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Alcohol and substance use are associated with altered metabolome in the first trimester serum samples of pregnant mothers

Short version of title:-Metabolic profile of substance abusing pregnant women

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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.

Objective: Our aim was to evaluate the effect of substance abuse, especially alcohol use, on the metabolic profile of pregnant women during the first trimester.

Study design: 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.

Keywords: alcohol, metabolomics, metabolism, pregnancy, prenatal diagnosis, smoking

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 the pregnancy is a major problem (1-4). 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 (5) pregnant women tend to underreport their alcohol consumption (6). Specific methods for detecting alcohol use, for example from whole-blood or hair samples, have been developed (7). 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 (8), 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 (9). 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 (10,11). These changes in the amino acid metabolism have, for example, been linked to chronic alcohol consumption caused neurobiological adaptations in key neurotransmitter systems, including increased glutamate levels and decreased serotonin levels, to compensate the effects of alcohol exposure and these changes are considered to be important for development of alcohol use disorder (12,13).

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 (14) 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 in order 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.

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) (5,15) 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 the 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 the pregnancy. The inclusion criterion for the tobacco smoking groups was five or more cigarettes per day during the 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 cranio-inferior) 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 (16). In brief, a 100μL aliquot of the first trimester screening fasting serum sample was mixed with 400μL of acetonitrile (VWR International), incubated on an ice bath for 15 min, and centrifuged. The supernatant was filtered through 0.2-μm polytetrafluoroethylene filters in a 96-well plate format. Quality control samples were made by mixing 2μL 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 × 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 demographic 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 Professional (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).

Results

Tables 1 and 2 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 the 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.

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 1, 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 1), 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, Figure 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 significantly decreased serum serotonin levels (mean abundance = 9034, SD = 6606, $d = -0.97$) as compared to controls (mean abundance = 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).

Comment

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 (Figure 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 (12). 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 (17). Alcohol induced increased glutamatergic tonus has been associated with apoptotic neurodegeneration in animal models of fetal alcohol exposure (18).

Our results are in line with the previous investigations of Ramadoss et al. (9) 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 ^{1}H magnetic resonance spectroscopy (19). Increased brain and cerebrospinal fluid glutamate levels have also been associated with chronic alcohol use in adults (20,21) and increased serum glutamate levels have been proposed as a biomarker for acamprosate treatment outcomes of alcohol dependence (22). Furthermore, a recent metabolic profiling study of young adults detected an association between decreased glutamine levels and alcohol consumption (23). Moreover, the significantly decreased asparagine levels in the alcohol using pregnant women (-21% when compared to controls, Figure 1) 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 1) 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 (24). 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 (25). 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 (19,26).

Moreover, we observed significantly decreased serotonin levels in the first trimester serum samples of alcohol using mothers when compared the controls (-43%, Figure 1). 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 (10,11). 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 (10,13,27-29). Furthermore, previous depression studies have revealed an association between depression, alcohol, caffeine, tobacco and illicit drug use during the pregnancy and furthermore, depression is associated with decreased levels of serotonin (30).

The decreased serum levels of glycerophosphocholine (Figure 1) could be linked with nicotine exposure, because glycerophosphocholine 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 phosphocholine levels has been reported in an *in vivo* ^{1}H magnetic resonance spectroscopy study of children with fetal alcohol exposure (19).

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 1). On the other hand, clear differences between alcohol using and tobacco-smoking mothers could be observed in glutamate, glutamine and serotonin levels (Figure 1). 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 (22-24). Furthermore, also other conditions could produce similar pattern of abnormal metabolites. However, for example diabetes and hypertension have been previously associated with increased levels of acylcarnitines, fatty acids and branched-chain amino acids, which were not associated with alcohol use in the present study (19,31,32).

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 consumption 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 1). 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.

Author's roles

H.S., L.M. and A.S. conceived of this study and provided expert knowledge. L.A. collected the data and the samples. H.K., A.S. and L.M. designed the mass-spectrometry analysis. K.O. and H.K. conducted the data preprocessing and analysis. K.O. and L.A. performed statistical analysis and wrote the first draft of the manuscript. All authors contributed to the writing and have approved the final version of the manuscript.

Conflict of interest

The authors report no conflict of interest.

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Condensation

Substance use is associated with a pattern of increased glutamate, and decreased glutamine and serotonin levels.

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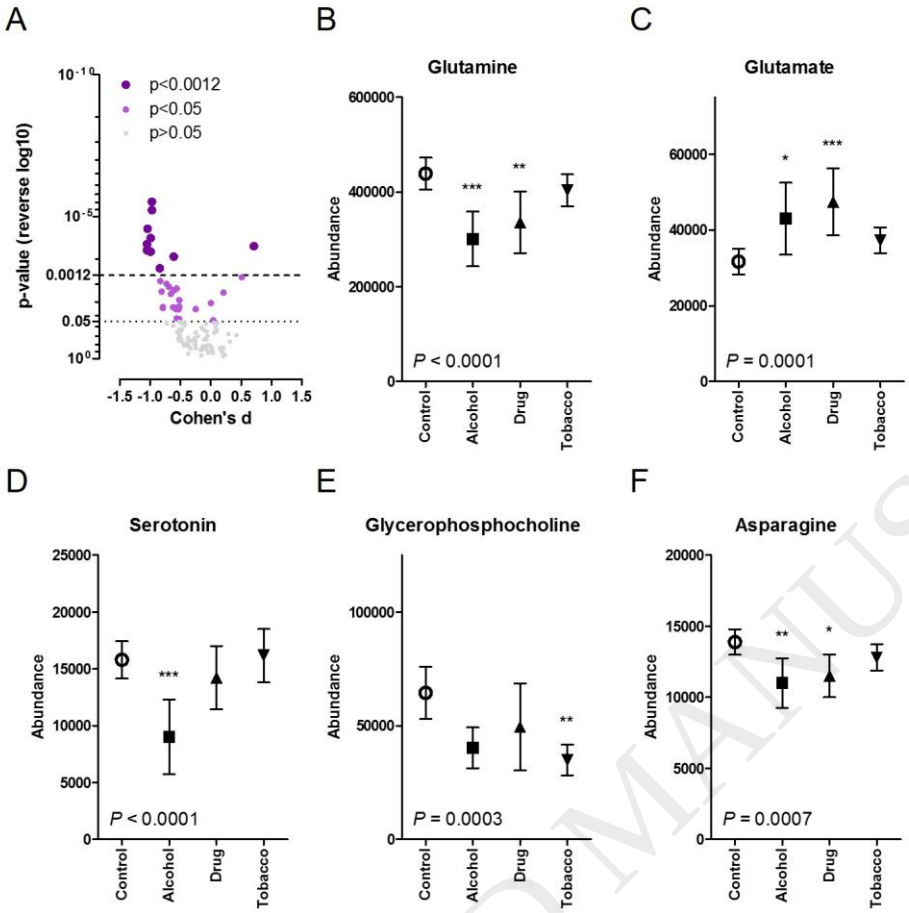
Figure 1: Main results from the metabolite profiling analysis of the first trimester serum samples of pregnant women.



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.

Table 1. 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	0(0%)		10 (52.6%)		**<0.001***	14 (58.3%)		**<0.001***	40(100%)		**<0.001***
Smoking during the	0(0%)		4 (21.1%)		**0.002***	9 (37.5%)		**<0.001***	40(100%)		**<0.001***
Alcohol use before	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	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	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	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	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	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)	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	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	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 2. 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	**0.393**
Birth weight (SD)	-0.28	0.7	-0.51	1.1	**0.401**	-0.35	0.9	**0.672**	-0.38	0.75	**0.527**
Birth head circumference (cm)	35.1	1.2	35.7	1.5	**0.11**	35.3	1.4	**0.485**	35.3	1.6	**0.494**
Birth head circumference (SD)	-0.01	0.88	0.5	1.1	**0.044***	0.15	1.0	**0.480**	0.11	1.14	**0.559**
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	**0.058**
Breech position (n/%)	0 (0%)		0 (0%)		**X**	3 (12.5%)		**0.028***	2 (5%)		**0.171**
Gender girl (n/%)	33 (61.1%)		8 (42%)		**0.176**	11 (45.8%)		**0.244**	15 (37.5%)		**0.050***
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%)		**0.306**

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 for the continuous values to compare differences between the study groups and controls.