaukee et al., 1998; Wetterling, Kanitz, Rumpf, Hapke, & Fischer, 1998). While elevated MCV is found after sustained and regular excessive drinking, the clinical utility of MCV in alcohol use identification, especially in pregnancy, is limited (Allen et al., 2000; Bearer, 2001; Mundle et al., 2000; Sillanaukee et al., 1998; Wetterling et al., 1998).

2.6.3.1.4 Acetaldehyde protein adducts

Acetaldehyde is the main product of oxidation in ethanol metabolism. Acetaldehyde is rapidly converted to acetate by acetaldehyde dehydrogenase. Due to its high reactivity, it is not suitable as an alcohol biomarker (Cederbaum, 2012). Acetaldehyde forms both stable and unstable adducts with various proteins (Conduah Birt, Shuker, & Farmer, 1998). Stable acetaldehyde-protein adducts (APAs) have been proposed as a biomarker of alcohol use, because they have a longer half-life than free acetaldehyde and remain measurable in blood for approximately a month after alcohol intake (Conduah Birt et al., 1998; Howlett et al., 2017; Magnusson et al., 2005). So far, the use of APAs as a biomarker of alcohol use remains unclear. Future studies to determine whether APAs are uselful tools to detect alcohol consumption are warranted.

2.6.3.1.5 Phosphatidylethanol

Phosphatidylethanol (PEth) is a group of compunds derived from phospholipids, formed in the presence of ethanol. PEth can be detected 2-

3 weeks after ethanol ingestion with a mean half-time of approximately 3 days. LC-MS/MS method for maternal and neonatal blood samples are used to analyse PEth. It is considered a sensitive indicator of heavy alcohol use (area under the receiver operating characteristic (AUROC) 0.69 for abstainers versus any alcohol consumption during pregnancy, sensitivity 40.7%, specificity 95.4%; AUROC 0.99 differentiating heavy drinkers from light, moderate and non-drinkers) (Kwak et al., 2014; Yang, J. et al., 2015).

However, low-to-moderate drinkers are not detected, and PEth has been found to be unstable for storage (Faller et al., 2013; Gnann, Weinmann, & Thierauf, 2012; Howlett et al., 2017; Schröck, Thierauf-Emberger, Schürch, & Weinmann, 2017).

2.6.3.1.6 First trimester trisomy screening paramaters: pregnancyassociated plasma protein A, free β-human chorionic gonadotropin and nuchal translucency thickness

Pregnancy-associated plasma protein A (PAPP-A) and free β-human chorionic gonadotropin (free β-hCG) are influenced by maternal and pregnancy variables such as smoking, gestational age and ethnic background (Kagan, Wright, Spencer, Molina, & Nicolaides, 2008). Additionally, PAPP-A is influenced by maternal weight, height, diabetes mellitus, method of conception, previous pregnancy with or without pre-eclampsia and birth weight Z-score of the neonate in the previous pregnancy (Wright, Silva, Papadopoulos, Wright, & Nicolaides, 2015). Previous experiments with human placental cell lines and extraction analyses using human placental samples demonstrated that ethanol exposure increased hCG production in a dose-dependent matter (Joya et al., 2015). Little is known about the effects of alcohol use on these first trimester screening parameters.

2.6.4 Summary of biomarkers for alcohol use

Figure 5 summarizes the time and detection window of used alcohol biomarkers. In this summary low drinking was defined as < 1drink/day and heavy use as ≥14 drinks/week or binge espisodes. Moderate drinking

varies among studies. A combination of several biomarkers increases accuracy to detect alcohol consumption, but proper validation of such combinations has not been performed in pregnant women (Bakhireva & Savage, 2011; Bearer, 2001; Cook, 2003; Niemelä et al., 2016). In conclusion, currently used alcohol biomarker tests are not sufficient to detect low or moderate alcohol use, and we lack sensitive and specific biomarkers for alcohol use during pregnancy (Cook, 2003; Hannuksela, Liisanantti, Nissinen, & Savolainen, 2007; Manich et al., 2012).

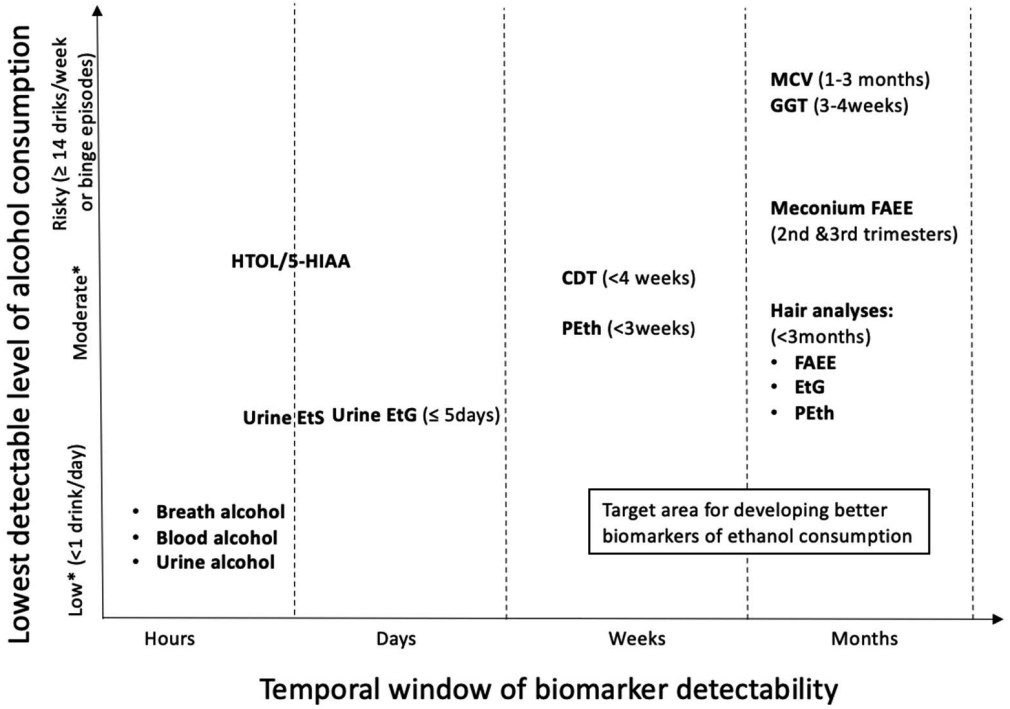

Figure 5. The period of time, or detection window, during which alcohol consumption can be detected and the lowest levels of alcohol consumption detectable by curren alcohol biomarkers. Abbreviations: EtS, Ethyl sulphate; EtG, ethyl glucuronide; CDT, carbohydrate-deficient transferrin; PEth, phosphatidylethanol; MCV, mean corpuscular volume; GGT, γ-glutamyltransferase; FAEE, fatty acid ethyl esters; HTOL/5-HIAA, 5-hydroxytryptophol/5-hydroxy-indole-acetic acid (discussed in paragraph 2.2.1.2) *The definition of low and moderate drinking in pregnant women greatly varies among studies. Modified from Bakhireva et al 2011 (Bakhireva & Savage, 2011).

2.7 TARGETED IMAGING FOR DETECTING ALCOHOL EXPOSURE

2.7.1 Obstetric ultrasonography

Obstetric ultrosonography has become a standard of care during the pregnancy. It is a safe, non-ionizing way to examine the fetus during pregnancy (Torloni et al., 2009). Ultrasonography is based on high-frequency sound waves (usuallu 0.5-40MHz). Sound waves can be described by their frequency, wavelength, period, amplitude, power, intensity and propagation speed. The sound waves progress in a medium, which can be solid, liquid or gas. Ultrasound is produced by the ultrasound probe, and the sound impulse progresses in tissues when sound waves are induced with the organs. Sound waves make the organ molecules oscillate. When sound travels through a medium, the molecules of that medium are alternately compressed (squeezed) and rarefied (stretched). Molecules oscillate but do not move as the sound wave passes through them. (Abuhamad & Chaoui, 2017). When the sound waves progress, they advance to different kind of layers and nonhomogenous tissues. This causes some of the ultrasound impulses to reflect back, and they can be registered by the probe. When the sound wave goes deeper into tissue the sound wave suppresses due to absorption and scattering of the sound wave (Sequeiros, Koskinen, Aronen, Lundbom, & Vanninen, 2017). The basics of the ultrasonography has remained the same during the years, but ultrasonography has become better in quality due to technical improvement. It also gives the opportunity to obtain 3D-images of the objects.

The benefits of the early ultrasonography are improvement in the early detection of multiple pregnancies and improved gestational dating, which may result in fewer inductions for post maturity. Routine scans also improve detection of major fetal abnormalities before 24 week of gestation (RR 3.46, 95% CI 1.67 to 7.14; participants=387; studies=2, moderate quality of evidence) (Whitworth, Bricker, & Mullan, 2015). However, another Cochrane review cocluded that based on existing evidence routine late pregnancy ultrasonography (after 24 weeks of gestation) in low-risk or

unselected populations does not confer benefit to the mother or baby (Bricker, Medley, & Pratt, 2015; Whitworth et al., 2015).

During the first trimester of pregnancy fetal nuchal translucency thickness (NTT) is measured to be used as part of aneuploidies detection. NTT is the sonographic appearance of a collection of fluid under the skin behind the fetal neck and back in the first trimester of pregnancy (Nicolaides, 2011). Appropriate training of sonographers and physicians and compliance with established standard ultrasound techniques is essential to ensure uniformity of NTT measurements among various operators. Semi-automated methods of measuring NTT have also been developed by several ultrasound manufacturers in order to reduce operator-dependent bias in NTT measurements (Abuhamad & Chaoui, 2017).

2.7.2 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is based to the behavior of the chemical elements in an external magnetic field. It is a non-ionizing imaging method that can produce image slices from almost any organ and tissue. MRIs employ powerful magnets which produce a strong magnetic field that forces protons in the body to align with that field. When a radiofrequency current is then pulsed through the patient, the protons are stimulated, and spin out of equilibrium, straining against the pull of the magnetic field. When the radiofrequency field is turned off, the MRI sensors are able to detect the energy released as the protons realign with the magnetic field. The time it takes for the protons to realign with the magnetic field, as well as the amount of energy released, changes depending on the environment and the chemical nature of the molecules. Physicians are able to tell the difference between various types of tissues on these magnetic properties. Contrast agents (often containing the element Gadolinium) may be given to a patient intravenously before or during the MRI to increase the speed at which protons realign with the magnetic field. The faster the protons realign, the brighter the image (Sequeiros et al., 2017).

Tesla (T) is a unit of magnetic strength unit equal to 10000 gauss. A 3T scanner has twice the strength of a 1.5T scanner. Higher strength is not always better quality. A different spinning frequency of hydrogen in water and fat causes a chemical shift which may cause artifact. Dielectric effects occur due to the radio frequency field (RF-field) component of the MRI. When carrying out an MRI, a coil will be placed over the body part being imaged and will work like an antenna to receive the signal from the body. Once the body is in the scanner, an RF pulse will be applied. This RF pulse is what excites the protons in the body. A dielectric effect is an interaction that can occur in certain tissues due to the electrical component of the RF field. It is more significant in 3T imaging and is most common in brain and abdominal imaging. Newer MRI software has developed ways to compensate for this artifact. (Abuhamad & Chaoui, 2017; Sequeiros et al., 2017).

The specific absorption rate is the estimated rate of energy that is being absorbed by a volume of tissue when RF energy is applied to the body during the MRI exam. This occurs in all MR scanners but will increase as the magnet strength increases. This means that while specific absorption rate is not an issue in a 1.5T scanner, it is an issue in a 3T scanner due to the increased magnetic field. The specific absorption rate means that the body can heat up when MRI is performed. 3T MRI is considered to be best when imaging orthopedic, neurologic and vascular targets (Sequeiros et al., 2017).

2.7.3 Single photon emission computed tomography

Single photon emission coputed tomography (SPECT) is a nuclear medicine tomography imaging techinque that uses gamma rays. The technique needs delivery of a gamma-emitting radioisotope into the patient. Imaging shows how the radiotracer flows to tissues and organs. It integrates computed tomography (CT) and a radioactive tracer. Several radioligands are used for the imaging of glutamate and serotonin receptors (Kim, J. H., Marton, Ametamey, & Cumming, 2020; Paterson, Kornum, Nutt, Pike, & Knudsen, 2013). Iodine-123-labeled nor-beta-CIT is a nonselective monoamine trasnporter SPECT ligand that has approximately equa affinity

for dopamine, serotonin and noradrenaline transporters. Despite its nonselectivity, β-[^{123}I]CIT has been used for imaging both dopamine (DAT) and serotonin transporters (SERT), by taking advantage of the differential localization of these transporters (striatum and midbrain, respectively) and the different tracer kinetics in these regions (Paterson et al., 2013).

2.7.4 Optical coherence tomography

Optical coherence tomography (OCT) is an imaging technique that gives a cross-sectional view of the retina, pigment epithelium and the surface of choroid. It is a simple, quick and non-invasive imaging method. OCT is indicated for investigating retinal diseases such as retinal degenerative diseases, macular holes, retinal detachment and diabetic retinopathy. An OCT image of the macula can detect the following retinal layers: nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external limiting membrane, ellipsoid zone (previously referred to as the IS/OS junction), interdigitation zone, and retinal pigment epithelium (Paterson et al., 2013; Seppänen, Kaarniranta, Setälä, & Uusitalo, 2018).

3 AIMS OF THE STUDY

The aim of this study was to investigate potential biomarkers to detect alcohol use during pregnancy. Furthermore, our additional aim was to investigate whether children exposed to alcohol during pregnancy show functional or structural brain findings and whether the eye abnormalities are detected by ophthalmological examination in young adults.

The specific aims of this study were as follows:

I. To investigate alcohol effects on first trimester screening parameters and first trimester metabolome (studies I and II)
II. To study fetal alcohol and drug effects during pregnancy and follow the growth and development of the child until 2.5 years of age (study III)
III. To assess structural and functional brain abnormalities in FAS/FAE by brain SPECT and MRI (study IV)
IV. To analyse ophthalmological findings and optic nerve thickness in FAS/FAE (study V)

4 FORMATION OF THE STUDY GROUPS

This thesis included three study populations. Figure 6 illustrates the study design and population. The first study population used in publications I and II (chapters 5 and 6) included approximately 2500 pregnant women attending first trimester screening for trisomy 21 in the Kuopio University Hospital area between June 2010 and June 2011. A total of 138 women were randomly selected for publication I (chapter 5) and 544 women for publication II (chapter 6).

The second study population for publication III (chapter 7) attended a longitudinal prospective study conducted in 2005–2008 in the maternity clinic at Kuopio University Hospital, Finland, and the primary healthcare center in the city of Kuopio. Subjects were recruited from patients at the maternity clinic at Kuopio University Hospital. The pregnant women followed up in the maternity clinic had been referred to the clinic by their general practitioners due to concerns about alcohol or drug abuse (n=23). The control subjects were recruited from two maternity clinics of the Kuopio city primary healthcare center (n=22).

The third study population for publications IV and V (chapters 8 and 9) included twelve children admitted to the Kuopio University Hospital for neurological or neuropsychological investigations, usually because of learning disabilities. All children were confirmed cases of intrauterine alcohol exposure and fulfilled the criteria for FAS or FAE. Ten out of these 12 participated as young adults in the study for publication V.

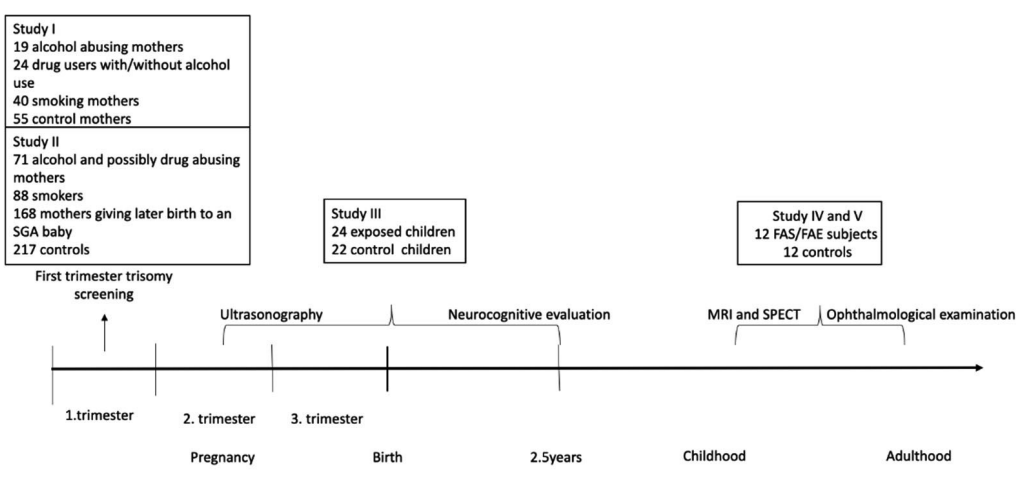

Figure 6. Formation of the study groups and their design in timeline.

5 ALCOHOL AND SUBSTANCE USE ARE ASSOCIATED WITH AN ALTERED METABOLOME IN THE FIRST TRIMESTER SERUM SAMPLES OF PREGNANT MOTHERS

5.1 ABSTRACT

Background: Although the effects of alcohol on metabolic processes in the body have been studied widely, there do not appear to be any previous reports clarifying how substance abuse changes metabolic profiles of pregnant women during the first trimester of pregnancy. Our aim was to evaluate the effect of substance abuse, especially alcohol use, on the metabolic profile of pregnant women during the first trimester.

Methods: We applied mass spectrometry based non-targeted metabolite profiling of serum collected during routine visit to the hospital between gestational weeks 9 + 0 to 11 + 6 from controls (n = 55), alcohol users (n = 19), drug users (n = 24) and tobacco smokers (n = 40).

Results: We observed statistically significantly differences among the study groups in serum levels of glutamate, glutamine, and serotonin (p-values 0.0001). The serum levels of glutamate were increased in alcohol and drug using mothers when compared to the controls, whereas levels of glutamine were decreased in alcohol and drug using mothers. In addition, serum levels of serotonin were decreased in alcohol using mothers when compared to the controls.

Conclusion: The present study shows that alcohol and drug use were associated with increased glutamate, and decreased glutamine levels, and alcohol use is associated with decreased serotonin levels. This study serves as a proof-of-concept that the metabolite profile of human first trimester serum samples could be used to detect alcohol exposure during pregnancy.

5.2 INTRODUCTION

One of the ongoing challenges for prevention, accurate diagnosis, and treatment of children with fetal alcohol exposure is the difficulty of confirming whether a mother is drinking alcohol during her pregnancy. The absence of reliable diagnostic methods to identify alcohol use during pregnancy is a major problem (Bakhireva & Savage, 2011; Cook, 2003; Hannuksela et al., 2007; Joya et al., 2012). Currently, clinical studies on alcoholism depend on information obtained from the subjects. Although questionnaires about alcohol use have been shown to be the best available way to detect alcohol use (Aertgeerts et al., 2002) pregnant women tend to underreport their alcohol consumption (Jacobson et al., 2002). Specific methods for detecting alcohol use, for example from whole-blood or hair samples, have been developed (Chiandetti et al., 2017). However, since these methods need sample types usually not collected during routine visits (e.g., hair), methods to detect alcohol use from serum samples would be preferred. Early identification of alcohol use as a part of the routine clinical screening during pregnancy would be beneficial in many ways; resources could be targeted more efficiently to support mothers to remain abstinent so that their babies will be born healthier (Forray, 2016), though with the current knowledge of the timing or dose of alcohol exposure needed for FASD to occur is not known.

The effects of alcohol on metabolic processes in the body have been studied widely. However, there is only a few studies about the effects of alcohol in pregnant women. In pregnant sheep, alcohol exposure has altered the amino acid balance, for example, it increased glutamate and decreased glutamine levels in the plasma (Ramadoss, Wu, & Cudd, 2008). Furthermore, alterations in metabolites of amino acids have also been associated with consequences of alcohol use during pregnancy. For example, prenatal alcohol exposure has been associated with a dysfunction of the serotonin (a metabolite of tryptophan) system (Riikonen, R. S. et al., 2005; Sari, Johnson, & Weedman, 2011). These changes in the amino acid metabolism have, for example, been linked to chronic alcohol consumption caused neurobiological adaptations in key

neuro- transmitter systems, including increased glutamate levels and decreased serotonin levels, to compensate the effects of alcohol exposure and these changes are considered important for development of alcohol use disorder (Koob & Volkow, 2016; Marcinkiewcz, Lowery-Gionta, & Kash, 2016).

As far as we are aware, there are no previous reports exploring metabolic profiles of pregnant women to identify alcohol use during the first trimester of pregnancy. In this study, we performed a non-targeted metabolite profiling analysis to examine the metabolic alterations in alcohol, alcohol and/or drug, and tobacco exposed pregnancies. In the current report, we focus mainly in investigating potential metabolites or groups of metabolites to identify, not to quantify, alcohol-exposed pregnancies. However, because drug use and especially tobacco smoking are common in persons with heavy alcohol use (Kalman, Morissette, & George, 2005) we also included these study groups to investigate whether metabolite profiles would be able to distinguish alcohol use from tobacco and drug use. The drug and tobacco groups were included to estimate the specificity of the seen metabolite changes to alcohol consumption, not to detect drug or tobacco use as such. The current report focuses mainly on investigating the metabolite profile associated which alcohol- exposed pregnancies.

5.3 MATERIALS AND METHODS

This is a retrospective cohort study of pregnant women attending first trimester screening for trisomy 21 and nuchal translucency in the Kuopio University hospital area. Pregnancies in these women who attended first trimester screening tests and routine care in June 2010–June 2011 in Kuopio University Hospital were searched from medical records. The pregnancy and the birth outcomes were evaluated and the 138 samples were collected out of approximately 2500 pregnancies in total. The study sample included 40 tobacco smoking mothers, 19 alcohol using mothers and 24 drug users with/without alcohol use, and 55 non-smoking control

mothers with appropriate for gestational age (AGA) infants (at delivery birth weight between the 10th and 90th percentile).

Alcohol and drug using pregnant women were followed in the maternity clinic; these women had been referred by general practitioners due to concerns about their alcohol or drug abuse. The Alcohol Use Disorder Identification Test (AUDIT) (Aertgeerts et al., 2002; Saunders et al., 1993) was used to identify the mothers with harmful patterns of alcohol consumption. The Alcohol Use Disorders Identification Test (AUDIT) is validated test used to determine if a person is at risk for alcohol abuse problems. AUDIT score of 8 or more indicates likelihood of harmful alcohol consumption. The inclusion criteria were a total AUDIT score of eight or more and/or alcohol use during pregnancy. The inclusion criteria for the drug user group were drug abuse before/during the ongoing pregnancy. Additionally, drug users could have used alcohol and/or tobacco during pregnancy. The inclusion criterion for the tobacco smoking groups was five or more cigarettes per day during pregnancy and they did not report any other substance use. The inclusion criteria for the control group were singleton pregnancy, a non-complicated vaginal birth and normal outcome: the mother or the newborn did not require any pre-, peri-, or postnatal follow-up, care, or interventions over and above what could be considered as routine. The controls were healthy women who did not have any other diagnosis at the time of the delivery, and these were spontaneous, normal parturitions. The women in the control group and tobacco group did not show alcohol or drug abuse as measured with AUDIT scores <8. They did not have any other diagnosis than normal parturition (Partus spontaneus, situs longitudinalis cranioinferior) according to ICD-10 criteria. One control baby received postnatal intensive care for a very short time due to the suspicion of a neonatal infection.

Serum samples were collected in maternity care units during the weeks 9 + 0 to 11 + 6. Blood samples were allowed to clot at room temperature for 30 min, centrifuged and stored at +4 °C. Serum samples were delivered to the Eastern Finland Laboratory Centre in Kuopio as cold or frozen specimens and stored at 20 °C. Samples were transferred to 70 °C during the spring 2012.

This study was approved by the Research Ethics Committee of Kuopio University Hospital. All study participants provided informed written consent.

LC–MS metabolite profiling analysis

The LC–MS metabolite profiling analysis utilized here has been described in detail elsewhere (Pekkinen et al., 2013). In brief, a 100 μL aliquot of the first trimester screening fasting serum sample was mixed with 400 μL of acetonitrile (VWR International), incubated on an ice bath for 15 min, and centrifuged. The supernatant was filtered through 0.2-mm polytetrafluoroethylene filters in a 96-well plate format. Quality control samples were made by mixing 2 μL aliquots of the serum samples. A solvent blank was prepared in the same manner.

The samples were analyzed by the liquid chromatography quadrupole time-of-flight mass spectrometry system (UHPLC- qTOF-MS, Agilent Technologies), which consisted of a 1290 LC system, a Jetstream electrospray ionization (ESI) source, and a 6540 UHD accurate-mass qTOF spectrometer. We used hydrophilic interaction (HILIC) chromatography (an Acquity UPLC BEH Amide column, 100 mm x 2.1 mm, 1.7 μm; Waters Corporation, Milford, MA) and positive ionization. This method was selected because we were interested in changes in the amino acid metabolism. The data acquisition software was MassHunter Acquisition B.04.00 (Agilent Technologies). The quality control and blank samples were injected at the beginning of the analysis and after every 12 samples. The order of the analysis of the samples was randomized. QC samples were used for the automatic data-dependent MS/MS analyses.

Data analysis

Demographic data management and the statistical analyses were performed using SPSS 21 (SPSS Inc., Chicago, IL, USA). In all of the analyses, a P-value of less than 0.05 was considered significant. An independent

sample t-test was used for continuous demo- graphic parameters, if they fulfilled the criteria for the parametric tests, otherwise Mann-Whitney test was used. Chi-square test was used to handle dichotomous variables and if there were fewer than five units in any of the classes, the Fischer's exact test was used.

The mass spectrometry data processing was performed using MassHunter Profinder B.06.00 (Agilent Technologies, USA). The batch recursive feature extraction function was used to extract ion to molecular features exhibiting isotopic peaks, dimers, and common adducts. Final alignment and quality control of peak spectra were done manually. The data were transferred as compound exchange format files into the Mass Profiler Profes- sional (MPP) software (version 13, Agilent Technologies) for statistical analysis.

Analysis of variance (ANOVA) was used to evaluate statistically significant differences among the study groups. Because of the correlative nature of metabolites in serum samples, principal component analysis was used to evaluate overall variance in the metabolic profiles of all subjects. The number of principal components needed to explain 95% of variance in the metabolic profiling data was used to adjust the α level for multiple test correction (Bonferroni's method). Furthermore, Bonferroni's method (when the study groups were compared to controls) was used as the post-hoc test for the molecular features which were statistically significant in the ANOVA comparison. Cohen's method (d) was used to calculate effect sizes and to compare the study groups to the control group. Statistically significantly altered metabolites were identified based on a comparison of accurate mass, isotope patterns and auto MS/MS spectra from molecular features to MS/MS spectra from chemical standards and Metlin database (https://metlin.scripps.edu).

5.4 RESULTS

Tables 2 and 3 show the anthropometric characteristics of the mothers and children, respectively, in the study groups. None of the mothers had diabetes prior to their pregnancy nor did they experience obstetric

cholestasis or polyhydramnion during pregnancy. Most of the Drug abusers were polydrug users. Drugs abused included amphetamine, different kind of opioids (including buprenorphine as a replacement therapy and abused agent), semisynthetic opioids, benzodiazepines, selective serotonin reuptake inhibitors, noradrenergic and specific serotonergic antidepressants and cannabinoids. Drug using mothers had a low prevalence of oligohydramnion and amnionitis in late pregnancy when compared to the controls (n = 1/4.2%, P = 0.280 for each comparison), this was not detected in the other study groups. There were no perinatal deaths in any of the groups.

In the metabolite profiling analysis, a spectral peak for cotinine (a nicotine metabolite, biomarker for nicotine exposure) was observed in the serum samples of 14 alcohol and 18 drug using mothers and in all the serum samples from tobacco smoking mothers. In contrast, no cotinine peak was observed in the serum samples from the controls (Supplementary Table 1), and therefore cotinine was excluded from further statistical analyses.

Table 2. Anthropometric characteristics of the alcohol and drug abusing and control mothers and their pregnancies

	Controls (n=55)		**Alcohol Abusers (n=19)**			**Drug Abusers (n=24)**			**Smokers (n=40)**		
	Mean	SD	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Marital status: married (n/%)	29(52.7%)		15 (78.9%)		**0.011***	16 (66.7%)		**0.074**	30(75.0%)		**0.022**
Smoking before pregnancy >5 cigarettes/day (n/%)	0(0%)		10 (52.6%)		**<0.001***	14 (58.3%)		**<0.001***	40(100%)		**<0.001***
Smoking during the pregnancy >5 cigarettes/day	0(0%)		4 (21.1%)		**0.002***	9 (37.5%)		**<0.001***	40(100%)		**<0.001***
Alcohol use before pregnancy (n/%)	22 (40.0%)		9 (47.4%)		**<0.409**	11 (45.8%)		**0.396**	15(37.5%)		**0.768**
AUDIT	3.1	1.6	19.5	8.3	**<0.001***	10.7	8.4	**<0.001***	5.7	5.0	**0.014***
Gestational diabetes (n/%)	0 (0%)		3 (15.8%)		**0.012***	2 (8.3%)		**0.076**	9(22.5%)		**<0.001***
Primigravida (n/%)	15 (27.2%)		13 (68.4%)		**<0.001***	16 (66.7%)		**<0.001***	13(32.5%)		**0.564**
BMI in the beginning of pregnancy	23.1	3.6	24.0	5.8	**0.557**	24.4	5.9	**0.366**	25.7	4.1	**0.002***
Mother´s weight at the beginning of pregnancy, kg	64.8	9.5	67.3	16.4	**0.570**	68.8	16.6	**0.323**	70.2	12.9	**0.024***
BMI in the end of the pregnancy	27.5	3.7	28.9	4.6	**0.289**	30.3	4.2	**0.017***	30.9	3.3	**<0.001***
Mother´s weight at the end of the pregnancy	76.9	9.5	79.5	14.1	**0.561**	84.8	13.2	**0.014***	84.4	11.4	**0.004***
Mother's age (at birth), years	29.2	4.7	26.4	7.0	**0.133**	24.5	4.4	**<0.001***	27.0	5.7	**0.048***
Height of the mother, cm	165.9	4.6	165.5	4.8	**0.746**	166.2	4.2	**0.793**	163.8	5.9	**0.057**
Number of previous pregnancies	1.9	1.7	1.2	1.9	**0.021***	1.52	1.9	**0.178**	2.1	1.9	**0.952**
Duration of pregnancy at birth (days)	280.5	6.3	282.4	10.4	**0.093**	282.3	6.8	**0.151**	278.9	9.8	**0.629**

Sample size may vary owing to missing values.

*statistically significant result, p<0.05

Chi square test was used to evaluate differences between the study groups and controls for dichotomous variables. If there were fewer than five units in any of the classes, the Fischer´s exact test was used.

Independent sample t-test was used compare differences between the study groups and controls for continuous variables. If parametric test criteria were not fulfilled, Mann-Whitney test was used.

Table 3. Anthropometric characteristics of the alcohol and drugs exposed and control children

	Controls (n=55)		**Alcohol Abusers (n=19)**			**Drug Abusers (n=24)**			**Smokers (n=40)**		
	Mean	SD	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Birth weight (g)	3550	350	3450	530	**0.433**	3540	440	**0.912**	3490	390	**0.39**
Birth weight (SD)	-0.28	0.7	-0.51	1.1	**0.401**	-0.35	0.9	**0.672**	-0.38	0.75	**0.52**
Birth head circumference (cm)	35.1	1.2	35.7	1.5	**0.11**	35.3	1.4	**0.485**	35.3	1.6	**0.49**
Birth head circumference (SD)	-0.01	0.88	0.5	1.1	**0.044***	0.15	1.0	**0.480**	0.11	1.14	**0.55**
Mean placental weight/birth weight ratio (%)	16.2	3.1	17.9	3.3	**0.054**	17.7	2.5	**0.062**	17.4	2.7	**0.05**
Breech position (n/%)	0 (0%)		0 (0%)		**X**	3 (12.5%)		**0.028***	2 (5%)		**0.17**
Gender girl (n/%)	33 (61.1%)		8 (42%)		**0.176**	11 (45.8%)		**0.244**	15 (37.5%)		**0.05**
SGA (<10th percentile) (n/%)	0 (0%)		2 (11%)		**0.053**	0 (0%)		**X**	0 (%)		**X**
Intensive care after birth (n/%)	1 (1.8%)		2 (11%)		**0.140**	6 (25%)		**0.002***	3 (7.5%)		**0.30**

Sample size may vary owing to missing values.

*statistically significant result, $p<0.05$

Chi square test was used to evaluate differences between the study groups and controls for dichotomous variables. If there were fewer than five units in any of the classes, the Fische exact test was used.

Independent sample t-test was used for the continuous values to compare differences between the study groups and controls.

A total of 104 molecular features were included into the statistical analysis (Supplementary Table 1). To account for multiple testing, the α level was adjusted to 0.0012 (Bonferroni's method), because 41 principal components were required to explain 95% of the variance in the metabolite profiling data. This resulted in the identification of ten molecular features, which were significantly altered among the study groups (Supplementary Table 1). With respect to the statistically significant features, we identified five main compounds (Figure 7, Supplementary file 1): serotonin ($P < 0.0001$), glutamate ($P = 0.0001$), glutamine ($P < 0.0001$), glycerophosphocholine ($P = 0.0003$), and asparagine ($P = 0.0007$). Other statistically significant molecular features were thought likely to be glutamine and serotonin adducts, because they had the same retention time and showed similar auto MS/MS fragmentation patterns.

In the post-hoc analyses (Figure 7), the alcohol (mean abundance = 42997, SD = 19204, d = 0. 71) and drug (mean abundance = 47431, SD = 20542, d = 0.99) using mothers had significantly elevated serum levels of glutamate when compared to the controls (mean abundance = 31647, SD = 12532, Fig. 1). The alcohol (mean abundance=301029, SD=116046, d=1.04) and drug (mean abundance = 335879, SD = 150900, d = 0.77) using mothers had significantly decreased serum glutamine levels when compared to the controls (mean abundance = 438772, SD = 124027). Moreover, the alcohol using mothers had signifi- cantly decreased serum serotonin levels (mean abundance = 9034, SD = 6606, d = 0.97) as compared to controls (mean abun- dance = 15796, SD = 6019). We also observed significantly decreased levels of asparagine in the alcohol using mothers (mean abundance = 11009, SD = 3517, d = 0.84) and the drug using mothers (mean abundance = 11521, SD = 3488, d = 0.69) when compared to the controls (mean abundance = 13888, SD = 3294). Finally, the tobacco smoking mothers (mean abundance = 34935, SD = 20875, d = 0.69) had significantly decreased serum levels of glycerophosphocholine when compared to controls (mean abundance = 81045, SD = 94903). The large variance in the glycerophosphocholine levels in the control group is explained by two samples with very high values (5.9 and 7.4 standard deviations higher than the mean of the control group). If these two values

are excluded from the analysis (new mean abundance = 64462, and SD = 41866, for the control group), the ANOVA comparison among the study groups is still statistically significant (P = 0.0007).

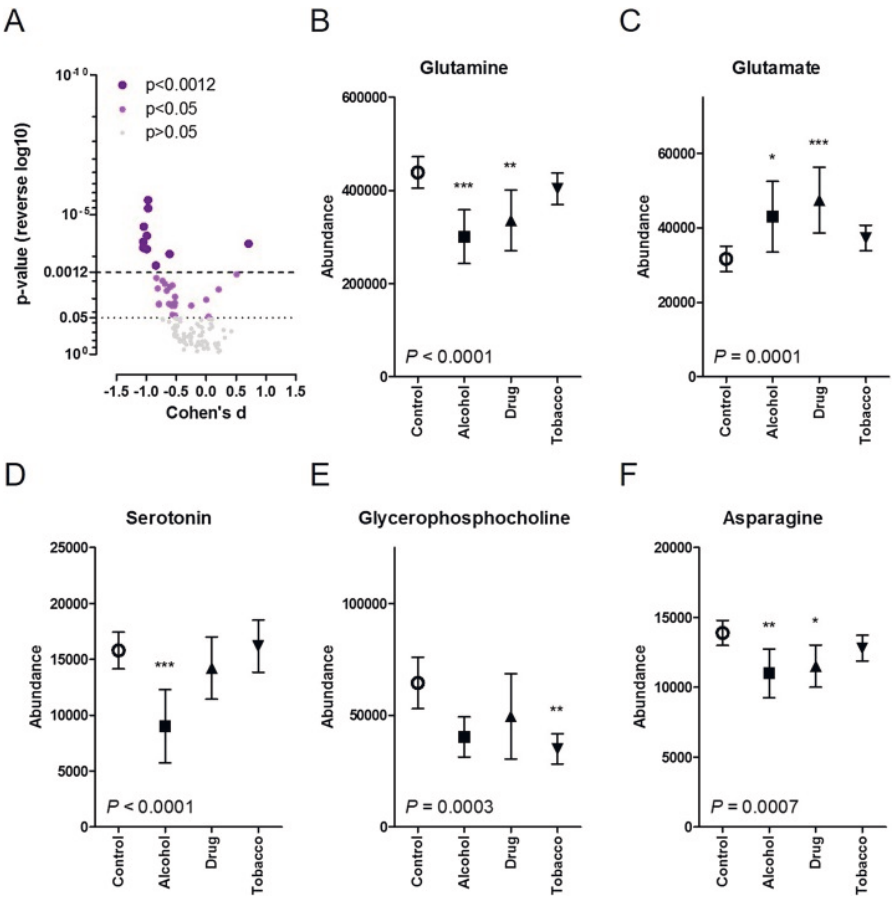

Figure 7. Main results from the metabolite profiling analysis of the first trimester serum samples of pregnant women

P-values (ANOVA among all study groups) and Cohen's d effect sizes (alcohol using mothers compared to the controls) are shown for the measured molecular features (A), of which 32 had P-values below 0.05, and ten had P-values below the Bonferroni adjusted α level of 0.0012. With this conservative statistical correction procedure, statistically significant differences among the study groups were observed in levels of glutamine (B), glutamate (C), serotonin (D), glycerophosphocholine (E) and asparagine (F) in first trimester serum samples of pregnant women.

Legend: Control, non-smoking control mothers; Alcohol, alcohol using mothers; Drug, drug using mothers with/without alcohol use; Tobacco, tobacco smoking mothers; P, P-value from ANOVA comparison; *, P < 0.05; **, P < 0.01 and ***, P < 0.001 after Bonferroni post-hoc correction when compared to the controls. Mean and 95% confidence intervals are shown for each group.

5.5 DISCUSSION

The aim of the present study was to examine whether there was a metabolic profile reflecting substance use, especially alcohol use, during pregnancy. Rather than observing a clear profile for alcohol use, we identified a metabolic profile for a risky pregnancy associated with substance use (alcohol and/or drug use). This serum risk profile displayed increased glutamate levels as well as decreased levels of glutamine and serotonin (Fig. 1).

Statistically significantly increased glutamate (+36% and +50%, respectively) and decreased glutamine levels (31% and 23%, respectively) in the serum samples of alcohol and drug using mothers can be considered to reflect dysfunctional glutamate metabolism. Glutamate is the major excitatory neurotransmitter in the brain and glutamine is produced from glutamate and ammonia by the enzyme glutamine synthetase (EC 6.3.1.2.). One of the main effects of alcohol consumption to the nervous system is inhibition of glutamatergic neurotransmission, which leads to increased production of glutamate when the alcohol consumption is chronic (Koob & Volkow, 2016). Glutamate can be made from glutamine by glutaminase (GLS1). Byproduct of this reaction is ammonia, which is a neurotoxic agent and could influence alcohol caused neurological problems in fetuses exposed to alcohol during pregnancy (Oja, Saransaari, & Korpi, 2017). Alcohol induced increased glutamatergic tonus has been associat- ed with apoptotic neurodegeneration in animal models of fetal alcohol exposure (Olney, 2002).

Our results are in line with the previous investigations of Ramadoss et al. (Ramadoss et al., 2008) which detected increased glutamate and decreased glutamine levels in plasma samples of alcohol exposed pregnant sheep when compared to controls. Moreover, elevated glutamate levels have been reported in the deep cerebellar nuclei of children with fetal alcohol exposure as measured in vivo with 1H magnetic resonance spectroscopy (du Plessis et al., 2014). Increased brain and cerebrospinal fluid glutamate levels have also been associated with chronic alcohol use in adults (Hermann et al., 2012; Umhau et al., 2010), and increased serum

glutamate levels have been proposed as a biomarker for acamprosate treatment outcomes of alcohol dependence (Nam et al., 2015). Furthermore, a recent metabolic profiling study of young adults detected an association between decreased glutamine levels and alcohol consumption (Würtz et al., 2016). Moreover, the significantly decreased asparagine levels in the alcohol using pregnant women (21% when compared to controls, Figure 7) can also be associated with glutamate metabolism, because asparagine synthesizing enzyme (asparagine synthetase, EC 6.3.5.4) produces glutamate in a reaction where aspartate is transformed to asparagine and glutamine to glutamate. Therefore, the significantly decreased asparagine levels in the alcohol using pregnant women (Figure 7) could be associated with reduced production of glutamate from glutamine by asparagine synthetase. Furthermore, these changes could also be associated with alcohol evoked alterations in the energy metabolism (mitochondrial function and the glycolysis and pentose phosphate pathways) e.g. in the brain, since these also lead to similar changes in amino acid metabolism (Meinhardt et al., 2014). Alterations in the glutamine and glutamate levels in the drug using mothers are also in line with previous research showing that chronic use of many drugs of abuse disrupt the glutamate system (Spencer, Scofield, & Kalivas, 2016). It can be speculated that since glutamate is important for normal brain function including cognition, memory and learning and because the fetal blood-brain barrier is incomplete, a dysfunctional glutamate system in the mother could influence the brain development of her fetus (du Plessis et al., 2014; Zhou & Danbolt, 2014).

Moreover, we observed significantly decreased serotonin levels in the first trimester serum samples of alcohol using mothers when compared the controls (43%, Figure 7). Importantly, smoking alone did not significantly alter serotonin levels during pregnancy. Dysfunction in the serotonin system, e.g. decreased serotonin transporter binding, has also been reported in children with fetal alcohol syndrome as well as in animal models of prenatal alcohol exposure (Riikonen et al., 2005; Sari et al., 2011). Serotonin modulates many brain functions, which have been associated with alcohol use, e.g. executive function, stress and reward

pathways. There are several reports indicating that deficient central serotonergic transmission plays a critical role in alcoholism (Karkkainen et al., 2015; Mantere et al., 2002; Marcinkiewcz et al., 2016; Sachs, Salahi, & Caron, 2014; Sari et al., 2011). Furthermore, previous depression studies have revealed an association between depression, alcohol, caffeine, tobacco, and illicit drug use during pregnancy and furthermore, depression is associated with decreased levels of serotonin (Field et al., 2007).

The decreased serum levels of glycerophosphocholine (Figure 7.) could be linked with nicotine exposure, because glycerophospho- choline is one of the major forms of choline storage in the body and as such, it is a precursor of acetylcholine, which is the endogenous ligand for the nicotinic acetylcholine receptors. Previously, a decreased combined signal for glycerophosphocholine and phos- phocholine levels has been reported in an in vivo 1H magnetic resonance spectroscopy study of children with fetal alcohol exposure (du Plessis et al., 2014).

It should be noted that most of the main findings in the serum samples from alcohol using mothers were fundamentally parallel to those seen in the drug using mothers when compared to the controls, only the effect size varied (Figure 7). On the other hand, clear differences between alcohol using and tobacco-smoking mothers could be observed in glutamate, glutamine and serotonin levels (Figure 7). These findings are in line with previous literature showing that heavy consumption of alcohol is associated with increased glutamate, and decreased glutamine and serotonin levels as a part of the neurobiological changes associated with development of addiction (Meinhardt et al., 2014; Nam et al., 2015; Würtz et al., 2016). Furthermore, also other conditions could produce similar pattern of abnormal metabolites. However, for example diabetes and hypertension have been previously associ- ated with increased levels of acylcarnitines, fatty acids and branched-chain amino acids, which were not associated with alcohol use in the present study (du Plessis et al., 2014; Nikolic, Sharman, Adams, & Edwards, 2014; Pallares-Mendez, Aguilar-Salinas, Cruz-Bautista, & Del Bosque-Plata, 2016).

The main limitation of the present study is the relatively low number of samples from alcohol and drug using mothers. Therefore, even though we

used the highly conservative Bonferroni correction to control for multiple testing, these present results should be confirmed with a larger number of samples from alcohol and drug using mothers. Future studies should investigate the correlations between timing and amount of alcohol consumed and the metabolite profile changes. The small sample size of the present study does not allow this to be studied reliably. It should be noted, that the possibility that the observed changes are a result of life-time use, rather that alcohol use only during pregnancy, cannot be ruled out in the present analysis. The strengths of the research include the fact that the serum samples were collected during a routine first trimester hospital visit and therefore should represent the variation one would expect to encounter in a clinical setting. Benefits of using pregnant population to study alcohol consump- tion caused changes in the metabolic profile include the regular monitoring of health during pregnancy and the fact that many pregnant women follow the guidelines and do not consume alcohol during pregnancy making selection of non-drinking control group more plausible.

In summary, this study demonstrates that alcohol consumption is associated with altered the metabolite profile in the plasma samples of pregnant women. This risk profile included increased levels of glutamate, as well as decreased levels of glutamine and serotonin (Figure 7). Future studies with larger cohorts should investigate if the changes in metabolite profile are on their own or in combination with traditional biomarkers of alcohol use, like gamma glutamyl transferase (GGT) levels, able to reliably detect alcohol use during early pregnancy in a prospective study setup and could be used for development of clinical biomarker panel.

5.6 ACKNOWLEDGEMENTS

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6 THE EFFECT OF MATERNAL ALCOHOL AND DRUG ABUSE ON FIRST TRIMESTER SCREENING ANALYTES: A RETROSPECTIVE COHORT STUDY

6.1 ABSTRACT

Background: The purpose of this study was to determine whether first trimester trisomy screening (FTS) parameters are affected by alcohol and drug use.

Methods: A routine combined FTS including measurements of maternal serum levels of free β-human chorionic gonadotropin subunit (free β-hCG) and pregnancy-associated plasma protein A (PAPP-A) were measured at 9-11 weeks of gestation, and fetal nuchal translucency thickness (NTT) at 11-13 weeks of gestation. In total 544 women with singleton pregnancies [71 alcohol and drug abusers, 88 smokers, 168 non-smokers delivering a small for gestational age (SGA) child, and 217 unexposed control women] were assessed.

Results: Free β-hCG levels were higher in alcohol and drug abusing than in unexposed pregnant women [mean 1.5 vs. 1.2 multiples of medians (MoM); P=0.013]. However, stepwise multiple linear regression analyses suggested that smoking could explain increased free β-hCG. Additionally, we observed lower PAPP-A levels in the smoking mothers (0.9 vs. 1.2 MoM; P=0.045) and in those giving birth to an SGA child compared to the controls (1.1 vs. 1.2 MoM; P<0.001). Fetal NTT did not differ significantly between any of the groups.

Conclusion: The present study shows increased free β-hCG levels in alcohol and drug abusers, but maternal smoking may explain the result. Maternal serum PAPP-A levels were lower in smoking than non-smoking mothers, and in mothers delivering an SGA child. However, FTS parameters (PAPP-A, free β-hCG and NTT) seem not to be applicable for the use as

alcohol biomarkers because of their clear overlap between alcohol abusers and healthy controls.

6.2 INTRODUCTION

One of the challenges for accurate detection and timely treatment of children with fetal alcohol exposure is the difficulty to confirm alcohol exposure during pregnancy, because we lack a reliable biomarker of alcohol abuse (Bakhireva & Savage, 2011; Cook, 2003; Hannuksela et al., 2007). The use of available biomarkers of alcohol consumption is hampered by a number of problems: the time window for detection of alcohol use is not sufficient, the biomarker may be insensitive or unspecific, the use of the biomarker has not been validated, or pregnancy itself affects the behaviour of the biomarker (Aertgeerts et al., 2002; Saunders et al., 1993). A combination of several biomarkers increases accuracy, but proper validation of such combinations has not been performed in pregnant women (Bearer, 2001; Cook, 2003; Niemelä et al., 2016).

A combined first trimester screening (FTS) test for chromosomal abnormalities includes measurements of pregnancy-associated plasma protein A (PAPP-A) and free β-human chorionic gonadotropin subunit (free β-hCG) from maternal serum, fetal nuchal translucency thickness (NTT), and recording the mother´s age. This combination identifies 85-95% of all fetuses with trisomies 21, 18 and 13, at a false positive rate of 5% (Nicolaides, 2011).

PAPP-A and free β-hCG are known to be influenced by maternal and pregnancy variables such as gestational age, maternal weight, smoking and ethnic background (Kagan et al., 2008). There is growing evidence that decreased PAPP-A is associated with a delivery of a small for gestational age (SGA) child at the end of pregnancy, even though the systematic review and meta-analysis of Morris et al. (Morris et al., 2008) showed that the sensitivity of PAPP-A to predict the birth of an SGA child remains low. Previous experiments with human placental cell lines and extraction analyses using human placental samples demonstrated that ethanol

exposure increased hCG production. Thus, hCG was suggested for a candidate surrogate biomarker of prenatal ethanol exposure (Joya et al., 2015). The influence of drug abuse on first trimester screening parameters has not been previously reported. However, it seems that maternal opioid use does not significantly affect second trimester free β-hCG levels (Scott, Holding, Purcell, Tutty, & Lindow, 2009).

To our knowledge, the influence of alcohol use on the first trimester screening parameters has not previously been reported. The aim of this study was to determine whether alcohol abuse has effects on the first trimester trisomy screening parameters (NTT, PAPP-A and particularly on free β-hCG) and whether any of these could be used as a biomarker of alcohol use during early pregnancy.

6.3 MATERIALS AND METHODS

This is a retrospective cohort study of pregnant women participating in routine combined FTS for trisomy 21 in the Kuopio University Hospital area in Central Finland. The pregnancies, recorded during FTS in routine maternal care between June 2010 and June 2011, were searched from the medical database. The pregnancy and birth outcomes were evaluated, and 544 study samples were selected out of all pregnancies during that time in the Kuopio University Hospital region (approximately 2500 pregnancies in total) (Figure 8). A risk ratio <1:250 was considered normal. The mothers having increased risk ratios (including trisomies, fetal abnormalities, vanishing twins) were excluded from the control group to keep it as "normal" as possible for revealing the possible effect of alcohol and drug exposure on screening parameters

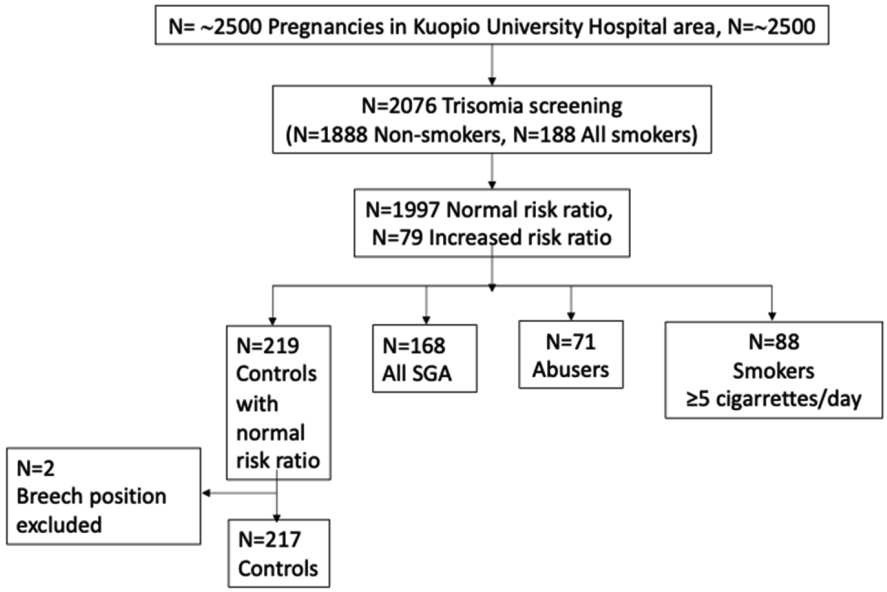

Figure 8. Flowchart showing the number of included and excluded subjects. The discrepancy in the number of the smokers in different boxes is explained by the definition of the final Smokers group (≥5 cigarettes/day). Control mothers having increased risk ratio were excluded. Every 8th mother was selected randomly out of all the criteria fulfilling non-smoking mothers to obtain an appropriate number of controls. Thirty-three (42.3%) out of the 71 Abusers were smoking (≥5 cigarettes/day) during pregnancy.

The final study group included 71 alcohol and possibly drug abusing mothers ("Abusers"), and for comparison all 88 Smokers (five or more cigarettes per day during pregnancy) having no history of alcohol or drug abuse and all 168 non-smoking (not alcohol or drug abusing) mothers having later given birth to an SGA child (defined here as birth weight below the 10th percentile for gestational age and) ("SGA mothers"). These comparison groups were included, since giving birth to an SGA baby and smoking are common among alcohol abusing mothers. We compared the study groups with 217 non-smoking control mothers having later delivered a normal-sized newborn (birth weight between the 10th and 90th

percentile) (“Controls”). The inclusion criteria for the Controls were singleton pregnancy, non-complicated vaginal birth (cephalic presentation), and normal outcome: the mother or the newborn did not require pre- or postnatal follow-up, care or interventions more than considered to be routine. The Controls were healthy women who did not have any other diagnosis at the time of the delivery than a spontaneous, normal parturition according to ICD-10 criteria. Every 8th mother was selected randomly out of all the criteria fulfilling non-smoking mothers to obtain an appropriate number of controls. All mothers were Caucasians by their ethnic background.

The alcohol and drug using pregnant women followed in the maternity clinic of the Kuopio University Hospital had been referred by general practitioners due to concerns aroused by alcohol or drug abuse. The alcohol Use Disorder Identification Test (AUDIT) (Aertgeerts et al., 2002; Saunders et al., 1993) was used to identify mothers with harmful patterns of alcohol consumption. The AUDIT questionnaire is a validated test used to determine if a person is at risk for alcohol abuse problems. The inclusion criteria for the Abusers were a total AUDIT score of eight or more, alcohol use during the ongoing pregnancy or any alcohol/drug abuse before or during the ongoing pregnancy. The previous risk users were defined as mothers having any previous use of IV drugs, rehabilitation due to drug abuse or long-term use of opioids, stimulants, or other drug abuse. Mothers who were Hepatitis C virus antibody positive, in drug or alcohol substitute treatment or committed themselves to drug and/or alcohol abstinence, and mothers who had quit all drug abuse when noting to be pregnant were also defined as previous risk users. After data and outcome collection we did not follow up the mothers or the children.

FTS was performed according to the recommendations of the Finnish ministry of Social Affairs and Health (Autti-Rämö, Koskinen, Mäkelä, Ritvanen, & Taipale, 2005). The screening parameters included PAPP-A and free β-hCG analyses from maternal serum, and fetal NTT measurements. Serum samples for PAPP-A and free β-hCG measurements were collected in maternity care units between gestational weeks 9+0 and 11+6. Blood samples were allowed to clot at room temperature for 30 min, centrifuged,

separated, and stored at +4 °C. Serum samples were delivered to the Eastern Finland Laboratory Centre in Kuopio refrigerated or frozen and stored at -20 °C. Serum concentrations of free β-hCG and PAPP-A were analyzed by time-resolved fluoroimmunoassay according to the manufacturer's instructions (PerkinElmer Life and Analytical Sciences, Wallac, Turku, Finland) and the routine first trimester aneuploidy screening protocol. The intra- and interassay coefficients of variation (CV) were <1.8 % and <3.7 % for PAPP-A, and <2.3 % and <4.1 % for free β-hCG, respectively. The CVs were determined in 20 aliquots of two serum pools analyzed in either the same or consecutive runs. The calibrators covered the ranges 10-2000 mU/L for PAPP-A and 2-200 ng/ml for free β-hCG. Serum samples were diluted 5-fold prior to the assay of PAPP-A.

The fetal NTT measurements were performed at healthcare centres or the Kuopio University Hospital Clinic by ultrasound-trained mid-wives and gynecologists between the gestational weeks 11+0 and 13+6. The crown rump length of the fetus obtained from the ultrasonography examination determined the gestational age. The concentrations of the serum markers and the fetal NTT measures were converted to multiples of medians (MoM). The total trisomy risk for trisomy 21, free β-hCG MoM, PAPP-A MoM and NTT MoM were calculated using LifeCycle software version 2.2 (PerkinElmer Life and Analytical Sciences). The correction factors for free β-hCG were maternal weight, diabetes, and smoking status, and for PAPP-A maternal weight and diabetes status. At the time of the analysis the smoking correction factor for free β-hCG was 0.82.

This study was approved by the Research Ethics Committee of Kuopio University Hospital. All study participants provided an informed written consent.

6.3.1 Statistical analyses

Data management and the statistical analyses were performed using SPSS 19 and 21 (SPSS Inc., Chicago, IL, USA). The continuous variables were tested with the independent samples t-test, if normally distributed. The study groups were compared with the Controls. PAPP-A, free β-hCG and NT showed normal distribution. The total trisomy risk showed normal

distribution after log10-transformation. The Chi-square test was used to analyze dichotomous variables. If there were fewer than five units in any of the classes, the Fischer´s exact test was used.

Stepwise multiple linear regression analysis was applied to explain free β-hCG and PAPP-A variation. P-value less than 0.05 was considered significant. The following factors were included in the analyses: confirmed maternal alcohol use, smoking, drug abuse, and giving birth to an SGA baby. In addition, a combination of 1) maternal alcohol use and smoking, and 2) maternal alcohol use and giving birth to an SGA baby were included in the regression analyses. The results of the regression analyses were shown as beta coefficients (B) and standardized coefficients (beta).

6.4 RESULTS

The main characteristics of the pregnancies, deliveries and newborns are depicted in Tables 4 and 5. The mean AUDIT score among the Abusers was 15.3 (SD 9.0) and smoking was highly prevalent (42.3%) in this group. The Abusers and Smokers were slightly younger, and the SGA mothers older than the Controls. The Smokers were somewhat heavier than the Controls prior to and at the beginning of the pregnancy. The SGA mothers and Abusers were more often nulliparous than the Controls. The SGA mothers were shorter and had more often arterial hypertension and preeclampsia than the Controls (Table 4).

Out of the 71 Abusers, 17 (24%) had confirmed use of alcohol, 25 (35%) used alcohol and/or drugs during the ongoing pregnancy, and 29 (41%) were previous risk users fulfilling the inclusion criteria. The mean duration of pregnancy was close to 280 days in all groups (Table 4). Altogether 16.9% of the Abusers gave birth to an SGA infant (Table 5). If alcohol exposure during the ongoing pregnancy was confirmed, an SGA birth was even more prevalent (6 out of 17 (35%)). Placental to birth weight ratio was highest in the Abusers (Table 5).

Serum free β-hCG and PAPP-A level comparisons among the Controls and the study groups showed two main findings. Firstly, significantly higher free β-hCG levels were found in the Abusers in comparison to the Controls.

Secondly, PAPP-A levels were significantly lower in the SGA mothers and Smokers compared with the Controls. Fetal NTT did not differ significantly between the groups (Table 6).

Table 4. Maternal and pregnancy characteristics of the study groups

	Controls (n=217)	Abusers (n=71)	*P*	Smokers (n=88)	*P*	SGA mothers (n=168)	*P*
Maternal age (years,mean ±SD)	29.1±4.5	25.6±6.1	<0.001	27.1±5.8	0.004	30.2±5.3	0.028
Maternal height (cm, mean ±SD)	165.4±5.4	165.2±4.7	0.803	164.1±5.8	0.053	163.4±5.8	0.001
Weight at the beginning of pregnancy (kg, mean ±SD)	64.2±11.4	66.7±14.0	0.140	70.7±16.2	0.001	66.4±37.5	0.423
BMI prior to pregnancy (kg/m^2, mean ±SD)	22.9±4.0	23.9±4.9	0.150	25.8±5.9	<0.001	24.5±15.4	0.162
Weight gain during pregnancy (kg, mean ±SD)	12.8±4.2	14.0±6.3	0.217	13.3±6.9	0.610	11.7±4.4	0.03
Nulliparous, n (%)	60 (27.6)	49 (69.0)	<0.001	29 (33.0)	0.356	97 (57.7)	<0.001
Arterial hypertension, n (%)	16 (7.4)	7 (9.9)	0.376	10 (11.4)	0.175	34 (20.2)	<0.001
Preeclampsia, n (%)	0 (0)	2 (2.8)	NA	6 (6.8)	NA	11 (6.5)	NA
Alcohol use before pregnancy, n (%)	90 (41.5)	38 (53.5)	0.081	41 (46.6)	0.432	76 (45.2)	0.452
Smoking during pregnancy (≥5 cigarrettes/day), n (%)	0 (0)	30 (42.3)	NA	88 (100)	NA	0 (0)	NA

Independent samples t-test was used for continuous variables and Chi-Square test for nominal values. If there were fewer than five units in any of the classes, the Fischer´s exact test was used. Sample size may vary owing to missing values. NA, not applicable, the parameter was excluded by definition from the Control group.

Table 5. Newborn characteristics in each study group

	Controls (n=217)	Abusers (n=71)	*P*	Smokers (n=88)	*P*	SGA mothers (n=168)	*P*
Male gender, n (%)	104 (47.9)	44 (62.0)	0.04	52 (59.1)	0.077	90 (53.6)	0.272
Birth weight (g, mean ±SD)	3540±370	3390±560	0.039	3310±550	0.001	2760±400	<0.001
SGA (birth weight <10 percentile), n (%)	0 (0)	12 (16.9)[a]	NA	16 (18.2)	NA	168 (100)	NA
Head circumpherence (cm, mean ±SD)	35.2±1.2	34.9±1.8	0.214	35.0±1.9	0.375	33.8±1.8	<0.001
Head circumpherence (<10th percentile), n (%)	9 (4.1)	14 (19.7)	<0.001	10 (11.4)	0.010	45 (26.8)	<0.001
Intensive care of the newborn, n (%)	0 (0)	15 (21.1)	NA	9 (10.2)	NA	13 (7.7)	NA
Placental weight (g, mean ±SD)	580±106	610±130	0.048	590±131	0.627	460±79	<0.001
Placental weight/birth weight ratio (%, mean ±SD)	16.3±2.5	18.1±2.9	<0.001	17.8±3.3	<0.001	16.8±3.0	0.079

Independent samples t-test was used for continuous variables and Chi-Square test for nominal values. If there were fewer than five units in any of the classes, the Fischer´s exact test was used. Sample size may vary owing to missing values. NA, not applicable, the parameter was excluded by definition from the Control group. [a]two of these drug abusers

Table 6. Free β-hCG, PAPP-A and NTT in the first trimester screening analysis

	Controls (n=217)			Abusers (n=71)					Smokers (n=88)					SGA mothers (n=168)				
	Median	Mean	SD	Median	Mean	SD	MD (95% CI)	P	Median	Mean	SD	MD (95% CI)	P	Median	Mean	SD	MD (95% CI)	P
β-hCG MoM	1.02	1.21	0.72	1.24	1.5	0.88	-0.29 (-0.50, -0.09)	0.013	1.18	1.45	1.06	-0.25 (-0.49, -0.01)	0.048	1.02	1.24	0.82	-0.03 (-0.18, 0.12)	0.700
PAPP-A MoM	1.04	1.20	0.76	0.90	1.14	0.78	0.06 (-0.15, 0.27)	0.197	0.83	0.90	0.47	0.31 (0.16, 0.45)	<0.001	0.90	1.08	0.64	0.12 (-0.02, 0.26)	0.043
NTT MoM	0.90	0.92	0.27	0.89	0.92	0.24	0.01 (-0.06, 0.08)	0.882	0.90	1.02	0.59	-0.09 (-0.19, 0.01)	0.145	0.91	0.98	0.38	-0.06 (-0.12, 0.01)	0.078

Independent samples t-test was used for the free β-hCG, PAPP-A and fetal NTT MoM values in comparison with the Controls. MD, mean difference; CI, 95% confidence interval of the difference; MoM, multiples of medians

Stepwise multiple linear regression analyses were performed separately for free β-hCG and PAPP-A levels to explain their variation. Both of these analyses were adjusted for confounding factors (listed in the Methods). Smoking remained the only explaining independent contributor (B=0.313, beta=0.15, P<0.001) to high free β-hCG level [F(1,539)=13.08, P<0.001, R2=0.024)]. Likewise, smoking (B=-0.21, beta=-0.12, P=0.005) and giving birth to an SGA baby (B=-0.17, beta=-0.12, P=0.007) were the only independent contributors to low PAPP-A levels [F(2,538)=6.38, P<0.05, R2=0.023].

Total trisomy risk was higher in the Abusers, Smokers and SGA mothers compared to the Controls (P=0.047, mean difference (MD) of the log10-transformed values -0.18, 95% CI (-0.35, -0.01), P=0.047; MD -0.28, 95%CI (-0.47, -0.09), P=0.001; MD -0.22, 95%CI (-0.38, -0.06), P=0.007, respectively).

6.5 DISCUSSION

In this study, we got two main findings. Firstly, we found increased free β-hCG levels in the Abusers. Secondly, we found decreased PAPP-A levels in the Smokers and SGA mothers. Fetal NTT did not differ between the groups.

Although our results suggested increased first trimester free β-hCG levels in women using alcohol, there was a significant overlap in free β-hCG levels between the alcohol-exposed and unexposed women. Forty-two % of the Abusers smoked during pregnancy and a multiple regression analysis revealed that smoking explained at least partly the increase in free β-hCG levels in the Abusers. This might be due to a stronger influence of smoking on free β-hCG levels than the correction factor in the FTS protocol predicted. Another possible factor explaining higher free β-hCG levels is that the placental size may have affected hCG production. The Abusers had higher placental weights than the Controls, which could partly explain increased free β-hCG levels. The increase in maternal serum free β-hCG levels in the Abusers could also be a direct effect caused by alcohol exposure, since ethanol treatment of trophoblast cells has been shown to increase hCG production *in vitro* (Joya et al., 2015; Karl & Fisher, 1993).

However, only a small difference and clear overlap between the Abusers and Controls, and the multiple regression analysis results indicated that the association of alcohol abuse with free β-hCG is weak and that free β-hCG cannot be used as a biomarker for alcohol use.

Fetal growth restriction is a classical feature of fetal alcohol syndrome and fetal alcohol effects (Astley, 2006), and the high percentage of SGA births among the Abusers in the present study (17%) is in line with literature (Dorrie, Focker, Freunscht, & Hebebrand, 2014; Kirkegaard, Henriksen, & Uldbjerg, 2011). Previous reports on the association between maternal serum free β-hCG levels and fetal growth restriction are conflicting. Fetal growth restriction has been associated with both decreased (Kirkegaard, Henriksen, Torring, & Uldbjerg, 2011; Kirkegaard et al., 2011) and elevated free β-hCG (Goetzinger et al., 2009). Nevertheless, in the present study, later SGA birth without alcohol or illicit drug exposure did not associate with altred free β-hCG levels.

Our findings of decreased PAPP-A levels in Smokers and SGA mothers are in line with previous studies. Both smoking and fetal growth restriction have previously been associated with decreased PAPP-A values, but the clinical utility of decreased PAPP-A to predict an increased risk to deliver an SGA baby is limited (Bestwick, Huttly, & Wald, 2008; Boucoiran et al., 2013; Dugoff et al., 2004; Krantz et al., 2004; Morris et al., 2008). However, regardless of the high prevalence of SGA births and smoking in the Abusers, serum PAPP-A levels of the Abusers were within the low normal range in this study.

The limitations of this study warrant explanation. The retrospective design of this study limits the consistency of the data, especially in the Abusers, where we rely on database information of the abused agents. The relatively small sample size is also a limitation in the study. On the other hand, the AUDIT questionnaire is excellent in identifying dependency, risk drinking, alcohol use disorder or risk drinking (Aertgeerts et al., 2002). The strengths of the study include the fact that the used serum samples were collected during routine first trimester hospital visits and therefore should represent the variation one would expect to see in clinical samples.

To our best knowledge, this is the first report on the association between alcohol use during pregnancy and increased FTS free β-hCG levels. Decreased PAPP-A among smokers and SGA mothers was in line with previous studies. The overlap between the study groups was remarkable and we conclude that free β-hCG is not applicable for the use as an alcohol biomarker.

6.6 ACKNOWLEDGEMENTS

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7 MATERNAL DRUG OR ALCOHOL ABUSE IS ASSOCIATED WITH DECREASED HEAD SIZE FROM MID-PREGNANCY TO CHILDHOOD

7.1 ABSTRACT

Background: Maternal alcohol abuse is poorly recognised and causes developmental problems. This study explored the fetal central nervous system (CNS), head circumference and psychomotor development of children exposed to drugs or alcohol during pregnancy up to 2.5 years of age.

Methods: We recruited 23 pregnant women referred to Kuopio University Hospital, Finland, by their family doctor because because of drug or alcohol abuse, and 22 control mothers. Fetal CNS parameters were measured by three-dimensional ultrasonography at the mean gestational age of 20 weeks and the Griffiths Mental Developmental Scales (GMDS), and anthropometric measurements were carried out at the mean ages of one and 2.5 years.

Results: The exposed fetuses had decreased biparietal and occipito-frontal distances and head circumferences, but unchanged cerebellar volume at 20 weeks, and decreased head circumferences and length and height at birth, one and 2.5 years of age. they scored lower than the controls on the GMDS general quotient and the hearing, language and locomotor subscales at 2.5 years of age.

Conclusions: Maternal alcohol or drug exposure was associated with decreased head size from mid-pregnancy to childhood and reduced development at 2.5 years. Fetal head circumference at mid-pregnancy was a useful indicator of substance abuse affecting the CNS.

7.2 KEY NOTES

- Fetal alcohol exposure causes numerous developmental problems. Many of them are poorly recognised.
- Fetal ultrasonography at 20 weeks of gestation revealed reduced biparietal and occipito-frontal distances and head circumference in alcohol-abusing mothers suggesting that these parameters are useful when looking for alcohol-mediated effects in mid-gestation.
- The exposed children had reduced height and head circumference but normal weight for height at the mean age of 2.5 years.

7.3 INTRODUCTION

Previous studies have shown that only a minority of pregnant women who abuse alcohol, drugs and medication are recognised by healthcare providers and receive the help they need (Aertgeerts et al., 2002; Pajulo, M., Savonlahti, Sourander, Helenius, & Piha, 2001; Sarkola, Kahila, Gissler, & Halmesmaki, 2007; Saunders et al., 1993). The prevalence of alcohol and drug consumption during pregnancy has also been shown to vary in different countries (U. S. Department of health and human services, 2012).

Fetal alcohol spectrum disorders (FASD) is the umbrella term for all fetal alcohol effects (Hoyme et al., 2005). The children at the severe end of this spectrum – the complete phenotype – have been defined as having fetal alcohol syndrome (FAS). FASD also includes partial fetal alcohol syndrome, alcohol-related birth defects and alcohol-related neuro- developmental disorder. FAS is the leading identifiable, nonhereditary cause of mental retardation in the Western world (National Center on Birth Defects and Developmental Disabilities Centers for Disease Control and Prevention Department of Health and Human Services in coordination with National Task Force on Fetal Alcohol Syndrome and Fetal Alcohol Effect American Academy of Pediatrics American College of Obstetricians and Gynecologists March of Dimes National Organization on Fetal Alcohol Syndrome, 2004). Although a diagnosis of FAS can be made on the basis of physical features, most of the children exposed prenatally to alcohol do not show these physical markers (Sampson et al., 1997).

True to form, the morphological effects of alcohol on the fetal central nervous system (CNS) vary from undetectable changes to difficult malformations. The morphological changes detected in children and adults after alcohol exposure during fetal life include microcephaly and cortical atrophy, cerebellar hypoplasia and dysmorphism of the corpus callosum, together with other mid-line structural abnormalities (Autti-Ramo et al., 2002; Donald et al., 2015). Furthermore, prenatal smoking exposure has been reported to be associated with a smaller frontal lobe and reduced cerebellar volumes in preterm infants (Ekblad et al., 2010). Only a few researchers have used fetal ultrasonography in studies of alcohol or drug-exposed foetuses. Kfir et al. (Kfir et al., 2009) demonstrated shorter caval–calvarial and fronto-thalamic distances during the second trimester and shorter fronto-thalamic and biparietal dis- tances during the third trimester in alcohol-exposed foe- tuses compared to nonexposed controls. Handmaker et al. (Handmaker et al., 2006) demonstrated a smaller transcerebellar diameter and a lower head-to-abdominal circumference ratio in alcohol- exposed foetuses at the mean gestational age of 27.3 weeks (range 18.0–41.7 weeks). Wass et al. (Wass, Persutte, & Hobbins, 2001) suggested a connection between the reduction in the fetal frontal brain size and alcohol exposure during pregnancy. In that study, the ultrasonographical examination time varied from 12 to 42 weeks of gestation. Exposure to illegal drugs has also been associated with physical birth defects and increased risk of regulatory and neuropsychological difficulties (Irner, Teasdale, & Olofsson, 2012; Moe, 2002).

Three-dimensional (3D) ultrasonography was introduced at the beginning of 1990s, and it has opened a new way to measure organ volumes quite accurately (Hata et al., 2000) . However, the potential of 3D ultrasonography to detect harmful consequences of alcohol abuse in CNS is unclear. At the same time, early identification of at-risk pregnancies is a strong protective factor for reducing harm from fetal alcohol exposure.

Prenatal alcohol exposure leads to a wide range of problems, and we lack reliable tools to detect early alcohol effects. Our aims were to investigate whether previously reported CNS changes associated with prenatal alcohol exposure were already visible at mid-pregnancy and to

evaluate head size, length and height and psychomotor development of exposed children up to the age of 2.5 years.

7.4 PATIENTS AND METHODS

The ultrasound measurements were performed as a part of a longitudinal prospective study conducted in 2005–2008 in the maternity clinic at Kuopio University Hospital, Finland, and the primary healthcare centre in the city of Kuopio. The study protocol was approved by the Research Ethics Committee of Kuopio University Hospital. All study participants provided informed, written consent.

7.4.1 Patients

Subjects were recruited from patients at the maternity clinic at Kuopio University Hospital (Table 7). The 23 pregnant women followed up in the maternity clinic had been referred to the clinic by their general practitioners due to concerns about alcohol or drug abuse. The Alcohol Use Disorder Identification Test (AUDIT) (Saunders et al., 1993) was used to identify women with harmful patterns of alcohol consump- tion. The inclusion criteria were total AUDIT scores of eight or more, alcohol use during the ongoing pregnancy or any drug abuse before or during pregnancy. The 22 control subjects were recruited from two maternity clinics of the Kuopio city primary healthcare centre. The inclusion criteria for the controls were non-smoking, healthy, an AUDIT score of less than eight before the pregnancy and no alcohol consumption during the ongoing pregnancy. All participants were Finnish Caucasians.

Table 7. Characteristics of the alcohol abusing and control mothers and their pregnancies

	Abusers n=23		Controls n=22		
	Mean	SD	Mean	SD	*P*
Number of previous pregnancies	1.3	0.58	1.3	0.79	0.867
Number of previous abortions	0.7	1.15	0.3	0.60	0.315
BMI before pregnancy (kg/m²)	22.9	2.17	22.8	4.54	0.575
Duration of gestation at the time of ultrasound (weeks)	19.5	0.63	20.3	0.89	**0.04***
Age at delivery (years)	25.0	5.02	29.7	3.09	**<0.01***
Duration of pregnancy at birth (weeks)	39.4	1.57	39.1	1.49	0.761

Mann-Whitney test was used to test differences between the groups. * Statistically significant difference, P<0.05

7.4.2 Ultrasound measurements

3D ultrasound images of the intracranial contents were obtained using a GE Voluson 730 Expert with a 4.0– 8.5-MHz RAB4-8L abdominal probe (General Electric Healthcare, London, UK) as part of a structural ultrasound examination at the gestational age of 18 + 3 to 21 + 6 weeks. The scans were performed by an experienced fetal medicine specialist (M-RO). All 3D volumes were saved with 4D View software version 6.2 (General Electric Healthcare) for offline examination. At least two volumes were collected for each evaluation from the axial 2D view at the transventricular level. The 3D volumes presenting clearer outlines of the cerebellum were selected for cere- bellar calculations. Virtual organ computer-aided analysis was used to measure the total cerebellar volume. The saved ultrasonographical data were analysed by two authors of this study (M-RO and AL).

To achieve a consistent orientation, the following adjustments were performed. In the coronal view, both hemi- spheres were set symmetrically across the perpendicular line. In the sagittal view, the cerebellum and the nasal bone were set at approximately the same horizontal line. In the transversal view, the hemispheres were set symmetrically across the axis going along the falx cerebri. The correct sagittal position was secured from the transversal view, where the thalamus and cavum septi pellucidi were

seen in the same section. This section was also the level for the measurements of the biparietal and occipitofrontal distances and the head circumference.

After the orientation correction, the transversal section at the level of mid-cerebellum was selected for the cerebellar volume and width measurements. After obtaining the ideal plane, the cerebellum was outlined for its external surface manually with the rotation angle of six degrees. By the end of the rotational process, the programme calculated the volume automatically and provided the reconstructed 3D image of the organ.

The maximal length of corpus callosum was obtained after the described orientation from the sagittal view, with the slice thickness set at 0.5 mm, and the volume contrast imaging switched on to optimise the visualisation.

7.4.3 Follow-up of the children

Midwives and registered nurses assessed the growth measures at birth. The children0s psychomotor development was assessed using the Griffiths Mental Develop- mental Scales (GMDS) 1996 Revision (Huntley, 1996) at the age of one year and the GMDS-Extended Revised (Luiz et al., 2006) at the age of 2.5 years. GMDS yields a general quotient of overall development. Standardised developmental sub- quotients were also created. The developmental domains that were tested were locomotor (gross motor), personal– social (self-care and social interaction), hearing and language, eye–hand coordination (fine motor) and performance (cognition, symbolic play and puzzles) at the age of one year. Depending on the child's skills, practical reasoning was also tested at the average age of 2.5 years (18). Growth measurements were performed at the mean ages of one and 2.5 years by one of the authors (AL). Sex- and age-specific standard deviation (SD) scores for length and height, birthweight and head circumference were calculated according to the Finnish growth reference (Karvonen, Hannila, Saari, & Dunkel, 2012; Saari et al., 2011). In addition, weight-for-length and height were determined at the mean ages of one and 2.5 years (Saari et al., 2011).

7.4.4 Data management and statistics

Data management and the statistical analyses were per- formed using SPSS 19 and 21 (SPSS Inc, Chicago, Illinois, USA). For all analyses, a p value of less than 0.05 was considered significant. To test differences between the study groups, univariate analysis by ANOVA was used for the ultrasonographical CNS parameters and the Mann–Whit- ney test for the GMDS and demographic parameters. The chi-squared test was used for dichotomous variables. The one-sample Wilcoxon signed rank test was used to assess the difference of the exposed children's head circumference, length and height (SD score) and weight (SD score at birth, weight-for-length and height percentage at the mean ages of one and 2.5 years) from the respective median values (SD score 0, 100% for weight-for-length and height) of the Finnish growth reference (Karvonen et al., 2012; Saari et al., 2011) .

7.5 RESULTS

7.5.1 Fetal ultrasound measurements

Only 11 subjects and 21 controls agreed to participate in the ultrasonography analyses at mid-pregnancy. Confirmed alcohol exposure was found in 64% and drug exposure in 82% of the subjects during the on-going pregnancy. The low quality of the ultrasonography images caused the exclusion of one subject and one control. Dichorionic twins were found in one subject. The head circumferences and the biparietal and occipito-frontal distances of the foetuses were significantly smaller in the exposed women than in the controls, when adjusted for gestational age and maternal smoking status. No significant difference in the corpus callosum or cerebellar measures was detected (Table 8).

Table 8. Fetal head and brain ultrasonographical measures of the study groups at mid-pregnancy

	Exposed fetuses (n=11)		Controls (n=20)		
Parameter	Mean	SD	Mean	SD	P
Biparietal distance (cm)	4.37	0.43	4.67	0.32	**0.03***
Occipitofrontal distance (cm)	6.04	0.40	6.37	0.39	**0.049***
Frontothalamic distance (cm)	3.08	0.34	3.40	0.27	0.62
Caval-calvarial distance (cm)	2.07	0.29	2.19	0.31	0.50
Head circumference (cm)	16.83	1.12	17.93	1.05	**0.04***
Head area (cm2)	22.24	2.88	25.35	3.13	0.10
Corpus callosum length (cm)	2.68	0.44	2.72	0.57	0.71
Corpus callosum height (cm)	1.10	0.12	1.25	0.19	0.75
Cerebellar volume (cm3)	0.84	0.17	1.08	0.28	0.56
Cerebellar width (cm)	1.79	0.13	1.88	0.15	0.81
Vermis width (cm)	0.67	0.10	0.72	0.10	0.98
Vermis height (cm)	1.10	0.12	1.25	0.19	0.44
Vermis depth (cm)	0.90	0.13	0.96	0.25	0.62

Univariate analysis by ANOVA was used to test differences between the groups.

P-values are adjusted for gestational age (GA) and smoking.

*Statistically significant difference, $P < 0.05$

7.5.2 Follow-up evaluation

The demographics of the mothers are given in Table 7. We carried out a follow-up evaluation of the children's growth characteristics and psychomotor development. At birth, the head circumference, length and weight were significantly smaller in the exposed children than the controls and the Finnish growth reference. At the mean ages of one and 2.5 years, head circumference and length and height remained smaller, but weight-for-length and height did not differ from the national reference. The head circumference to length–height ratio did not differ between the study groups at any age (Table 9).

The GMDS scores did not differ significantly between the exposed and controls at the mean age of one year. Although the exposed children scored lower than the controls in the general quotient and in the subscales of hearing and language and locomotor at the mean age of 2.5 years, their mean scores were close to the 50th percentile of the standard with a remarkable individual variation (Table 10).

Table 9. Anthropometric characteristics of the alcohol-exposed and control children

	Exposed (n=24)		**Controls (n=22)**		
	Mean	SD	Mean	SD	*P*
At birth					
Length (cm)	48.8	2.75	50.0	1.70	**0.01***
Length (SD scores)	-1.3	1.59	-0.1	0.88	**<0.01*/<0.01***
Weight (g)	3070	489.50	3490	446.70	**<0.01***
Weight (SD scores)	-1.2	0.84	-0.1	0.99	**<0.01*/<0.01***
Head circumference (cm)	34.2	1.54	34.9	1.48	**0.02***
Head circumference (SD scores)	-0.8	0.91	0.3	1.03	**0.01*/<0.01***
Head circumference/length (%)	70.1	1.04	69.8	3.11	0.74
Placental weight (% of birth weight)[o]	17.0	1.93	15.8	2.27	0.05
At the mean age of 1 year (n=23/19)					
Length (cm)	76.2	2.70	76.9	3.07	0.20
Length (SD scores)	-0.5	0.71	-0.1	1.28	0.14/**<0.01***
Weight (kg)	9.8	1.09	10.2	1.33	0.31
Weight for length (%) [y]	99.1	7.55	101.2	7.97	0.69/0.42
Head circumference (cm)	46.3	1.05	47.1	1.24	0.06
Head circumference (SD scores)	-0.8	0.89	-0.2	0.89	0.20/**<0.01***
Head circumference/length (%)	60.8	2.32	61.3	2.59	0.85
At the mean age of 2½ years (n=19/18)					
Height (cm)	91.3	2.42	91.6	3.68	0.93
Height (SD scores)	-0.7	0.66	-0.4	1.00	0.32/**<0.01***
Weight (kg)	13.7	1.60	13.9	2.06	0.53
Weight for height (%) [y]	102	8.79	103	8.43	0.82/0.53
Head circumference (cm)	49.0	1.00	49.6	1.57	0.19
Head circumference (SD scores)	-0.8	0.82	-0.6	0.99	0.35/**<0.01***
Head circumference/height (%)	53.7	1.70	54.2	2.10	0.39

[o] The pair of twins excluded; [y] Percent of the median weight for length/height.

The Mann-Whitney test was used to test differences between the study groups (the left-sided P-values) and the one sample Wilcoxon signed rank test to assess differences between the exposed children and the Finnish growth reference (the right-sided P-values). * Statistically significant difference, P<0.05

Table 10. Psychomotor development of the alcohol-exposed and control children evaluated by the Griffiths developmental scales

	Exposed children (n=19)		Control children (n=19)		
	Mean	SD	Mean	SD	*P*
At the mean age of 1 year					
Locomotor (subquotient)	101.1	10.0	93.8	15.1	0.20
Personal-social (subquotient)	100.3	15.3	96.7	16.5	0.54
Hearing and language (subquotient)	98.7	17.8	93.6	9.8	0.58
Eye and hand (subquotient)	100.3	19.9	97.7	15.9	0.52
Performance (subquotient)	105.5	13.9	106.1	21.4	0.70
Total subquotient	101.5	10.9	97.8	13.1	0.42
At the mean age of 2½ years (n=19/16)					
Locomotor (percentile)	51.6	31.8	76.9	25.9	**0.04***
Personal-social (percentile)	62.6	29.1	67.9	25.9	0.50
Hearing and language (percentile)	41.2	30.8	66.2	34.3	**0.02***
Eye and hand (percentile)	48.0	28.8	56.3	26.5	0.45
Performance (percentile)	41.5	29.3	54.1	29.2	0.16
Practical reasoning (percentile)	54.0	24.5	66.6	27.0	0.12
General quotient (percentile)	49.1	28.3	69.2	30.9	**0.03***

Mann-Whitney test was used to test differences between the groups

*Statistically significant difference, $P < 0.05$

7.6 DISCUSSION

In our study, alcohol exposure during pregnancy was already associated with decreased fetal head circumference at mid-pregnancy, but cerebellar changes were not detected. Follow-up of the exposed children showed that their head circumference and height were still found to be decreased at up to 2.5 years of age when compared with the Finnish growth reference. Although the exposed children scored lower than the controls in the general quotient and in the subscales of hearing and language and locomotion at the mean age of 2.5 years, their mean scores were close to the 50th percentile of the standard.

Our results were in line with previous studies showing that prenatal alcohol exposure was associated with decreased head circumference at birth (Carter, Jacobson, Sokol, Avison, & Jacobson, 2013; Ortega-Garcia et al., 2012). Head circumference was shown to be a good predictor of brain volume in FASD (Treit, Zhou, Andrew, Chudley, & Beaulieu, 2015). However, our study is apparently the first to show that decreased head circumference was already detectable at mid-pregnancy in alcohol-exposed foetuses. In addition to smaller head circumferences, the exposed foetuses had smaller biparietal and occipito-frontal diame- ters than the nonexposed ones at the mean gestational age of 20 weeks. To our knowledge, 3D ultrasonographically measured cerebellar volumes have not previously been reported in alcohol or drug-exposed foetuses. We did not find changes in fetal cerebellar volumes at the mean gestational age of 20 weeks, although cerebellar changes are common in alcohol-exposed children who have problems at school (Autti-Ramo et al., 2002).

Given the pervasive nature of FASD, efforts have been made to identify a typical neurocognitive profile of prenatally alcohol-exposed children. Children with FAS suffer from widespread cognitive impairment, and prenatally alcohol-exposed individuals demonstrate impairments in multiple neurocognitive domains, with little evidence of a specific profile (Davies et al., 2011; Ervalahti et al., 2007; Irner et al., 2012; Quattlebaum & O'Connor, 2013). It is evident that the development of neurocognitive problems becomes detectable over time, usually at school age, although the profile varies. Even though we were not able to show an association between reduced fetal head circumference and developmental problems in infancy, we found that decreased head circumference was a risk factor for intellectual and behavioral problems. Stoler-Poria et al. (Stoler-Poria, Lev, Schweiger, Lerman-Sagie, & Malinger, 2010) showed more behavioral problems in two- to six-year-old children who were diagnosed with microcephaly in utero than in control children.

The limitations of our study warrant explanations. Commitment to the follow-up study was an obvious limiting factor for the participation in the study, and therefore, the sample size remained regrettably small. Pregnant mothers encounter confusing feelings due to their pregnancy, and guilt

about alcohol or drug abuse is evidently present. There were also alcohol abusers who were referred to the hospital at a later stage of pregnancy and could therefore not participate in the second-trimester ultrasonography. The reluctance of pregnant mothers to report prenatal drinking is a general challenge, and therefore, estimations of the degree of exposure may vary considerably (Manich et al., 2012). The mixed use of different substances has become a part of everyday life. In this study, we could not differentiate the potential synergism of alcohol and drugs or the effects of different drugs among the abusers. Adjustment for smoking was carried out for the fetal parameters, but not for the postnatal parameters. The majority of the foetuses were also exposed to smoking, which hampers head and body growth during pregnancy (Salihu & Wilson, 2007).

The methodological issues also caused limitations. GMDS has been shown to be a useful tool as a general indicator of subsequent development, but it only has a limited value as a predictor of hearing and speech in children below two years of age (Sutcliffe, Soo, & Barnes, 2010). Normal scores in the early years cannot preclude later neurological or cognitive problems. In addition, the investigators in this study could not be blinded for the alcohol or drug abuse of the mothers.

7.7 CONCLUSION

In summary, we found that a decrease in head circumference and occipito-frontal and biparietal distances could already be seen in alcohol or drug-exposed foetuses at the gestational age of 20 weeks. Cerebellar volume changes were not detected at this stage of pregnancy. The head circumferences and height of the exposed children remained reduced compared to the population reference at 2.5 years of age, while the weight-for-height and the head circumference to height ratio was normal. Early detection of fetal CNS changes in alcohol-exposed foetuses could be used to motivate maternal abstinence from alcohol and to reduce further detrimental alcohol-induced consequences.

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7.10 CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

8 DEEP SEROTONERGIC AND DOPAMINERGIC STRUCTURES IN FETAL ALCOHOL SYNDROME: A STUDY WITH NOR-BETA-CIT-SINGLE-PHOTON EMISSION COMPUTED 8 TOMOGRAPHY AND MAGNETIC RESONANCE IMAGING VOLUMETRY

8.1 ABSTRACT

Background: In prenatally alcohol exposed children, the relationship between brain structure and function is highlighted to be important to study.

Methods: We studied 12 children with fetal alcohol syndrome (FAS) and fetal alcohol effects (FAE) by magnetic resonance imaging volumetry and by single-photon emission computed tomography with iodine-123 labeled 2beta-carbomethoxy-3beta-(4-iodophenyl) ([123I]nor-beta-CIT) and related these findings to those from neuropsychological and psychiatric tests.

Results: The absolute volumes of studied nuclei, including the brain volume, were significantly smaller in FAS/FAE children than in control patients. After normalization of volumes, significant differences were not found. Left hippocampus was smaller than the right ($p<.003$) but did not significantly differ from the control subjects. The children with FAS/FAE showed reduced serotonin ($p=.02$) in the medial frontal cortex and slightly increased striatal dopamine transporter binding. All FAS/FAE children had attention-deficit/hyperkinetic disorder (ADHD). None had depression. The internalization scores correlated with dopamine transporter binding ($r=-.65$; $p=.03$).

Conclusions: The results indicate that the serotonin (5-HT) system may be vulnerable to the effects of ethanol in utero. The high dopamine

transporter levels may correlate with the ADHD findings. Reduced serotonin and increased binding of dopamine transporter are also seen in type 2 alcoholism. Some behavioral problems of FAS/FAE might be preventable by early intervention and treatment.

8.2 INTRODUCTION

Alcohol exposure during fetal life may result in a number of disturbances ranging from growth retardation to behavioral abnormalities later in life. The developing brain is particularly susceptible to prenatal exposure to alcohol. Those affected may present mental and motor retardation, hyperactivity, and poor attention span.

Brain imaging with magnetic resonance imaging (MRI) has shown a spectrum of anomalies associated with fetal alcohol syndrome (FAS)/fetal alcohol effects (FAE), including reduced brain volumes and reduced basal ganglia (Mattson et al., 1996; Sowell et al., 2001; Sowell, Thompson et al., 2001). Several studies have also reported hypoplastic or agenetic corpus callosum, hypoplastic cerebelum, and reduction of the basal ganglia (Autti-Ramo et al., 2002; Johnson et al., 1996; Mattson et al., 1996; Riikonen et al., 1999; Roebuck, Mattson, & Riley, 1998; Sowell et al., 2001). Swayze et al. (Swayze et al., 1997) showed a high frequency of midline anomalies. The wide spectrum of anomalies is in agreement with the fact that since the exposure during pregnancy is diverse and the timing of exposure during brain morphogenesis is likely to be crucial, the phenotype is likewise a very heterogeneous one. The exact mechanism by which these effects of alcohol are produced is still unclear.

Animal experiments suggest ethanol exposure in utero may alter serotonin (5-hydroxy-tryptophan [5-HT]) neurotransmission in discrete brain regions permanently (Zafar, Shelat, Redei, & Tejani-Butt, 2000). Prenatal ethanol exposure has also been demonstrated to reduce dopamine (DA) neurotransmission in the rat midbrain (Shen, Hannigan, & Kapatos, 1999). Prenatal alcohol exposure can alter permanently neurotransmitters in mature central nervous system. This might play an important role in affective and motivated behavior.

Single-photon emission computed tomography (SPECT) enables us to investigate both dopamine and serotonergic neurotransmission in vivo. When 2ß-carbomethoxy-3ß-(4- iodophenyl) (nor-ß-CIT) is used as a tracer (labelled with ^{123}I), it binds specifically, but not selectively, to 5-HT and DA transporters (SERT and DAT) and has a high rate of specific versus nonspecific binding. Nor-ß-CIT has the highest binding rate to DAT and is therefore ideal for investigation of striatal dopamine system.

The aim of this study was to 1) study by dynamic SPECT the dopamine and serotonin metabolism in children prenatally exposed to alcohol and correlate the findings with behavioral symptoms; 2) study by MRI the absolute and relative brain volumes; and 3) correlate brain structure and function.

8.3 MATERIALS AND METHODS

Twelve children included in this study were admitted to the Kuopio University Hospital for neurological or neuropsychological investigations, usually because of learning disabilities. All were confirmed cases of alcohol exposure. This required either direct admittance of the mother, positive breath test during delivery, or direct observation of the mother's alcohol consumption during pregnancy by a third party (obstetric health care or social service workers or the father). All of the children were in foster care or in an adoptive home.

Diagnosis of FAS was given only when all of the following three criteria were met: 1) either prenatal or postnatal growth retardation could be confirmed from patient records, 2) perma nent central nervous system (CNS) involvement could be demonstrated; and 3) two or more characteristic physical anomalies could be found. Growth criteria were met when either length or weight was below -2 SD or -10%, respectively, or the occipitofrontal head circumference (HC) was below -2 SD. Permanent CNS involvement meant in this study that either mental retardation or significant learning disabilities could be demonstrated. Characteristic physical anomalies were the following: microcephaly (HC <-2 SD), short palpebral fissure, hypoplastic philtrum, and shallow upper lip and/or

flattening of the maxillary area. When only two of the three criteria were met, a diagnosis of FAE was given. Ten patients had a normal MRI. Subject 7 had periventricular atrophy due to neonatal asphyxia (birth weight 830 g, Apgar scores 3, 4, and 5 at 2, 5, and 10 minutes). Subject 10 had hypoplasia of corpus callosum. The diagnostic evaluation of the FAE/FAS cases is shown in Table 11.

Table 11. Clinical Cahracteristics of the Patientes with FAS/FAE

Subject	Age (Years)	Gender	Length (SD)	Weight (%)	HC (SD)	IQ	Handedness	Diagnosis
S1	5	M	1	−.5	−1	100	Left	FAE
S2	6	F	−2	−17	−1	84	Right	FAE
S3	8	M	−2	−15	−2.5	100	Right	FAS
S4	8	F	−3	−20	−1.5	72	Right	FAS
S5	9	M	−.6	−9	−2.8	97	Left	FAS
S6	11	M	0	64	−2	53	Right	FAS
S7	12	F	−1	−10	−3	56	Right	FAS
S8	12	F	−3	−25	−1	89	Left	FAS
S9	12	F	−1.5	0	−4	83	Right	FAS
S10	13	F	−1	−10	−2	100	Right	FAS
S11	14	M	−3	−20	−2.5	39	Left	FAS
S12	16	F	−3	−15	0	41	Right	FAS

All patients had ADHD. Abbreviations: FAS: fetal alcohol syndrome; FAE: fetal alcohol effects; SD: standard deviation; M: male; F: female; IQ: intelligence quotient, total score in Wechsler Intelligence Scale for Children; HC: head circumference; ADHD: attention/deficit hyperkinetic disorder.

8.3.1 Control subjects

Because of ethical reasons, we could not do any MRI or SPECT studies for normal children. For MRI volumetry, the sex- and age-matched control subjects had the following diagnoses: cephalalgia (*n* = 4), unknown motoric disturbance (*n* = 1), visual symptoms (*n* = 1), tremor (*n* = 2), convulsions (*n* = 1), and tiredness (*n* = 1) The control subjects were retrospectively verified not to have been prenatally exposed to heavy alcohol and had normal growth measurements, normal facial appearance, and no difficulties at

school. All control children had normal MRI findings. For dynamic SPECT, there were 10 age-matched control patients. The patients had the following diagnoses: tremor and cephalgia (*n* = 1), tremor (*n* = 3), long-lasting tiredness (*n* = 1), epilepsy (*n* = 1), cephalgia (*n* = 2), unexplained feeling of odd being (*n* = 1), and motor disturbance (*n* = 1).
The SPECT of control patients was done to evaluate the DAT and SERT function of deep brain structures in the patients, where all careful investigations failed to give any etiology. None of these patients received any medication affecting serotonin or dopamine metabolism during the SPECT study or for the 2 months preceding it. Most control patients were investigated by MRI for etiology before SPECT study, and all MRI findings were normal. Because of achieving system, only four control patients' images were available for volumetric study (Tables 12 and 13).

Table 12. Clinical Data of the Control Subjects for MRI Volumetric Study

Subject	Age (Years)	Gender	Length (SD)	Weight (%)	HC (SD)	Handedness	Diagnosis
C1	7	M	−1.5	3	−1.5	Left	Tremor
C2	7	F	.5	30	1	Right	Tiredness
C3	10	M	.5	20	0	Left	Cephalgia
C4	10	F	1	0	0	Right	Tremor
C5	11	M	1	0	0	Right	Convulsiones
C6	12	F	0	80	0	Right	Tremor
C7	12	F	1	0	0	Right	Cephalgia
C8	12	F	0	.5	0	Left	Motor disorder, Klippel-Feil syndrome
C9	13	F	1	0	0	Right	Visual symptoms
C10	14	M	1	20	0	Right	Visual symptoms

Underlined subjects served also as control subjects for SPECT. Abbreviations: MRI: magnetic resonance imaging; HD: head circumference; SD: standard deviation; M: male; F: female; SPECT: single-photon emission computed tomography.

Table13. Clinical Data of the Control Subjects for SPECT Study

Subject	Age (Years)	Gender	Length (SD)	Weight (%)	HC (SD)	Handedness	CDI Score	Diagnosis
C1	7	M	− 1.5	3	− 1.5	Left	1	Tremor
C2	7	M	− 1	− 5	− 1	Right	4	Tremor
C3	7	F	.5	30	1	Right	4	Tiredness
C4	7	M	2	5	4	Right	0	Tremor
C5	10	F	.5	30	3	Right	12	Vertigo
C6	10	F	1	0	0	Right	2	Tremor
C7	12	M	− 1	10	− .5	Right	4	Cephalgia
C8	12	F	− 1	0	− 1	Right	2	Absence attacks
C9	12	F	0	.5	0	Left	0	Motor disorder, Klippel-Feil syndrome
C10	14	M	0	0	1	Right		Cephalgia

Children's Depression Inventory (CDI, Kovacs 1992).SPECT, single-photon emission computed tomography; SD, standard deviation; HC, head circumference; CDI, Children's Depression Inventory; M, male; F,female.

8.3.2 Neuropsychological and psychiatric assessment

The Wechsler Intelligence Scale for Children-III (WISC-III) was used as a measure of psychometric intelligence (Wechsler, 1995; Wechsler, 1999). For assessment of attention and executive functions, subtests from Neuropsychological Investigation for Children (NEPSY) (Korkman, Kirk, & Kemp, 1998) were used. Naming skills were assessed by the Boston Naming Test (Kaplan, Goodglass, Weintraub, & Segal, 1997). The Cognitive Assessment System (CAS) (Basic Battery) was used to assess information processing skills (Naglieri & Das, 1997). The behavior of the children was assessed by using standardized questionnaires. Two scales were used: The Fetal Alcohol Behavior Scale (FABS) (Streissguth, Bookstein, Barr, Press, & Sampson, 1998) and the Child Behavior Checklist (CBCL) (Achenbach, 1991). The FABS, which was filled in by the guardians, is a 36-item questionnaire used as a diagnostic aid in FAS. Twelve or more points indicate behavior typical for subjects with FAS. The CBCL was filled in by the guardians and used to screen the children for psychiatric prob- lems. The CBCL consists of 118 items concerning behavioral/ emotional problems, is widely used in screening for childhood psychiatric symptoms and disorders, and has good reliability and discriminative validity. The children themselves filled in the Children's Depression Inventory (CDI) (Kovacs, 1992), developed to uncover depression in childhood. The CDI consists of 27 items rated on a scale of 0 to 2. If the child concerned had any difficulties in reading, the nurse was asked to read the items aloud. The protocol of the study aimed to find especially depressive disorders among the participants. All children who scored nine points or more on the CDI or six or more points on the depression/anxiety scale of the Child Behavior Checklist- Parent Form (CBCL-P) were interviewed using Kiddie-Sads Interview Schedule (Chambers et al., 1985) to assess current and past depressive episodes.

8.3.3 MRI Image acquisition and analysis

The cases and the control subjects underwent MRI scanning on a Siemens Vision 1.5 Tesla scanner (Siemens Medical Systems Inc., Erlangen, Germany). Three-dimensional T1-weighted ana- tomical images were obtained using the Magnetisation-Prepared Rapid Gradient Echo (MPRAGE) sequence. The oblique coronal slices (thickness 2.0 mm) included the whole cerebrum. Standard neuroanatomical landmarks (such as the line from anterior to posterior commisure, hemispheres, and orbits) were used to reconstruct slices (thickness 2 mm) perpendicular to the long axis of the left hippocampus. Blind assessment and confidentiality were maintained by using subject numbers only.

We used EasyMeasure software (Magnetic Resonance and Image Analysis and Research Centre, University of Liverpool, United Kingdom) for volume estimation of the hippocampus, the amygdala, the nucleus caudatus, and the putamen. This point- counting method consists of overlaying a systematic array of the test points (one point per nine pixels) completely over each slice (in random overstation) (Figures 9 and 10). The instances in which a point is within the nucleus are recorded by clicking a computer mouse (Light, Roberts, Whitehouse, & Edwards, 1995; Roberts, Puddephat, & McNulty, 2000). The total number of the test points is then multiplied by the volume of the test point (17.17 mm3). Boundaries of these nuclei were identified through the use of the neuroanatomy atlas (Martin, 1996) and previously published research (Watson et al., 1992). The intraclass correlation coefficients for intrarater reliability were .94 for the amygdala and .88 for the hippocampus measured from 10 patients; both left and right sides were measured.

The coronal intracranial area was measured at the level of the anterior commissure (the reference slice). This intracranial area is known to give a reliable correlation to the whole brain volume (Pennanen et al., 2004). To remove the influence of the head size to the volumes of nuclei, the volumes were normalized by dividing the volume of the nucleus with the intracranial volume of the reference slice (Laakso et al., 1998) using the formula:

(volume of the structure/intracranial volume in reference slice) x 100.

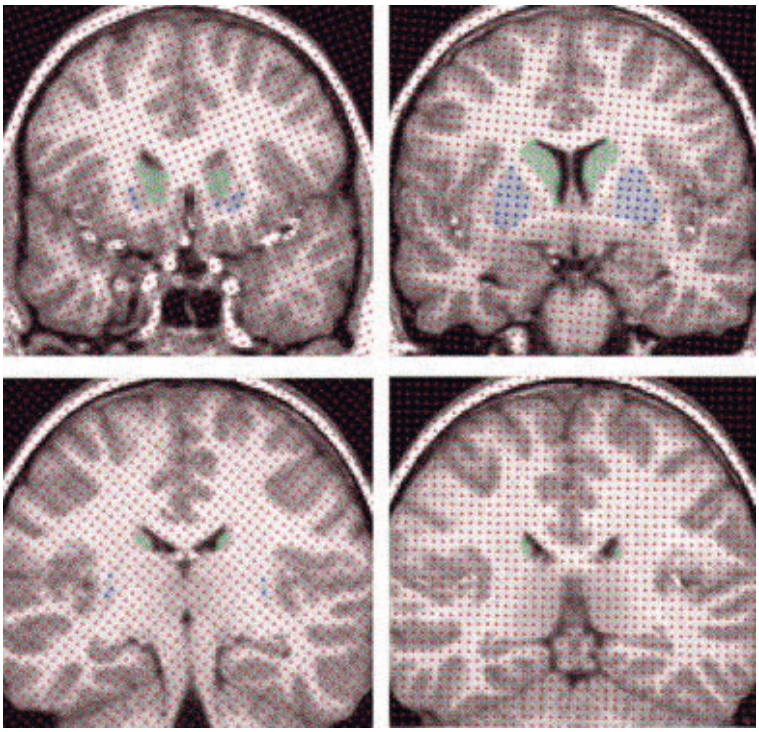

Figure 9. Images from volumetric software, EasyMeasure. This point-counting method consists of overlaying a systematic array of the test points: putamen (blue) and caudatus (green) defined from anterior to posterior. The most anterior slice including both putamen and caudatus is seen in upper left panel. The most posterior slice including putamen is seen in lower left panel. The most posterior slice including caudatus is shown in lower right panel.

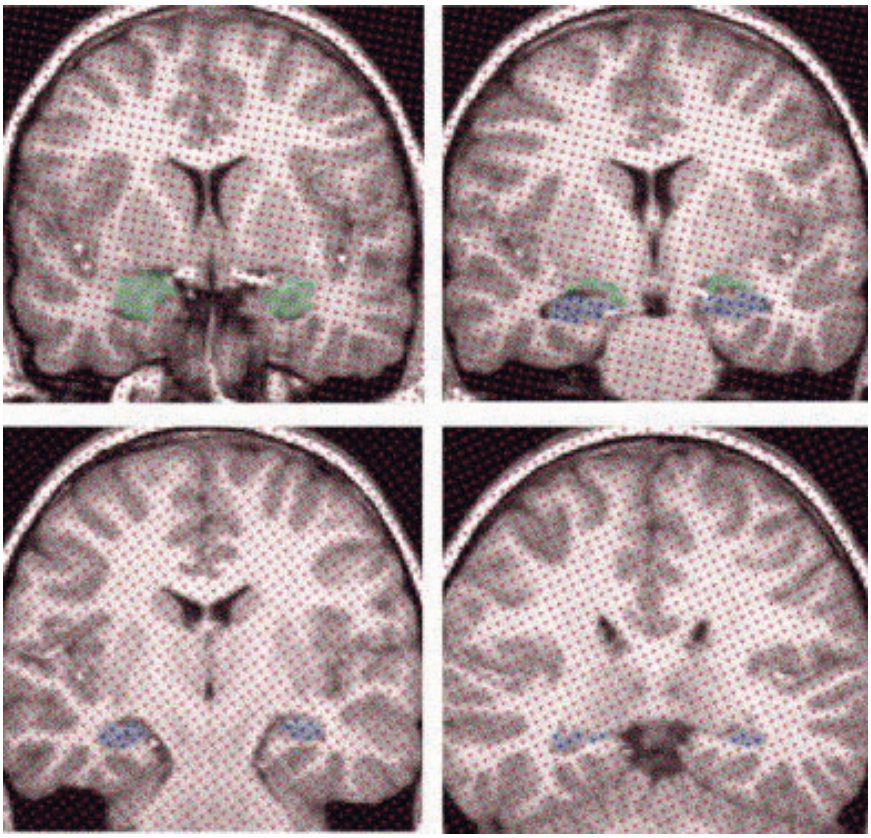

Figure 10. Images from volumetric software, EasyMeasure. This point-counting method consists of overlaying a systematic array of the test points: amygdala (green) and hippocampus (blue) defined from anterior to posterior.

8.3.4 SPECT

We used SPECT with the radioligand 123I-labelled nor-β-CIT, which specifically labels 5-HT and DA transporters. Nor-β-CIT has a tenfold higher affinity for the SERT transporter than the β-CIT (Hiltunen et al., 1998). A dose of 80 to 150 MBq of [123I]nor-β-CIT (3 MBq/kg of body weight) was injected into the right antecubital vein in a dimly lit and quiet room. Effective dose for a study subject was 4 to 6 mSv. Serial SPECT scans (5 minutes, 6 hours, and 24 hours after injection) were performed using a Siemens MultiSPECT 3 gamma camera (Siemens Medical Systems, Inc.,

Hoffman Estates, Illinois) with fan-beam collimators (Kuikka, Tenhunen-Eskelinen, Jurvelin, & Kiilianen, 1993).
The SPECT scans were decay-corrected and reconstructed with Butterworth-filtered backprojection in a 128 x 128 matrix with a pixel size of 3 x 3 mm, and were attenuation-corrected with a Chang's algorithm (μ = .11 cm^{-1}) The imaging resolution was 8 to 9 mm. The SPECT slices were consecutively summarized to the thickness of 6 mm and realigned using a semiautomatic brain quantification program (Brain Quantification, Siemens Medical Systems Inc., Hoffman Estates, Illinois) with Talairach coordinates (Talairach & Tournoux, 1993) (Figure 11A). Regions of interest (ROIs) were midbrain, medial frontal cortex (MFC), temporal poles, and the striatum. The cerebellum served as a reference region. In each subject, fixed-size circular ROI was used to compose the midbrain as well as the medial frontal cortex. For the striatal and the temporal poles, fixed-size irregular regions were placed onto the right side and then mirrored onto the left. No brain size or partial volume corrections were applied.
The specific binding in mL/mL for SERT (midbrain, temporal poles, and MFC) and DAT (striatum) was calculated using a graphical plot (Acton et al., 1999). The slope of this plot is equal to the distribution volume ratio: (Region − Cerebellum)/Cerebellum = V − 1) (Figure 11B). The striatal uptake was pooled. The nuclear medicine specialist (J.T.K.), who drew the ROIs and performed analysis, was not aware of the clinical status of the study subjects. As in the MRI analyses, only subject numbers were used.

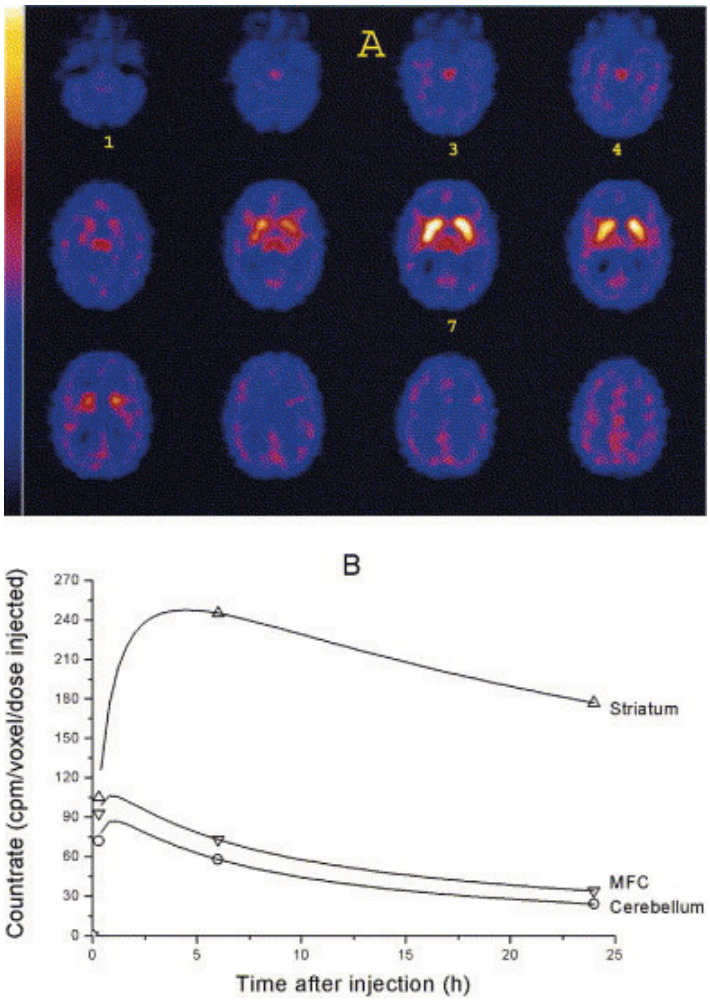

Figure 11. Transaxial slices (6 mm thick) 5.7 hours after injection of 80 MBq of [123I]nor-β-CIT in an 8-year-old girl with fetal alcohol syndrome (A). The highest uptake of the medial frontal cortex (MFC) and striatum are best visible on the slice number 7. The striatal uptake mainly reflects DAT binding and the uptake in the MFC SERT binding, respectively. The slice 1 refers tothe cerebellum and the slices 3 and 4 to the midbrain and temporal poles. The color bar is shown on the left. The image right is the patient's left. (B) Time-activity curves are illustrated for the striatum, medial frontal cortex, and cerebellum. The time-activity curves were smoothed by the log-normalfunction (OriginTM6, Microcal Software Inc., Northampton, Massachusetts).

8.3.5 Statistical analysis

All values are expressed as mean ± standard deviation (SD). Student *t* test was applied for variables with normal distribution for group comparisons. Differences were considered to be statistically significant at a two-tailed value of $p < .05$.
Both normalized and absolute volumes were analyzed to assess the differences between the FAS patient and control subjects. The variance test with repeated measurements was used to determine if lateralization of the structure (left or right) had an effect on the volumes and the unpaired measurements variance test for gender (female/male) or the group (FAS/FAE vs. control) analysis.

8.3.6 Ethics

Written informed consent was obtained from each subject or by the safeguards in case of younger children prior to participation, and all procedures were carried out in accordance with the Ethical Committee in Kuopio University Hospital. The tests were individually tailored to each subject's clinical presentation and were developed with their cooperation. Control conditions were matched with respect to age and gender. The control patients had usually transitory neurological symptoms, which needed etiological studies. The Ethical Committee of the Hospital approved the study.

8.4 RESULTS

8.4.1 Neuropsychological and psychiatric assessment

Tables 11, 12, and 13 show the characteristics of FAS/FAE cases and matched control subjects. The mean intelligence quotient (IQ) of the children was 76.2 ± 30.5 (SD), varying between 39 and 100. Verbal IQ was slightly higher (79.4 ± 31.6) than the performance IQ (73.7 ± 30.8). Two children could be classified as having mild and two as having moderate mental retardation, and all four had FAS. Their performance in subsets of attention and executive functions was poorer than the average, especially

in impulse inhibition and auditory attention. Their performance in CAS subsets was significantly poorer than that of the control group, especially in processes of attention and planning. The CBCL total scores varied between 4 and 83 points, and the mean scores were 35.4 ± 24.9 for total, 8.3 ± 8.6 for internalising, and 11.3 ± 9.1 for externalizing score (Table 14). The CBCL-P syndrome scale mean scores were 3.0 ± 3.1 for withdrawal, 1.7 ± 2.4 for somatic complaints, 3.8 ± 4.1 for anxiety/depression, 4.8 ± 3.8 for social problems, 1.4 ± 2.0 for thought problems, 6.2 ± 4.3 for attention problems, 2.8 ± 2.6 for delinquent behavior, and 8.5 ± 6.9 for aggressive behavior. The score of the CDI varied between 0 and 11 points, and the mean score was 6.0 ± 4.9 (Table 14). Altogether, five children were interviewed and none fulfilled the diagnostic criteria of current depression. One child (Subject 7), however, fulfilled diagnostic criteria for past depression. Furthermore, one of the children (Subject 10) was heavily traumatized and had many psychiatric symptoms fulfilling the diagnostic criteria of posttraumatic stress disorder. None of the control subjects for SPECT fulfilled the criteria of depression.

Table 14. Neuropsychological Findings in Subjects with FAS/FAE

Subject	Total Score	Internalizing	Externalizing	CDI Score	FABS Score
S1	39	6	15	4	16
S2	24	6	8	4	21
S3	34	2	17	16	10
S4	5	0	4	3	0
S5	67	8	29	6	30
S6	37	15	10	15	5
S7	52	10	18	6	12
S8	8	2	0	5	ND
S9	4	0	1	0	10
S10	87	31	20	7	34
S11	39	13	14	5	11
S12	29	7	0	1	17

Tested by Child Behavior Check List (CBCL; Achenbach 1991): total internal and external scores; Children's Depression Inventory (CDI; Kovacs 1992); and Fetal Alcoholic Behavior Scale (FABS; {{642 Streissguth 1998;}} Streissguth et al 1998). CDI for the control subjects scored from 0 to 13 (mean 3.1). FABS total score in the Fetal Alcohol Behavior Scale when 12 or more points where gained. Abbreviations: ND: no data; FAS: fetal alcohol syndrome; FAE: fetal alcohol effects; CDI: Children'sDepression Inventory; FABS: Fetal Alcoholic Behavior Scale

8.4.2 MRI volumetry

Table 15 shows the absolute and relative volumes of the groups. The intracranial volume was significantly smaller in the FAS/FAE cases than in the control subjects ($p = .000$). When laterality (left/right) in different groups (FAS/FAE vs. control subjects) was analyzed with analysis of variance (ANOVA), the following significant differences were found: the absolute volumes of all nuclei were smaller in the FAS/FAE group than in the control group (amygdala $p = .001$, hippocampus $p = .002$, nucleus caudatus $p = .006$, putamen $p = .001$). There was a significant difference in hippocampal volumes between the left and right hemisphere; the right hippocampus was larger than the left one in both groups. There were also no statistical differences in this asymmetry between groups.

There were also no significant differences between the groups or gender after normalization. Importantly, the right hippocampus was larger than the left after normalization in both groups ($p = .003$).

Table 15. The absolute and Relative Volumes of Different Brain Structures in FAS/FAE Patients and in Control Subjects

	Absolute Volumes, Mean (SD), mm3			Relative Volumes, Mean (SD) %		
	FAE/FAS	Control Subjects	*p* Value	FAE/FAS	Control Subjects	p Value
Amygdala, Right	1782.0 (218.5)	2091.4 (230.8)	.003	19.8 (3.5)	20.9 (2.2)	n.s.
Amygdala, Left	1799.8 (265.0)	2193.0 (335.6)	.004	19.9 (2.8)	21.8 (2.8)	n.s.
Caudatus, Right	3157.8 (467.1)	3730.8 (492.8)	.008	34.8 (4.3)	37.2 (4.4)	n.s.
Caudatus, Left	3028.2 (354.7)	3722.2 (734.5)	.007	33.4 (3.5)	37.0 (6.2)	n.s.
Hippocampus, Right	2543.1 (324.5)	2944.0 (325.3)	.006	28.2 (3.9)	29.4 (3.3)	n.s.
Hippocampus, Left	2402.8 (241.0)	2829.6 (321.8)	.001	26.6 (3.2)	28.2 (2.8)	n.s.
Putamen, Right	4230.4 (310.9)	4898.1 (521.6)	.001	46.8 (3.5)	49.0 (6.3)	n.s.
Putamen, Left	4230.4 (257.9)	4948.2 (416.3)	.000	46.8 (3.6)	49.5 (5.5)	n.s.

n.s. denotes nonsignificant. *p*-values from group comparison with independentttest. Abbreviations: FAS: fetal alcohol syndrome; FAE: fetal alcohol effects; SD: standard deviation

8.4.3 SPECT

Serotonin and dopamine transporter binding in different brain areas in the FAS/FAE patients and in control children are shown in Table 16. In the MFC, SERT binding was lower than in control subjects ($p = .02$), Figure 12. In basal ganglia, DAT binding was higher than in the control subjects ($p = .03$) (Table 16, Figure 13).

There was a significant negative correlation between internal- ization score and striatal DAT specific binding ($r = –.65$; $p = .02$) (Figure 14). Figure 15 shows the regression analysis between DAT specific binding and normalized striatal volume. No significant correlation was found.

Table 16. Serotonin (SERT) and Dopamine Transporter (DAT) Bindings in Different Brain Areas of Children with FAE/FAS and in Control Children

	[^{123I}]nor-B-CIT: SERT+ (mL/mL)				DAT (mL/mL)
	Midbrain	Temp Right	Temp Left	MFC	Striatum
Patients					
S1	1.13	.40	.29	.28	3.18
S2	1.11	.28	.24	.23	3.34
S3	1.36	.27	.27	.16	2.93
S4	1.33	.35	.33	.20	.3.29
S5	1.37	.30	.31	.22	3.22
S6	1.2	.32	.28	.20	2.95
S7	1.37	.29	.24	.26	2.64
S8	1.23	.30	.17	.35	3.26
S9	1.38	.32	.26	.22	3.11
S10	1.02	.08	.08	.15	2.39
S11	1.14	.31	.29	.34	3.28
S12	1.33	.28	.24	.25	2.71
Mean	1.25	.29	.25	.24	3.02
SD	.13	.08	.07	.06	.31
Control Subjects					
C1	1.10	.16	.16	.20	2.35
C2	1.26	.21	.16	.26	2.42
C3	1.02	.21	.25	.31	3.09
C4	1.24	.22	.22	.25	2.26
C5	1.36	.36	.32	.36	3.04
C6	1.19	.54	.5	.45	2.77
C7	1.33	.27	.29	.32	2.46
C8	1.38	.47	.49	.43	3.24
C9	1.11	.23	.24	.30	2.84
C10	1.22	.22	.22	.28	2.87
Mean	1.22	.29	.29	.32	2.73
SD	.12	.13	.12	.08	.34
P value				p = .02	p = .03

SERT+ = minor amount of DAT included. Midbrain (≈ Dorsal raphe nuclei). Student t-test was used in statistical comparisons between groups. Abbreviations: SERT: serotonin transporter; DAT: dopamine transporter; FAE: fetal alcohol effects; FAS: fetal alcohol syndrome; Temp: temporal lobe; MFC: medial frontal cortex (anterior part of cyrus cinguli); SD: standard deviation

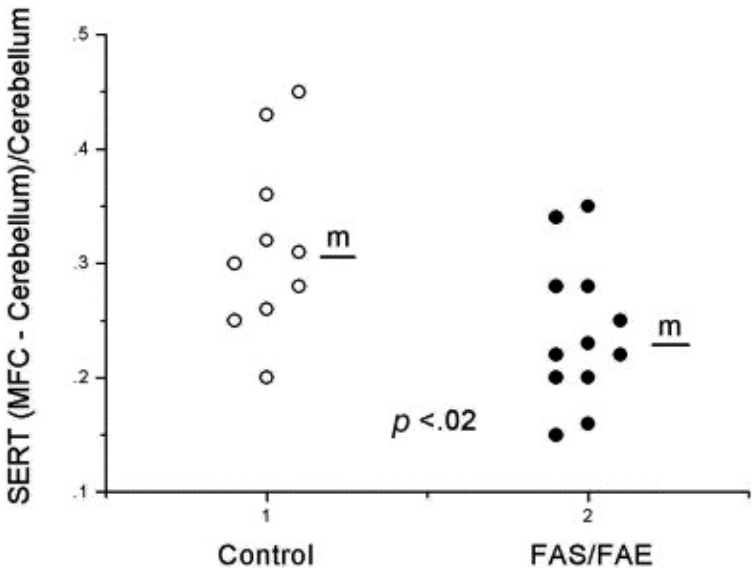

Figure 12. Serotonin transporter binding in children with FAS/FAE and control children. SERT was significantly lower than in the patients compared with control subjects (p=.02). Abbreviations: M: mean value; FAS: fetal alcoholsyndrome; FAE, fetal alcohol effects

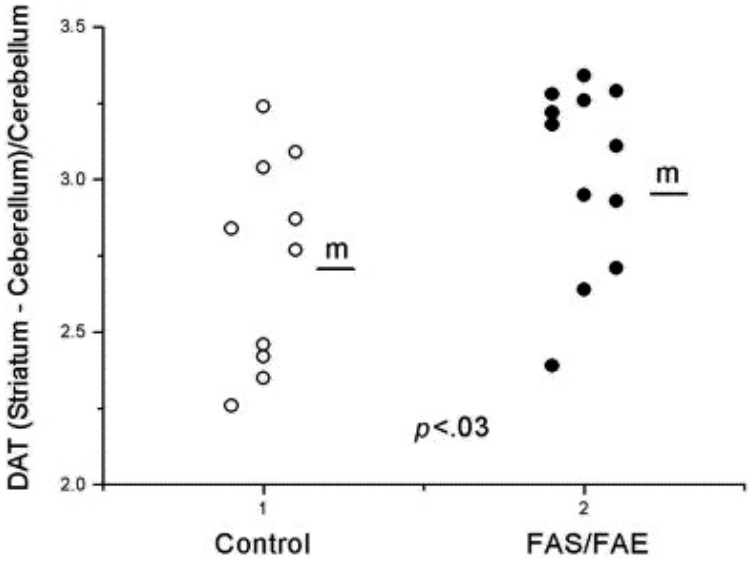

Figure 13. Dopamine transporter binding in children with FAS/FAE and in the control children. DAT was slightly higher (p=.03) in the patients compared with control subjects. Abbreviations: M: mean value; FAS: fetal alcohol syndrome; FAS: fetal alcohol effects.

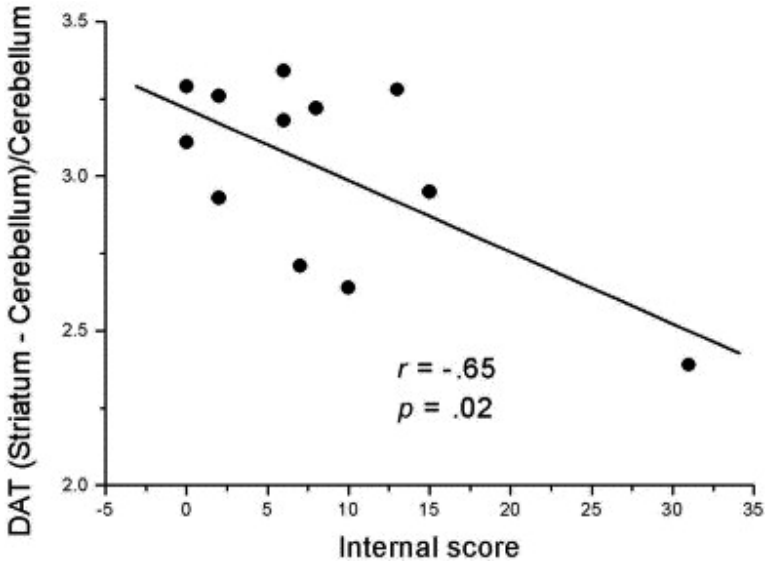

Figure 14. Linear regression between the DAT binding and internal score.

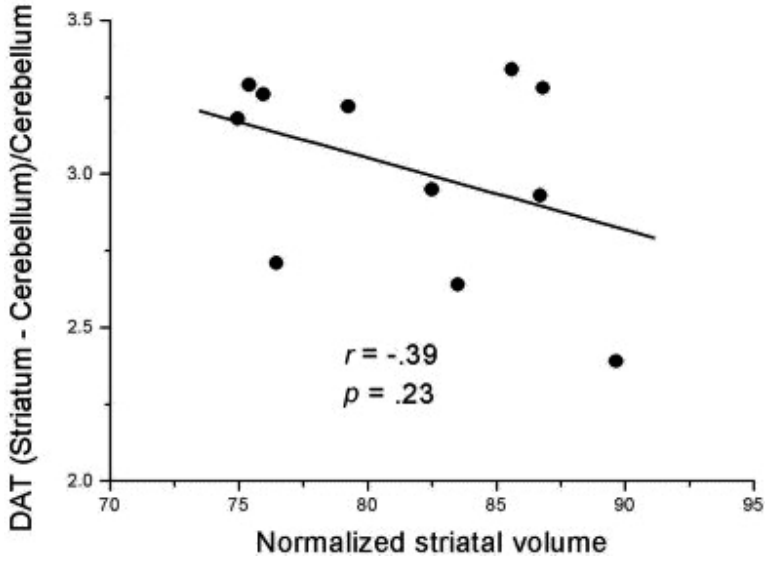

Figure 15. Linear regression between the striatal DAT binding and normalized volumes. Although the correlation did not reach a statistical significance in this small patient population, it indicates that the normal striatal DAT binding is involved in greater (=normal) striatal volume.

8.5 DISCUSSION

This study included 12 children aged 5 to 16 years who were exposed to ethanol before birth. All had dysfunction of the CNS. The combination of tests clearly demonstrated permanent CNS involvement. The overall range of IQ was between 39 and 100. Mild to moderate mental retardation was seen in four children. All had attention-deficient disorder.

8.5.1 MRI volumetry

All children with FAS/FAE had a small absolute volume of the brain. The absolute volumes of midbrain nuclei, amygdala, nucleus caudatus, and putamen were smaller than the control subjects. When relative volumes were compared with the control subjects, however, no significant differences were found. Hippocampal asymmetry was seen in the sense that the left h pocampus was smaller than right ($p < .003$). This asymmetry was seen also in the control group, and there were no significant differences between groups in this asymmetry. The left-right asymmetry was larger than normally seen in adults (up to 10%) (Kalviainen et al., 1998; Pennanen et al., 2004). Riley et al. (Riley, E., Barron, & Hannigan, 1986) suggested that many behavioral deficits resembling attentiondeficit/hyperkinetic disorder (ADHD) commonly seen in FAS/ FAE children may be attributed to the morphological changes in the hippocampus. The left-right dominance was lacking in an earlier brain perfusion SPECT study in children with FAS (Riikonen et al., 1999) and in the study of Autti-Rämö et al. (Autti-Ramo et al., 2002) in which 3 of 17 children had small hippocampus on the left side. This would mean an impairment of the function of the left hemisphere. However, our present study did not confirm this hypothesis, and in the recent study of Archibald et al. (Archibald et al., 2001), no changes in the temporal lobe in the subjects mixed with FAS and FAE could be seen. In the FAS group, excluding FAE, hippocampal volumes were even proportionally larger than in control subjects.

8.5.2 SPECT

To our knowledge, this is the first report of serotonin and dopamine transporter binding in FAS/FAE children. Our main finding was that FAS/FAE children had decreased serotonin (−20%) and slightly increased dopamine transporter binding. Serotonin binding was decreased especially in the medial frontal cortex (anterior cingulus). Nor-β-CIT binding to serotonin transporters may be affected, either due to a difference in the density of transporters or a difference in endogenous serotonin concentrations in the synapse. Low 5-HT concentrations in the synapse and high 5-HT transporter ability would have similar effects on radioligand binding (Heinz, Higley et al., 1998; Heinz, Ragan et al., 1998).

Serotonin dysregulation has been implicated in depression (Risch & Nemeroff, 1992). Decreased levels of 5-HT transporters have been observed in the midbrain regions of subjects with major depression (Staley, Malison, & Innis, 1998). We did not observe any correlation between the midbrain 5-HT transporter density and depression scores. In fact, none of the FAS/FAE or control children were considered to have a depression at the time of SPECT study.

Monoamine neurotransmitters such as serotonin, dopamine, and norepinephrine appear to function as maintenance growth factors because they must be present to produce their maturational actions (Azmitia, 2001). Diminished serotonergic function in FAS children might be due to vulnerable effects of prenatal alcohol exposure. Animal studies demonstrated deficits of serotonin in the brain stem as early as the 15th day of gestation (Druse, Kuo, & Tajuddin, 1991). Animal experiments suggest that ethanol may alter serotonin neurotransmission in discrete brain regions permanently (Azmitia, 2001; Zafar et al., 2000). Decreased serotonin availability can lead to behavioral symptoms frequently associated with decreased 5-HT transmission (Mazer et al., 1997).

The highest levels of axonal DAT are found in striatum (Donnan et al., 1991; Kaufman, Spealman, & Madras, 1991). In the human brain, the nigrostriatal dopaminergic system originates in the midbrain (substantia nigra) and terminates in the caudate nucleus and putamen. Radiolabeled β-CIT and nor-β-CIT have high binding to the dopamine transporter system

(Kuikka et al., 1995) and can also be used as radioligands in the investigation of the dopamine system. In our study, regression analysis between DAT specific binding and normalized striatal volume showed a nonsignificant correlation ($r = -.39$). Similarly, there was a significant negative correlation between internalization and DAT specific binding ($r = -.65$; $p = .02$).

Dougherty et al. (Dougherty et al., 2025) and Krause et al. (2000) (Krause, Dresel, Krause, Kung, & Tatsch, 2000) have reported increased DAT densities in the basal ganglia of adults with ADHD studied by SPECT. Vies et al (Vles et al., 2003) have shown that there is a 74.7% down-regulation of DAT in striatum after 4 weeks methylphenidate medication in children. All the FAS children in our study had ADHD. The characteristic deficits of ADHD, namely impaired attention, excessive motor activity, and impulsivity, may therefore result from the selective deficiency in the availability of dopamine at the synaptic level.

Type 2 alcoholism is characterized by antisocial personality traits from teenage and persistent seeking of alcohol and other substances for their euphoric effects. There is early onset of instability to abstain entirely from alcohol, impulsiveness, fighting, and arrests when drinking and antisociality (Cloninger et al., 1988). Type 1 alcoholism is characterized by anxious (passive-dependent) personality traits and rapid development of tolerance and dependence on the antianxiety effects of alcohol. This type includes 80% of all alcoholics. Type 2 alcoholics have apparently higher and type 1 alcoholics lower DAT densities than healthy control subjects (Tiihonen et al., 1995). Type 2 alcoholics have also a serotonergic defect (Cloninger, 1987; Virkkunen & Linnoila, 1990).

Our patients clearly had a decreased SERT and increased DAT binding, both characteristic of type 2 alcoholics. It is unknown whether it is a reflection of genetic background or due to a deficit in the balance of DA and serotonergic neuroregulation. Impaired serotonergic function in FAS children might partly explain why the FAS children cannot respond appropriately to stress or other stimuli or modulate their affective states. Serotonin agonists can prevent some of the adverse effects of ethanol on the development of the serotonin system (Eriksen, Gillespie, & Druse,

2000). Some behavioral aspects of FAS might be preventable by early intervention and treatment.

9 OPTICAL COHERENCE TOMOGRAPHY SHOWS DECREASED THICKNESS OF RETINAL NERVE FIBRE LAYER AMONG FETAL ALCOHOL EXPOSED YOUNG ADULTS IN A CASE-CONTROL STUDY

Fetal alcohol spectrum disorder is an umbrella term that describes all the fetal alcohol effects. We lack a reliable biomarker to detect fetal alcohol exposure and a uniform way to diagnose fetal alcohol spectrum disorder (Coles et al., 2016; Cook, 2003). The eye is a sensitive indicator of prenatal adverse events and the eyes and visual system are often damaged by maternal alcohol misuse during pregnancy (Stromland & Pinazo-Duran, 2002). Among the most frequent abnormalities are optic nerve hypoplasia and increased tortuosity of retinal vessels (Abdelrahman & Conn, 2009). The aim of the present study was to evaluate peripapillary retinal nerve fibre layer (RNFL) thickness using optical coherence tomography (OCT) among fetal alcohol exposed young adults.

We invited twelve previously studied (Riikonen et al., 2005) young adults suffering from fetal alcohol syndrome (FAS) and fetal alcohol effects (FAE) to take part in the ophthalmological examination including OCT. Ten out of the twelve subjects accepted the invitation and they were examined in the Ophthalmology Clinic of Kuopio University Hospital (Finland) during 2012-2013. All subjects were previously confirmed cases of fetal alcohol exposure. The diagnostic criteria of FAS and more detailed characteristics of the subjects have been described previously (Riikonen et al., 2005). The control subjects were recruited from the circle of acquaintances of the personnel and from medical students at University of Eastern Finland. They were healthy non-smoking and not exposed to alcohol during pregnancy. The study included one on-site visit in the the Ophthalmology Clinic of Kuopio University Hospital. A standard ophthalmological examination including indirect ophthalmoscopy and measurement of the vision was

performed to exclude eye diseases. OCT was performed using OTI OCT-SLO (Ophthalmic Technologies Inc., Toronto, Canada). The study protocol was approved by the Ethics Committee of Kuopio University Hospital. All study participants provided an informed written consent.

Data management and the statistical analyses were performed using SPSS 24 (SPSS Inc., Chicago, IL). The mean age of the FAS/FAE group was 20.7 years (SD 3.3) and that of the control group 20.5 years (SD 4.3). Six out of ten in both study groups were females. One person in the FAS/FAE group had missing value of the right eye OCT. We found that the mean peripapillary RNFL thickness was smaller in the FAS/FAE group compared to the controls and in all peripapillary segments except for the right eye nasal quadrant (Table 1).

OCT with RNFL thickness measurement is a potentially useful tool to evaluate the effects of preceding alcohol exposure during pregnancy. To our knowledge, only one study has previously investigated optic nerve thickness with OCT in FAS and our results are in line with that study (Menezes et al., 2016; Riikonen et al., 2005). We suggest that this finding should be studied in a larger sample and with more advanced equipments to evaluate whether RNFL thickness could be used as a detection tool for fetal alcohol exposure.

Table 17. Mean peripapillary RNFL thickness (μm) in quadrants of the right and left eye

	Controls (n=10)		FAS/FAE (n=10)		
	Mean	SD	mean	SD	*P*
Right eye					
Nasal	82,0	18,7	62,8	24,6	0.053
Temporal	72,2	8,6	48,9	12,8	<0.001
Inferior	140,3	15,0	85,3	18,3	< 0.001
Superior	125,3	17,0	91,0	15,0	0.001
Mean	105,0	10,8	72,0	9,3	< 0.001
Left eye					
Nasal	77,6	13,5	62,2	25,9	0.009
Temporal	73,2	5,6	50,4	7,3	< 0.001
Inferior	138,0	12,3	91,6	17,0	< 0.001
Superior	124,4	13,3	85,6	18,4	< 0.001
Mean	103,3	8,1	72,5	8,1	< 0.001

Mann-Whitney U test was used to compare differences between the FAS/FAE and control groups.

10 GENERAL DISCUSSION

10.1 SUMMARY

10.1.1 First trimester metabolomics and trisomy screening

The metabolic profile of serum samples from pregnant women during the first trimester showed that alcohol and drug use were associated with increased glutamate and decreased glutamine levels, and alcohol use was associated with decreased serotonin levels. Decreased glutamine in pregnant women during the first trimester of pregnancy is in accordance with previous studies on alcohol consumption during pregnancy (Bajaj et al., 2017; Harada et al., 2016; Mittal & Dabur, 2015; Würtz et al., 2016). Interestingly, Harada et al. (Harada et al., 2016) suggested that an increased glutamate/glutamine ratio could be a good alcohol consumption indicator, because this ratio had stronger relation to serum GGT, AST and ALT than glutamate and glutamine alone. In our study glutamate/glutamine ratio (unpublished results) was also significantly increased in the samples from alcohol-exposed pregnancies (Welch's t-test p-value = 0.0042, Cohen's d = 0.71), and illicit drug-exposed pregnancies (p = 0.0061, d = 1.10) when compared to the samples from healthy control pregnancies. The sample from tobacco-exposed pregnancies had a trend towards an increased glutamate/glutamine ratio, when compared to the samples from healthy pregnancies, but this difference was not significant (p = 0.0579, d = 0.23).

First trimester trisomy screening parameters showed increased free β-hCG levels in mothers exposed to alcohol and drugs. The differences found in this study were too small for β-hCG to be useful as a biomarker. Maternal PAPP-A levels were lower in smoking than non-smoking mothers. The software for calculating the trisomy risk used smoking adjustment for PAPP-A. This study showed that the correction factor was not sufficient at the time of the analysis, but thereafter the correction factor has been modified.

10.1.2 Ultrasonography and follow-up findings

Decreased fetal head circumference was detected already during the second trimester of pregnancy in children exposed to alcohol (exposed fetuses mean=16.83 cm, SD=1.12 cm; controls mean 17.93 cm, SD=1.05 cm). Decreased head circumference in children exposed to alcohol was also present at birth (mean=-0.8 SD-scores, SD=0.89, Finnish growth reference) and was still detactable at 2.5 years of age (mean=-0.8 SD-scores, SD=0.82, Finnish growth reference). However, the head circumferences of the exposed children were mainly in the normal range of the Finnish growth reference.

During pregnancy the etiology of the growth restriction can be categorized broadly into maternal, fetal, and placental (American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics and the Society forMaternal-FetalMedicin, 2019). After birth growth is determined more by an individual´s own genetic factors and environmental factors. In concordance with this idea, the prevalence of the small head circumference associated with fetal alcohol exposure decreases with age (Jacobson et al., 2021). Therefore, the best timepoint to evaluate fetal alcohol exposure associated growth restriction is at birth or in the early years of life.

The alcohol exposed children in our study scored lower (but within the normal range) than the controls in the Griffiths subscales of locomotor, hearing and language and general quotient at the age of 2.5 years. The Griffiths scales do not evaluate attention, social skills or behaviour. Despite results in the normal range at the age of 2.5 years, develoment of neurological or cognitive problems later in life cannot be precluded.

10.1.3 MRI and SPECT imaging

Children diagnosed with FAS/FAE showed increased dopamine binding and reduced serotonin transporter binding, indicating that the serotonin (5-HT) system is vulnerable to the effect of ethanol in utero. Increased dopamine transporter binding may correlate with ADHD findings. Additionally, our findings of decreased maternal serotonin levels during the first trimester

of pregnancy suggest that maternal serotonin system is also vulnerable to the effects of ethanol. However, the results of the low maternal serum serotonin levels and reduced serotonin transporter binding in FAS/FAE children are not comparable. Previous studies indicate that placental hyposerotonemia may impair subsequent sensory, motor, and cognitive abilities (Rosenfeld, 2021; Sato, 2013; Yang et al., 2014), which are common findings in FAS/FAE children. Further studies are needed to get a better understanding of serotonin in maternal-placental-fetal axis.

Our study showed that FAS/FAE children had significantly smaller absolute volumes of the amygdala, hippocampus, caudatus and putamen and total brain volume than control children. After normalization of volumes, significant differences were not found. The left hippocampus was smaller than the right ($p<.003$), but did not significantly differ from the control subjects. These findings indicate that FAS/FAE children have an overall smaller brain, and the structures studied are decreased in the same proportion. None of the structures studied are safe from the teratogenic effects of ethanol.

10.1.4 OCT findings in FAS/FAE

Mean peripapillary RNFL thickness was reduced in FAS/FAE adolescents. Our findings are in accordance with the previous study of Menezes et al. (Menezes et al., 2016). Furthermore, Orum et al. (Orum & Kalenderoglu, 2020) found reduced RNFL sectors in persons with alcohol use disorder. The present results of reduced thickness of RNFL indicate that RNFL thickness could be a potential indicator of alcohol exposure. However, in all studies the sample size has been limited and therefore the use of RNFL thickness needs to be studied in a larger sample size.

10.2 STRENGTHS AND LIMITATIONS OF THE PRESENT STUDY

Our primary aim was to study and follow up prospectively alcohol using mothers and their pregnancies and children up to 2.5 years of age. Low compliance and commitment of the patients to this study was a major challenge. Due to the low compliance and high dropout rate of the

participants we ended up studying the effects of alcohol on a broader spectrum. Finally, we had three different sources to investigate alcohol use during the pregnancy. This can be considered as a strength and a limitation of the study at the same time.

The limitations of this study include our inability to define the exposure agents precisely. Therefore, dose-response conclusions are impossible to make. We could not either reliably separete the exposure agents properly (mixed use of drugs, smoking and alcohol). These limitations are generally recognized challenges in the field of alcohol research and are also a reason to carry out this study. The strengths of this research include the fact that these findings should present the variation one would expect to encounter in a clinical setting. Lastly, since this was a study about associations, conclusions in terms of causality cannot be drawn.

10.3 FUTURE DIRECTIONS

In an optimal study design, exposure agents could be defined more reliably and dose-outcome relationship could be defined more precisely. However, when studing living human beings, this never can be optimally obtained. The outcome of the alcohol exposed fetuses is multifactorial, in which genetic, epigenetic and environmental features have their own role. We still lack a reliable way to detect alcohol use during pregnancy. Moreover, an international consensus of the diagnostic criteria for FAS/FAE has not been reached.

However, further research on metabolomics in FAS/FAE children and adults might give a better understanding of permanent metabolite changes after fetal alcohol exposure. It would be also interesting to investigate serotonin, glutamate and glutamine metabolism in a longitudinal follow-up in which maternal, placental and fetal measurements could be followed by later investigations of children and adults exposed prenatally to alcohol. Furthermore, a bigger sample size and wider range of FASD outcomes are needed to confirm whether RNFL thickness could be used as an indicator of fetal alcohol exposure.

11 CONCLUSIONS

The conclusions of this study are as follows

1) Alcohol and drug abuse during the first trimester of pregnancy is associated with
    a. increased glutamate and decreased glutamine and serotonin serum levels among pregnant mothers
    b. increased levels of free β-hCG and decreased PAPP-A levels among pregnant women. Increased free β-hCG was considered clinically irrelevant due to significant overlap with the controls. Decreased PAPP-A was probably explained by smoking.
2) The detrimental effect of fetal alcohol and drug exposure is already detected in mid-pregnancy ultrasonography as a decrease in fetal head size. These exposed children had decreased head size also at the age of 2.5 years, eventhough the changes were relatively small.
3) Children diagnosed with FAS/FAE have reduced serotonin binding and increased dopamine binding to their transporters in the brain. This finding, together with decreased maternal serotonin, indicates that maternal alcohol use causes long-lasting effects on the serotonin system.
4) Peripapillary RNFL thickness is reduced in young adults diagnosed with FAS/FAE as children, suggesting that RNFL thickness might be a potential indicator for fetal alcohol exposure.

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APPENDICES

Supplementary file 1 Main compunds and other metabolome findings (Study I)

Compound ID	CAS	Composite Spectrum	Mass	RT	ANOVA p-value	Control Mean	Control SD	Alcohol Mean	Alcohol SD	Alcohol d	Drug Mean	Drug SD	Drug d	Tobacco Mean	Tobacco SD	Tobacco d
Cotinine	486-56-6	(199.0883, 92.09)(177.1022, 49175.99)(178.1053, 4807.18)(179.1087, 406.76)(180.1059, 25.76)	176.0952	0.61		ND		130025	108003		202167	133659		169360	107161	
Glutamine	56-85-9	(169.0582, 40605.14)(170.0612, 2477.94)(315.1269, 3092.56)(316.1302, 350.4)(185.0321, 1828.66)(147.0767, 92969.64)(148.0794, 5026.94)	146.0693	6.06	<0.0001*	438772	124027	301029	116046	-1.04	335879	150900	-0.77	403820	103747	-0.26
Serotonin	50-67-9	(177.1023, 5674.82)(178.1057, 621.87)	176.0947	1.69	<0.0001*	15796	6019	9034	6606	-0.97	14222	6435	-0.23	16181	7169	0.06
Serotonin - OH		(160.0755, 5302.02)(161.0788, 632.53)	159.0682	1.69	<0.0001*	15616	6009	9013	6541	-0.97	13913	6534	-0.25	15780	6896	0.02
Glutamate	56-86-0	(170.0422, 196.77)(148.0606, 20758.0)(149.0638, 1092.97)	147.0532	6.31	0.0001*	31647	12532	42997	19204	0.71	47431	20542	0.99	37276	10565	0.35
Glutamine - OH		(152.0318, 2016.42)(153.034, 43.52)(130.05, 19872.05)(131.053, 978.32)	129.0427	6.06	0.0001*	73450	19775	52142	19105	-0.99	57925	25024	-0.72	68651	16926	-0.22
Glutamine + 2Na - H		(191.0402, 4700.65)(192.0429, 315.05)	190.0328	6.06	0.0001*	28762	3233	24415	5619	-1.05	25594	3941	-0.77	27970	3365	-0.19
2 x Glutamine + ACN + 3H		(359.0918, 3798.83)(360.0938, 464.35)(695.194, 1471.52)(696.1963, 353.79)(337.109, 896.54)	336.1021	6.06	0.0002*	23435	4467	18113	5960	-1.05	18881	4633	-0.90	21978	3997	-0.29
Unknown 1		(527.143, 3795.31)(528.1457, 692.45)	526.1352	6.07	0.0002*	13256	2686	10261	3543	-0.99	10572	2680	-0.89	12571	2481	-0.23
Glycerophosphocholine	28319-77-9	(553.1689, 117.19)(280.0924, 33024.67)(281.0953, 2629.51)(282.0967, 405.71)(537.1954, 12056.47)(538.198, 1991.64)(539.2, 382.01)(296.0661, 1606.04)(258.1101, 21260.51)(259.1132, 2089.94)(260.1144, 289.59)	257.1026	6.18	0.0003*	81045	94903	40291	18127	-0.61	49484	44056	-0.47	34935	20875	-0.69
L-Asparagine	70-47-3	(155.0425, 151.17)(133.0607, 3800.06)(134.0637, 179.65)	132.0534	6.17	0.0007*	13888	3294	11009	3517	-0.84	11521	3488	-0.69	12801	2810	-0.32
Trigonelline	535-83-1	(275.1024, 300.98)(160.037, 12010.37)(161.0403, 875.21)(297.0845, 892.2)(176.0107, 1146.02)(138.0553, 319133.4)(139.0583, 20012.59)(140.061, 1590.12)	137.0471	4.29	0.0014	98743	172812	325312	397755	0.51	396698	476505	0.67	491493	578376	0.88
Butyrylcarnitine	25576-40-3	(232.1546, 24993.62)(233.1577, 3065.9)(234.1593, 369.43)	231.1472	1.69	0.0019	82115	34355	55335	26506	-0.83	70024	31699	-0.37	78800	27772	-0.10
Creatinine	60-27-5	(227.1262, 12445.3.66)(228.1283, 11061.27)(229.1306, 1100.89)(136.0478, 3375.36)(137.0501, 98.18)(249.1069, 790.08)(152.0216, 165.29)(114.0668, 372596.06)(115.0693, 15861.06)(116.0715, 997.56)	113.0598	1.32	0.0023	2171767	402021	1840076	433821	-0.73	1823012	408825	-0.77	2086000	476220	-0.19
2 x Creatinine		(227.1257, 168886.89)	226.1185	1.32	0.0030	444703	102720	365747	108326	-0.69	356825	99322	-0.77	422210	119102	-0.20
Propionylcarnitine	20064-19-1	(218.139, 25538.4)(219.1421, 2706.46)(220.1443, 277.45)	217.1314	2.21	0.0034	122533	34391	100863	40950	-0.56	116142	38426	-0.17	134781	37582	0.32
Unknown 2		(246.1701, 4327.32)(247.1732, 493.92)	245.1629	1.39	0.0039	21110	6401	16790	6526	-0.60	19179	7252	-0.27	23061	7415	0.27
Unknown 3		(963.6395, 525.07)(964.6421, 226.79)(504.3061, 1614.35)(505.309, 446.07)(985.6227, 248.59)(520.2786, 78.81)(482.3254, 28705.91)(483.3281, 6343.83)(484.3302, 1143.6)(485.3328, 118.36)	481.317	1.44	0.0044	132914	35419	103595	25043	-0.81	130097	34596	-0.08	138515	37168	0.15
Unknown 4		(516.3058, 3519.47)(517.309, 987.41)(532.2795, 189.93)(494.3249, 82588.99)(495.3275, 19078.2)	493.3179	1.43	0.0048	197745	59691	215481	59758	0.21	249944	65121	0.63	248516	113401	0.61
Unknown 5		(249.0609, 4222.48)(250.0638, 546.28)	248.0536	4.05	0.0053	25020	3078	22593	3934	-0.66	23585	3744	-0.39	25581	3705	0.15
Unknown 6		(182.0785, 15108.33)(183.0815, 1273.63)(341.1682, 1806.6)(342.1713, 297.84)(198.0522, 1242.84)(160.0967, 47590.06)(161.0998, 3815.26)	159.0892	4.62	0.0088	56144	84985	12920	16716	-0.52	53403	91819	-0.03	32442	88625	-0.29
Unknown 7		(186.1124, 2643.76)(187.1147, 270.35)	185.1051	4.04	0.0112	9720	2506	9720	2590	0.00	10297	2471	0.20	11699	3288	0.68
Unknown 8		(137.0711, 27458.45)(138.0741, 2105.82)	136.0636	2.33	0.0145	74293	44872	51778	44156	-0.52	51592	41589	-0.52	59147	37850	-0.35
Unknown 9		(121.0713, 749.53)(61.0402, 3203.72)	60.0329	1.02	0.0154	15228	3006	12994	2345	-0.79	13799	2415	-0.50	14342	2669	-0.31
Unknown 10		(254.1721, 2610.45)(255.1744, 307.22)	253.1655	1.32	0.0159	9364	2224	7871	2384	-0.62	7834	2135	-0.63	8967	2524	-0.16
Unknown 11		(188.0707, 17776.07)(189.0737, 2254.05)(190.0763, 179.41)	187.0633	4.05	0.0163	71947	12413	63040	13020	-0.60	68082	17238	-0.26	74398	15939	0.16
Unknown 12		(150.0583, 3768.17)(151.0611, 265.09)	149.051	0.91	0.0167	11180	3881	8609	2172	-0.79	9596	3034	-0.48	9544	2322	-0.50
L-Carnitine		(323.2176, 623.86)(184.0944, 17740.84)(185.0977, 1281.76)(345.1998, 736.49)(200.0679, 655.41)(162.113, 825693.94)(163.1161, 54469.55)(164.1175, 5298.14)	161.1057	4.81	0.0181	4246070	603898	4068244	682822	-0.25	4340452	705006	0.14	4615222	730844	0.53
Tryptophan	73-22-3	(409.187, 132.61)(227.079, 5585.29)(228.082, 728.73)(431.1683, 336.48)(243.0527, 2660.41)(244.0561, 309.46)(205.0975, 69100.89)(206.1002, 7668.32)(207.1028, 708.62)	204.09	4.05	0.0184	261822	50440	227369	52729	-0.57	248394	67928	-0.22	273228	64087	0.19
Pipecolic acid		(152.0681, 648.23)(130.0867, 115903.34)(131.0896, 6430.82)	129.0792	3.90	0.0185	152893	202107	61504	40337	-0.53	154459	158751	0.01	105715	167282	-0.27
Unknown 13		(226.0448, 3536.76)(227.0481, 321.18)	225.0374	5.06	0.0383	12897	2571	11406	2652	-0.56	11971	2603	-0.35	13039	2563	0.05

					ANOVA	Control		Alcohol			Drug			Tobacco		
Compound ID	CAS	Composite Spectrum	Mass	RT	p-value	Mean	SD	Mean	SD	*d*	Mean	SD	*d*	Mean	SD	*d*
Unknown 14		(144.0806, 4453.37)(145.0846, 429.93)	143.0733	4.06	0.0409	20334	3281	18350	3422	-0.52	19098	4048	-0.33	20870	4071	0.14
Unknown 15		(263.1463, 9154.69)(264.1489, 991.02)(154.0587, 32750.49)(155.0614, 1458.61)(285.1283, 6103.66)(286.131, 574.89)(287.1327, 61.18)(170.0326, 1458.77)(132.0771, 187037.67)(133.0798, 8279.52)	131.0695	5.52	0.0450	585604	281672	596052	243873	0.04	674736	265361	0.31	738209	299628	0.53
Unknown 16		(161.1075, 3048.26)(162.1114, 316.18)	160.0999	4.06	0.0512	13845	2075	12499	2405	-0.52	13274	2859	-0.22	14310	2868	0.18
Unknown 17		(144.0629, 229.29)(122.0813, 58398.75)(123.0847, 2660.02)	121.0738	5.20	0.0530	132379	46715	111970	44179	-0.43	147936	64333	0.32	120064	33050	-0.26
Unknown 18		(110.0715, 4383.15)(111.0736, 294.55)	109.0643	6.73	0.0546	25747	4845	22433	3672	-0.73	24801	4677	-0.21	24930	4050	-0.18
Unknown 19		(136.0481, 7040.38)(137.0506, 353.22)(114.0664, 391.82)	113.0587	5.52	0.0551	20983	10459	21532	9167	0.05	24757	10143	0.36	26554	10257	0.53
Unknown 20		(287.197, 19749.94)(288.2, 2814.11)(289.2024, 327.55)(325.1532, 69.75)(166.0836, 5888.39)(167.0871, 420.71)(309.1784, 2586.55)(182.0577, 1297.63)(144.1023, 1030431.0)(145.1054, 65242.12)(146.1076, 3718.59)	143.0948	3.69	0.0565	4233809	4882165	2122150	2295413	-0.48	3802084	4365632	-0.10	2443463	4170600	-0.41
Unknown 21		(260.186, 10675.93)(261.1891, 1638.73)(262.1922, 155.47)	259.1781	1.14	0.0571	11152	9388	12003	6934	0.10	15051	10168	0.45	13375	6908	0.26
Histidine	71-00-1	(178.0586, 7081.71)(179.062, 492.68)(333.1282, 489.01)(194.0327, 134.56)(156.0768, 45159.52)(157.0798, 3225.87)(158.0817, 288.4)	155.0695	6.73	0.0584	388384	72128	338440	54573	-0.72	368240	69431	-0.29	371099	65098	-0.25
Unknown 22		(161.1284, 7509.04)(162.131, 766.19)	160.1211	6.76	0.0597	6875	3814	6462	4681	-0.08	6906	4748	0.01	9577	7354	0.49
Unknown 23		(160.1334, 49939.32)(161.1365, 4018.52)(162.1379, 354.32)	159.126	2.23	0.0682	266261	78407	228721	91632	-0.43	255650	99655	-0.12	280069	80438	0.16
Unknown 24		(60.0812, 8573.77)(61.0844, 383.84)	59.0737	1.58	0.0693	41859	11269	35496	8062	-0.62	37778	9362	-0.40	37898	9147	-0.39
Unknown 25		(192.0738, 440.66)(170.0925, 12025.35)(171.0955, 978.62)	169.0849	6.30	0.0817	25697	9366	26581	9826	0.08	32151	15501	0.56	30390	11304	0.41
Unknown 26		(229.1546, 3635.53)(230.1577, 499.07)	228.1472	4.19	0.0820	29342	6139	28248	10187	-0.15	25344	7358	-0.54	27834	7188	-0.20
Unknown 27		(140.0682, 1389.99)(118.0864, 52088.45)(119.0896, 2747.42)	117.079	4.82	0.0928	176248	38229	155056	40226	-0.48	172379	50028	-0.09	183387	45840	0.16
Tyrosine	60-18-4	(220.0308, 181.4)(182.0812, 11296.07)(183.0845, 1091.79)	181.0739	5.06	0.0945	36686	8697	31975	7339	-0.52	35548	9963	-0.13	37724	8831	0.12
Unknown 28		(142.0475, 177.77)(120.0654, 9629.68)	119.0581	5.72	0.0982	29479	5924	28642	6509	-0.12	30657	8956	0.17	32819	6828	0.47
Unknown 29		(146.1176, 20922.46)(147.1206, 1689.28)	145.1104	3.56	0.1016	158496	32308	143140	24963	-0.48	153459	29570	-0.16	165241	33870	0.21
Unknown 30		(991.6763, 108708.95)(992.6789, 52879.63)(993.6805, 13165.67)(994.6822, 2345.27)(995.6845, 413.61)(1029.6243, 329.28)(1030.6277, 321.16)(1031.6332, 178.88)(1013.6547, 8081.97)(1014.6576, 3947.18)(534.2959, 1843.19)(535.2994, 481.05)(536.2966, 149.35)(496.3432, 1303649.6)(497.3464, 320632.1)(498.3481, 47583.13)(499.3499, 5132.97)	495.3348	1.38	0.1058	12375692	1987437	12566020	1424160	0.10	13287518	1813131	0.49	13036664	1707859	0.36
Unknown 31		(307.1096, 981.13)(176.04, 1720.6)(154.0587, 29713.12)(155.0615, 1411.97)	153.0511	5.57	0.1100	39947	32603	37620	19763	-0.07	45901	30310	0.17	53944	44996	0.39
Unknown 32		(86.0967, 29145.8)(87.1001, 1461.71)	85.0894	4.02	0.1154	100428	27258	85882	17354	-0.55	100394	30823	0.00	102393	24817	0.07
Unknown 33		(86.0966, 20732.41)(87.0998, 1010.78)	85.0896	4.24	0.1168	42324	14148	34550	7659	-0.52	44180	19881	0.12	43129	13949	0.05
Unknown 34		(164.0289, 3515.39)(165.0319, 188.87)	163.0222	5.73	0.1208	19341	2384	18677	2741	-0.25	19618	2851	0.11	20339	2528	0.38
Unknown 35		(625.3023, 142.68)(335.1367, 926.52)(336.1392, 150.28)(313.1552, 21787.27)(314.1581, 4118.36)(315.1606, 469.42)	312.1475	1.83	0.1225	62060	27864	63837	34508	0.06	63739	26953	0.06	72789	27299	0.37
Leucine	61-90-5	(154.0846, 183.35)(132.1023, 146733.89)(133.1052, 9848.34)	131.0946	4.02	0.1341	500402	139360	428988	90602	-0.52	500305	158599	0.00	511492	127233	0.08
Unknown 36		(595.2966, 868.78)(596.2995, 275.34)(573.3149, 17499.62)(574.3174, 5390.26)(575.3197, 1008.53)	572.3071	6.06	0.1352	15371	17554	22587	22551	0.29	26070	25506	0.42	29301	31764	0.55
Isoleucine	73-32-5	(154.0841, 221.43)(132.102, 35169.55)(133.105, 2672.79)(134.107, 188.84)	131.0948	4.24	0.1497	179340	61530	146867	33325	-0.51	186472	84960	0.11	181931	60184	0.04
Unknown 37		(72.0812, 19065.01)(73.0845, 793.56)	71.0738	4.82	0.1512	66406	13822	58598	11844	-0.51	65904	18236	-0.03	67525	15423	0.07
Unknown 38		(304.212, 5152.97)(305.2154, 883.05)	303.2043	1.64	0.1514	8940	7195	12190	10415	0.42	12094	7377	0.41	10891	6373	0.25
Unknown 39		(291.0453, 17695.57)(292.0481, 1651.87)(293.0494, 209.27)	290.0383	1.31	0.1597	72501	18894	71427	14002	-0.06	63804	18008	-0.49	68550	16484	-0.22
Unknown 40		(129.066, 7387.67)(130.0688, 396.1)	128.0585	5.65	0.1662	12697	6400	11358	6882	-0.21	14063	6808	0.22	11112	5261	-0.25
Lysine		(169.0942, 586.41)(147.1128, 32115.87)(148.116, 2230.77)(149.1174, 195.15)	146.1053	7.06	0.1855	98704	41689	77804	23043	-0.53	81373	35024	-0.44	93397	41213	-0.14
Unknown 41		(226.9519, 11668.43)(227.955, 419.72)	225.9449	4.43	0.1872	165941	18564	159507	14070	-0.37	169333	18484	0.19	169259	15647	0.19

					ANOVA	Control		Alcohol			Drug			Tobacco		
Compound ID	CAS	Composite Spectrum	Mass	RT	p-value	Mean	SD	Mean	SD	*d*	Mean	SD	*d*	Mean	SD	*d*
Unknown 14		(144.0806, 4453.37)(145.0846, 429.93)	143.0733	4.06	0.0409	20334	3281	18350	3422	-0.52	19098	4048	-0.33	20870	4071	0.14
Unknown 15		(263.1463, 9154.69)(264.1489, 991.02)(154.0587, 32750.49)(155.0614, 1458.61)(285.1283, 6103.66)(286.131, 574.89)(287.1327, 61.18)(170.0326, 1458.77)(132.0771, 187037.67)(133.0798, 8279.52)	131.0695	5.52	0.0450	585604	281672	596052	243873	0.04	674736	265361	0.31	738209	299628	0.53
Unknown 16		(161.1075, 3048.26)(162.1114, 316.18)	160.0999	4.06	0.0512	13845	2075	12499	2405	-0.52	13274	2859	-0.22	14310	2868	0.18
Unknown 17		(144.0629, 229.29)(122.0813, 58398.75)(123.0847, 2660.02)	121.0738	5.20	0.0530	132379	46715	111970	44179	-0.43	147936	64333	0.32	120064	33050	-0.26
Unknown 18		(110.0715, 4383.15)(111.0736, 294.55)	109.0643	6.73	0.0546	25747	4845	22433	3672	-0.73	24801	4677	-0.21	24930	4050	-0.18
Unknown 19		(136.0481, 7040.38)(137.0506, 353.22)(114.0664, 391.82)	113.0587	5.52	0.0551	20983	10459	21532	9167	0.05	24757	10143	0.36	26554	10257	0.53
Unknown 20		(287.197, 19749.94)(288.2, 2814.11)(289.2024, 327.55)(325.1532, 69.75)(166.0836, 5888.39)(167.0871, 420.71)(309.1784, 2586.55)(182.0577, 1297.63)(144.1023, 1030431.0)(145.1054, 65242.12)(146.1076, 3718.59)	143.0948	3.69	0.0565	4233809	4882165	2122150	2295413	-0.48	3802084	4365632	-0.10	2443463	4170600	-0.41
Unknown 21		(260.186, 10675.93)(261.1891, 1638.73)(262.1922, 155.47)	259.1781	1.14	0.0571	11152	9388	12003	6934	0.10	15051	10168	0.45	13375	6908	0.26
Histidine	71-00-1	(178.0586, 7081.71)(179.062, 492.68)(333.1282, 489.01)(194.0327, 134.56)(156.0768, 45159.52)(157.0798, 3225.87)(158.0817, 288.4)	155.0695	6.73	0.0584	388384	72128	338440	54573	-0.72	368240	69431	-0.29	371099	65098	-0.25
Unknown 22		(161.1284, 7509.04)(162.131, 766.19)	160.1211	6.76	0.0597	6875	3814	6462	4681	-0.08	6906	4748	0.01	9577	7354	0.49
Unknown 23		(160.1334, 49939.32)(161.1365, 4018.52)(162.1379, 354.32)	159.126	2.23	0.0682	266261	78407	228721	91632	-0.43	255650	99655	-0.12	280069	80438	0.16
Unknown 24		(60.0812, 8573.77)(61.0844, 383.84)	59.0737	1.58	0.0693	41859	11269	35496	8062	-0.62	37778	9362	-0.40	37898	9147	-0.39
Unknown 25		(192.0738, 440.66)(170.0925, 12025.35)(171.0955, 978.62)	169.0849	6.30	0.0817	25697	9366	26581	9826	0.08	32151	15501	0.56	30390	11304	0.41
Unknown 26		(229.1546, 3635.53)(230.1577, 499.07)	228.1472	4.19	0.0820	29342	6139	28248	10187	-0.15	25344	7358	-0.54	27834	7188	-0.20
Unknown 27		(140.0682, 1389.99)(118.0864, 52088.45)(119.0896, 2747.42)	117.079	4.82	0.0928	176248	38229	155056	40226	-0.48	172379	50028	-0.09	183387	45840	0.16
Tyrosine	60-18-4	(220.0308, 181.4)(182.0812, 11296.07)(183.0845, 1091.79)	181.0739	5.06	0.0945	36686	8697	31975	7339	-0.52	35548	9963	-0.13	37724	8831	0.12
Unknown 28		(142.0475, 177.77)(120.0654, 9629.68)	119.0581	5.72	0.0982	29479	5924	28642	6509	-0.12	30657	8956	0.17	32819	6828	0.47
Unknown 29		(146.1176, 20922.46)(147.1206, 1689.28)	145.1104	3.56	0.1016	158496	32308	143140	24963	-0.48	153459	29570	-0.16	165241	33870	0.21
Unknown 30		(991.6763, 108708.95)(992.6789, 52879.63)(993.6805, 13165.67)(994.6822, 2345.27)(995.6845, 413.61)(1029.6243, 329.28)(1030.6277, 321.16)(1031.6332, 178.88)(1013.6547, 8081.97)(1014.6576, 3947.18)(534.2959, 1843.19)(535.2994, 481.05)(536.2966, 149.35)(496.3432, 1303649.6)(497.3464, 320632.1)(498.3481, 47583.13)(499.3499, 5132.97)	495.3348	1.38	0.1058	12375692	1987437	12566020	1424160	0.10	13287518	1813131	0.49	13036664	1707859	0.36
Unknown 31		(307.1096, 981.13)(176.04, 1720.6)(154.0587, 29713.12)(155.0615, 1411.97)	153.0511	5.57	0.1100	39947	32603	37620	19763	-0.07	45901	30310	0.17	53944	44996	0.39
Unknown 32		(86.0967, 29145.8)(87.1001, 1461.71)	85.0894	4.02	0.1154	100428	27258	85882	17354	-0.55	100394	30823	0.00	102393	24817	0.07
Unknown 33		(86.0966, 20732.41)(87.0998, 1010.78)	85.0896	4.24	0.1168	42324	14148	34550	7659	-0.52	44180	19881	0.12	43129	13949	0.05
Unknown 34		(164.0289, 3515.39)(165.0319, 188.87)	163.0222	5.73	0.1208	19341	2384	18677	2741	-0.25	19618	2851	0.11	20339	2528	0.38
Unknown 35		(625.3023, 142.68)(335.1367, 926.52)(336.1392, 150.28)(313.1552, 21787.27)(314.1581, 4118.36)(315.1606, 469.42)	312.1475	1.83	0.1225	62060	27864	63837	34508	0.06	63739	26953	0.06	72789	27299	0.37
Leucine	61-90-5	(154.0846, 183.35)(132.1023, 146733.89)(133.1052, 9848.34)	131.0946	4.02	0.1341	500402	139360	428988	90602	-0.52	500305	158599	0.00	511492	127233	0.08
Unknown 36		(595.2966, 868.78)(596.2995, 275.34)(573.3149, 17499.62)(574.3174, 5390.26)(575.3197, 1008.53)	572.3071	6.06	0.1352	15371	17554	22587	22551	0.29	26070	25506	0.42	29301	31764	0.55
Isoleucine	73-32-5	(154.0841, 221.43)(132.102, 35169.55)(133.105, 2672.79)(134.107, 188.84)	131.0948	4.24	0.1497	179340	61530	146867	33325	-0.51	186472	84960	0.11	181931	60184	0.04
Unknown 37		(72.0812, 19065.01)(73.0845, 793.56)	71.0738	4.82	0.1512	66406	13822	58598	11844	-0.51	65904	18236	-0.03	67525	15423	0.07
Unknown 38		(304.212, 5152.97)(305.2154, 883.05)	303.2043	1.64	0.1514	8940	7195	12190	10415	0.42	12094	7377	0.41	10891	6373	0.25
Unknown 39		(291.0453, 17695.57)(292.0481, 1651.87)(293.0494, 209.27)	290.0383	1.31	0.1597	72501	18894	71427	14002	-0.06	63804	18008	-0.49	68550	16484	-0.22
Unknown 40		(129.066, 7387.67)(130.0688, 396.1)	128.0585	5.65	0.1662	12697	6400	11358	6882	-0.21	14063	6808	0.22	11112	5261	-0.25
Lysine		(169.0942, 586.41)(147.1128, 32115.87)(148.116, 2230.77)(149.1174, 195.15)	146.1053	7.06	0.1855	98704	41689	77804	23043	-0.53	81373	35024	-0.44	93397	41213	-0.14
Unknown 41		(226.9519, 11668.43)(227.955, 419.72)	225.9449	4.43	0.1872	165941	18564	159507	14070	-0.37	169333	18484	0.19	169259	15647	0.19

					ANOVA	Control		Alcohol			Drug			Tobacco		
Compound ID	CAS	Composite Spectrum	Mass	RT	p-value	Mean	SD	Mean	SD	*d*	Mean	SD	*d*	Mean	SD	*d*
Unknown 65		(203.0528, 90418.34)(204.0558, 5112.9)	202.0453	5.19	0.5492	1117782	238957	1120229	218425	0.01	1256490	277219	0.52	1159485	305283	0.16
Unknown 66		(502.3272, 1628.75)(503.3293, 437.26)(480.3454, 24805.27)(481.3486, 6310.9)(482.3512, 1040.82)(483.353, 127.34)	479.3378	1.16	0.5773	111617	35288	117432	27936	0.18	111130	26754	-0.01	106604	33515	-0.15
Unknown 67		(504.342, 2799.83)(505.3455, 759.03)(482.3611, 49684.61)(483.3639, 11327.33)(484.3662, 1857.99)(485.3697, 223.34)	481.3519	1.45	0.6243	193064	55597	194925	51128	0.03	204610	50925	0.21	189490	58503	-0.06
Unknown 68		(537.169, 1674.94)(538.1718, 366.61)(575.1244, 149.55)(291.0704, 47403.96)(292.0731, 5181.71)(293.0748, 722.27)(559.1514, 10172.03)(560.1538, 2287.24)(561.1558, 430.02)(307.044, 1812.06)(308.0464, 117.52)(269.0882, 13545.5)(270.0911, 1514.14)(271.0927, 157.0)	268.0805	2.63	0.6273	64713	121030	60577	136555	-0.04	66905	131003	0.02	40192	58969	-0.22
Unknown 69		(1039.6716, 6161.51)(1040.6746, 3126.96)(542.3242, 13649.12)(543.3271, 3778.99)(1061.6533, 553.03)(1062.658, 333.05)(558.2613, 609.59)(559.2676, 197.54)(520.3414, 341475.75)(521.3444, 85682.53)	519.3347	1.34	0.6794	1643174	580249	1447285	438412	-0.37	1611404	632769	-0.06	1496069	355035	-0.28
Unknown 70		(225.1323, 190.94)(203.1502, 8542.46)(204.1527, 805.51)	202.1429	6.35	0.7383	36353	9007	38546	8463	0.23	38760	10651	0.25	37632	10371	0.13
Unknown 71		(286.2018, 27489.63)(287.2047, 4185.04)(288.2104, 683.16)	285.1938	1.01	0.7723	79401	43805	72920	36638	-0.15	79424	36436	0.00	86000	44022	0.16
Unknown 72		(192.1592, 12526.83)(193.1623, 1303.88)(194.1636, 124.65)	191.152	1.10	0.8183	53988	20474	61581	43577	0.21	59980	35806	0.16	62487	47570	0.23

Legend: *, statistically significant at corrected α level 0.0012 (Bonferroni's method); RT, retention time, d, Cohen's effect size when compared to the control group; SD, standard deviation; ND, not detected

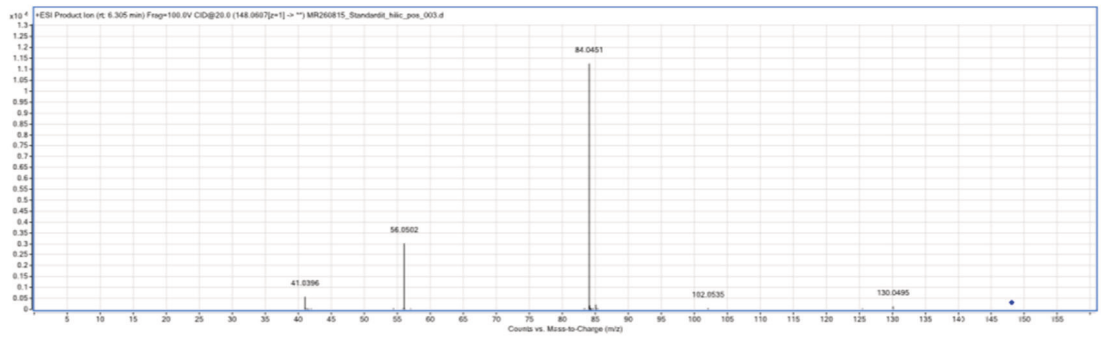
Supplementary file 2: Identification of significantly altered molecular features

Glutamate

Chemical standard:

Retention time 6.3 min

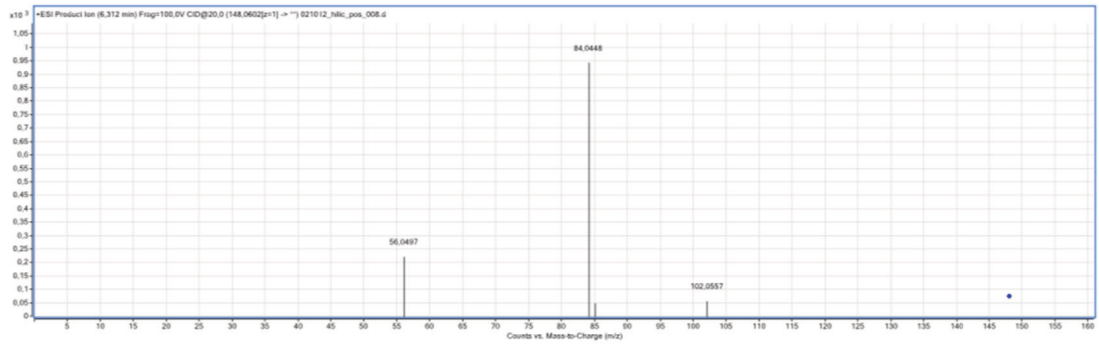
MS/MS spectrum (20 V):



Experimental sample:

Retention time: 6.3 min

Auto MS/MS spectrum (20 V):

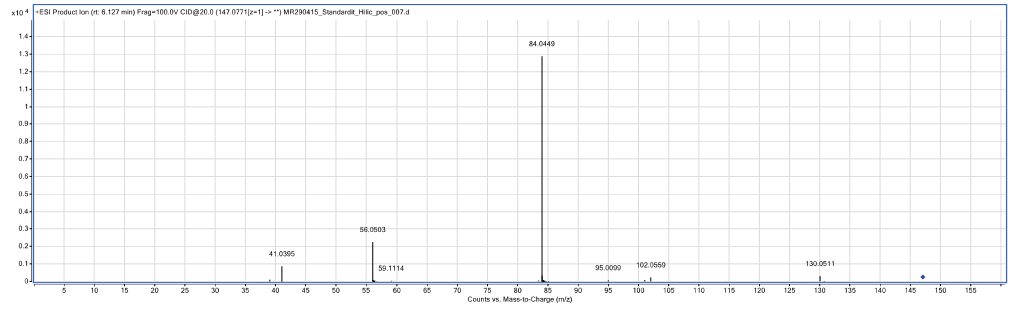


Glutamine

Chemical standard:

Retention time 6.1 min

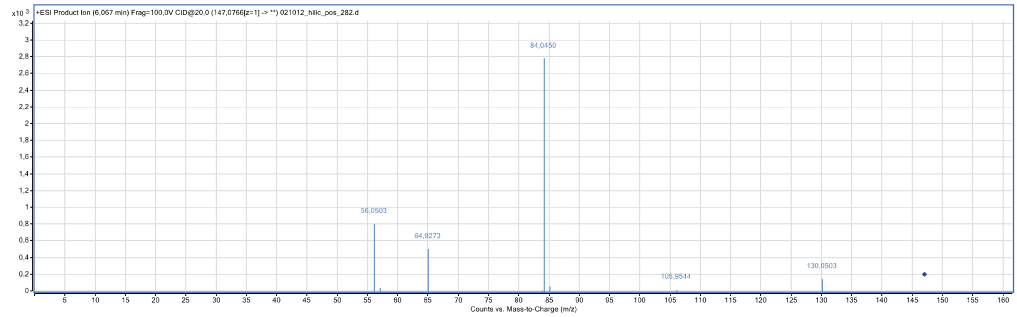
MS/MS spectrum (20 V):



Experimental sample:

Retention time: 6.1 min

Auto MS/MS spectrum (20 V):

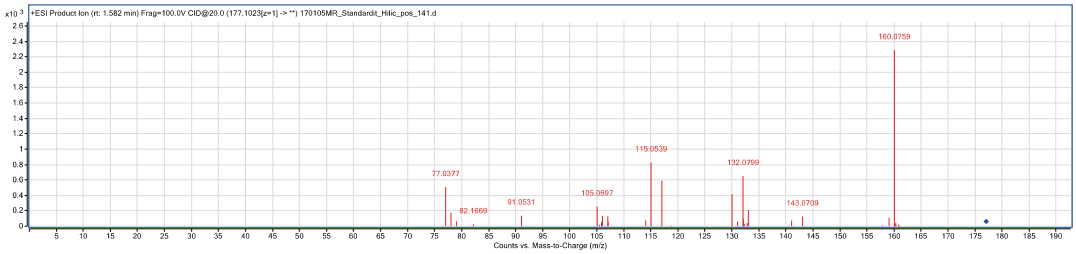


Serotonin

Chemical standard:

Retention time 1.6 min

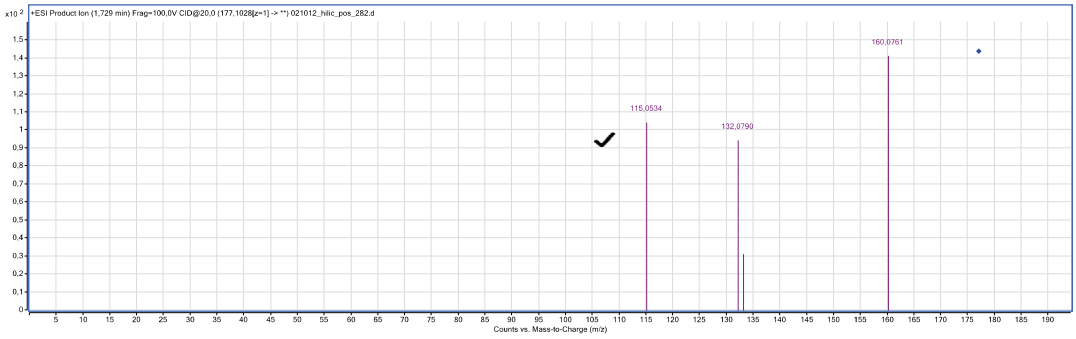
MS/MS spectrum (20 V):



Experimental sample:

Retention time: 1.7 min

Auto MS/MS spectrum (20 V):

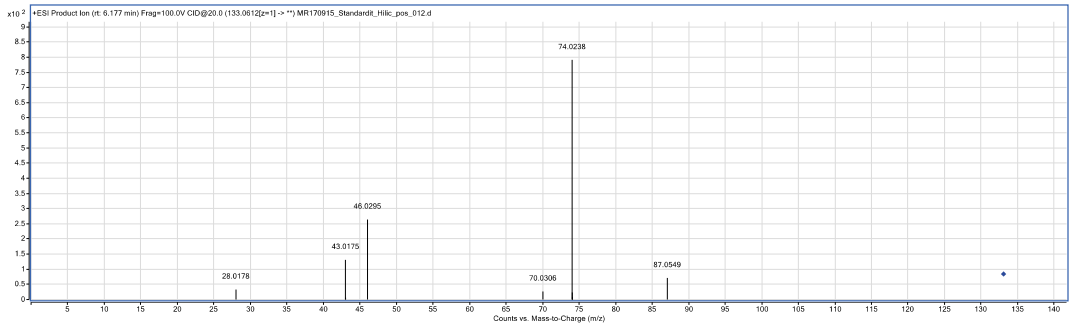


Asparagine

Chemical standard:

Retention time 6.1 min

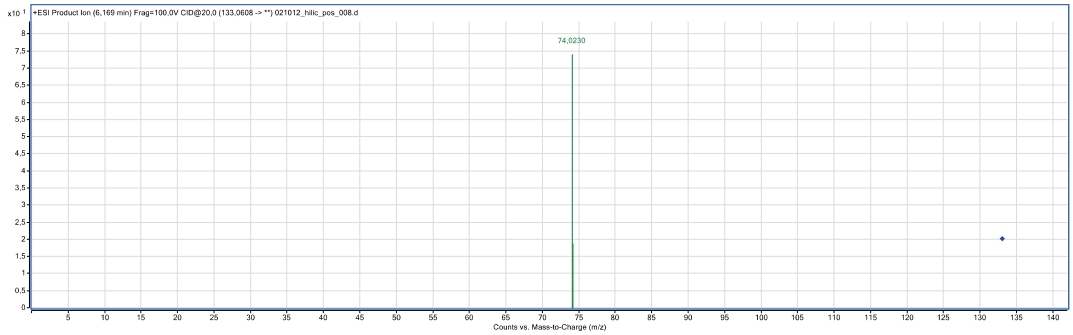
MS/MS spectrum (20 V):



Experimental sample:

Retention time: 6.2 min

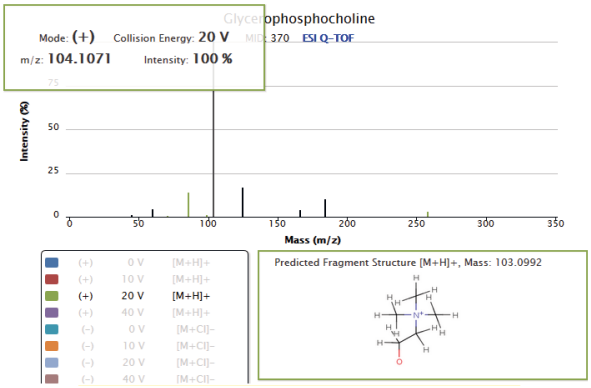
Auto MS/MS spectrum (20 V):



Glycerophosphocholine

Metlin database:

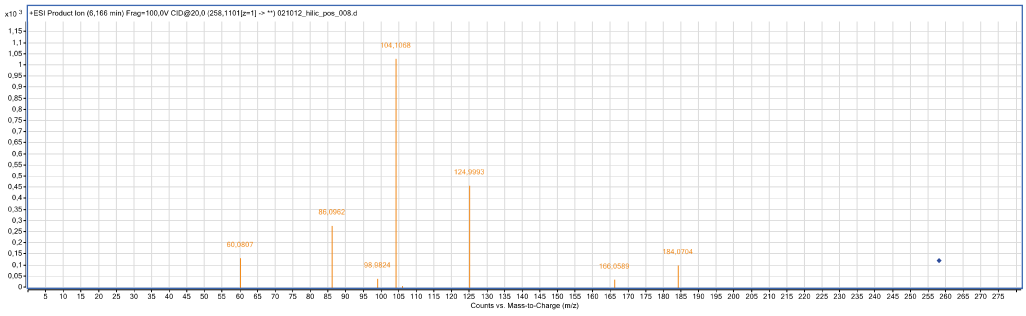
MS/MS spectrum (20 V):



Experimental sample:

Retention time: 6.2 min

Auto MS/MS spectrum (20 V):



ANNI LEHIKOINEN

The aim of this study was to investigate long-term sequelae of alcohol use during pregnancy. We studied serum markers of alcohol-abusing pregnant mothers during the first trimester, investigated alcohol-exposed fetuses during the second trimester by ultrasonography, and followed their outcome until the age of 2.5 years. Furthermore, we performed MRI and SPECT imaging studies in 5 to16 years old children and ophthalmological examination in young adults diagnosed with FASD as children.

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