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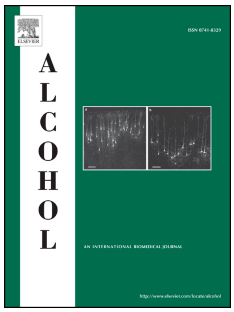

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Differential correlations between maternal hair levels of tobacco and alcohol with fetal growth restriction clinical subtypes

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Abbreviations:

- AGA: Appropriate for gestational age.
- IUGR: Intrauterine growth restriction.
- SGA: Small for gestational age.
- NIC: nicotine
- EtG: ethyl glucuronide

S. Sabra: study design, samples collection, data analysis, interpretation of results, writing the manuscript.

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Abstract

Maternal exposure to tobacco and alcohol is a known cause among others for fetal growth restriction (FGR). Clinically, FGR can be subclassified into two forms; intrauterine growth restriction (IUGR) and small for gestational age (SGA), based on the severity of the growth retardation, and abnormal uterine artery Doppler or cerebro-placental ratio. This study aimed at investigating any differential correlation between maternal exposures to these toxins with the two clinical forms of FGR. Therefore, a case-control study was conducted in Barcelona, Spain. 64 FGR, which was further subclassified into IUGR (n=36) and SGA (n=28), and 89 matched appropriate for gestational age (AGA) were included. The levels of nicotine (NIC) and ethyl glucuronide (EtG), biomarkers of tobacco and alcohol exposure, respectively, were assessed in the maternal hair in the third trimester. Our analysis showed 65% of the pregnant women consumed alcohol, 25% smoked and 19% did both. The ORs of IUGR was 21 times versus 14 times for being SGA with maternal heavy smoking, while with alcohol consumption the ORs for IUGR was 22 times versus 37 times for the SGA group. The differential correlations between these toxins with the two subtypes of FGR suggest different mechanisms influencing fetal weight. Our alarming data of alcohol consumption during pregnancy should be considered for further confirmation among Spanish women.

Keywords ethyl glucuronide, nicotine, fetal growth restriction, IUGR, hair, biological matrices.

Introduction

Birth weight is a strong predictor of pregnancy outcomes, as well as estimating neonatal survival rates, achievement of developmental milestones and health risks ((Bernstein, Horbar, Badger, Ohlsson, & Golan, 2000). The health risks associated with FGR are prematurity, sudden infant death syndrome, various metabolic and neurological complications and delayed effects into adulthood life (Bamfo & Odibo, 2011; Brodsky & Christou, 2004; Cosmi, Fanelli, Visentin, Trevisanuto, & Zanardo, 2011; Hunt, 2007; Ismail & Chang, 2012; Longo et al., 2013; Markel, Engelstad, & Poindexter, 2014). Still, the etiology of FGR is largely unknown. Therefore, investigating factors causing FGR is crucial due to its impact on human health.

Prenatal tobacco and alcohol consumption are considered among the leading causes of FGR despite of being also the most modifiable (Almond, Chay, & Lee, 2005; Hammoud et al., 2005; Ismail & Chang, 2012). In Spain, about 25% of females above the age of 16 smoke (How Tobacco Smoke Causes Disease, 2010). The prevalence rate of women consuming alcohol during pregnancy is 12% to 15%, with 3% to 4% reporting binge drinking; this data is mostly obtained by questionnaires (Bhuvaneswar, Chang, Epstein, & Stern, 2007). However, Garcia detected that 45% of his cohort of pregnant women, in Barcelona, consumed alcohol using biological matrices (Garcia-Algar et al., 2008).

During gestation, the assessment of maternal smoking and alcohol consumption is either by interviewing the mother or by completed questionnaires in the first prenatal visit. Alcohol consumption is mostly recorded as the number of drinks consumed however; various alcoholic drinks contain different concentrations of alcohol. The same concept can be applied for smoking. Furthermore, the majority of pregnant women rarely disclose their smoking and drinking habits for the misapprehension that small amounts of alcohol are inconsequential or fear of stigma or any legal implications (Stone, 2015) or living in a society like Spain. In Spain, drinking is more mundane and integrated into everyday life with high tolerance of high levels of alcohol consumption. Since, there is a high chance of false and/or under reporting, then estimating the association between FGR with the exposure to these toxins is challenging.

Yet, various studies established a correlation between FGR with the exposure of these toxins (Hellerstedt, Himes, Story, Alton, & Edwards, 1997; Meis et al., 1997). It was shown that prenatal smoking increased the rates of FGR and preterm delivery (Hammoud et al., 2005; Magee, Hattis, & Kivel, 2004; Windham, Hopkins, Fenster, & Swan, 2000). For alcohol consumption during pregnancy, the risk for FGR was found to be directly proportional with the amount of alcohol intake (Mariscal et al., 2006). Also, Lundsberg documented increased risk of preterm delivery with maternal alcohol use, which was associated with major comorbidities including FGR (Lundsberg, Bracken, & Saftlas, 1997).

Recently, NIC and EtG are used as reliable biomarkers to detect tobacco and alcohol exposure, respectively, in human hair (Bakhireva & Savage, 2011; Florescu et al., 2009;

Silins & Hogberg, 2011). In the growing hair, these toxins are delivered and retained for long-term compared to the other biological samples such as blood and urine, which cover only few days prior to testing (Jacob, Yu, Shulgin, & Benowitz, 1999; Mistriotis & Andreadis, 2013; Pragst & Balikova, 2006; Uematsu, 1993).

Cotinine, which is a major metabolite of nicotine, has been used as a biomarker distributed in various body fluids including the blood, saliva and urine (Jung et al., 2012). However, most studies on pregnant women used either blood, saliva or urine samples to detect cotinine levels (Bowker, Lewis, Coleman, Vaz, & Cooper, 2014; Polanska, Hanke, Laudanski, & Kalinka, 2007). Cotinine concentration in body fluids of pregnant women has been shown to differ from the normal adult population (Haddow, Knight, Palomaki, & McCarthy, 1988). Dempsey documented that during pregnancy, the metabolic clearance of cotinine was significantly higher (140%) compared to nicotine (60%), the half-life of cotinine was shorter, the plasma levels of cotinine were lower compared to nicotine (Dempsey, Jacob, & Benowitz, 2002).

Rebagliato et al found marked differences between antenatal and postnatal cotinine concentrations in smokers after controlling for tobacco consumption. The salivary cotinine levels during pregnancy was significantly decreased compared with postnatal levels, which suggest an altered metabolism and distribution of nicotine and cotinine during pregnancy, with higher clearance rates of cotinine compared with non-pregnant women (Rebagliato et al., 1998).
Therefore, we have reason to believe that during pregnancy, cotinine may not be the optimal marker used for smoking.

Furthermore, investigations concerning birth weight have led to a new hypothesis, which states that FGR, which is defined as estimated fetal weight (EFW) $<10^{th}$ centile, may present with two different clinical forms during gestation. The first, with an abnormal uterine artery Doppler or cerebro-placental ratio, and/or growth centile below the 3^{rd} centile, with poorer perinatal outcome is called “intrauterine growth restriction” (IUGR). The second, with normal Doppler studies, with near-normal perinatal outcome, is termed “small for gestational age” (SGA) (Figueras & Gratacos, 2014).

Therefore, a prospective case-control study was carried out to evaluate maternal exposure to smoking and alcohol, determined by the levels of NIC and EtG, in the maternal hair, in a Southern European population. Also, to explore any differential correlations between the detected levels of these compounds with the two different clinical subtypes of FGR.

Materials and methods

Study population

Pregnant women were recruited from Barcelona Center of Maternal-Fetal and Neonatal Medicine, Hospital Sant Joan de Déu and Hospital Clínic, University of Barcelona, Spain. Exclusion criteria included multiple gestations, fetuses with congenital anomalies, large for

gestational age (LGA), defined by birth weight >95th centile, maternal diagnosis with any morbidity related or unrelated to pregnancy including chronic hypertension, diabetes, autoimmune disorders, cardiac and renal health problems. The institutional Hospital Ethical Committee (PIC-86-14) approved the research protocol for this study. 153 patients were enrolled in the study. Each patient signed an informed consent after receiving a thorough explanation of the study, prior to sample collection.

After delivery for cases ascertainment, birth weights were recalculated in centiles, adjusted for the gestational age at delivery and neonatal sex using reference curves (Figueras et al., 2008). The patients were divided into three groups: AGA, IUGR and SGA based on centiles, the cerebro-placental ratio and uterine artery pulsatility index values in the last ultrasound in the third trimester.. The AGA group is defined as the EFW >10th centile, while IUGR is determined as the EFW is <3rd or <10th centile with cerebro-placental ratio <5th and/or mean uterine artery pulsatility index >95th centile pathological Doppler, while SGA group is the EFW between 3rd and 10th centile with normal feto-placental Doppler. The total number of participants were 153, AGA; n = 89, IUGR; n = 36 and SGA; n = 28.

Sample collection

Hair samples were collected from the recruited patients in their late third trimester of pregnancy. Approximately 100-200 mg of hair was collected by cutting as close to the scalp as possible from the vertex on the back of the head. Each cm of the scalp hair reflects approximately one month of past exposure (Uematsu, Mizuno, Nagashima, Oshima, & Nakamura, 1995). The proximal 1 cm of the hair represents last month's exposure, therefore the whole length of the hair may replicate the average dose of exposure during pregnancy. The minimum hair length collected was 9 cm. The end closest to the scalp was clearly marked and stored at room temperature until analysis. Hair samples were gathered independently of the patient's disclosure of tobacco and alcohol consumption.

Hair analysis

Analytes under investigation were measured by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Briefly, total segments were washed with 2.5 mL dichloromethane and 2.5 mL methyl alcohol and after dried, reduced in short cuts and processed using a ball mill (mixer mill MM 200; Retsch, GmbH & Co., Haan, Germany) for 30 min at 30 amplitude units until obtaining a fine grey powder. 25 milligrams of this powder were weighed in a glass tube and were added to 10 ng of N-ethylnorcotine (25 μg/mL) and 10 pg of EtG-d5 (0.05 μg/mL). Internal standards used for NIC and EtG determination, respectively, and treated with 500 μL M_3 buffer reagent (Comedical, Trento, Italy) for 1 h at 100 °C. Then, the treated samples were cooled at room temperature and a sample volume of 10 μL was injected in UHPLC-MS/MS. For EtG analysis, M_3 buffer reagent extract was directly injected while for NIC, 100 μL of the M_3 buffer reagent extract was diluted with 900 μL of water before injection. The separated analytes were detected with

a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode via positive electrospray ionization. MRM transitions were m/z 163.1 > 117.0, 163.1 > 132.6 for NIC, and m/z 221.1→75.0, 221.1→85.0, for EtG and m/z 226.0→75.0, 226.0→85.0 for EtG-d5. The validated method showed a limit of quantification at 0.1 ng/mg and a limit of detection at 0.03 ng/mg for NIC and a limit of quantification at 7 pg/mg, and limit of detection at 3 pg/mg for EtG.

NIC levels were divided into three categories: Category 1; low exposure (<0.9 ng/mg), Category 2; active smokers (1 – 3 ng/mg) and Category 3; heavy smokers (>3 ng/mg) (Pascal Kintz, Russell, Baber, & Pichini, 2015). Likewise, EtG levels were distributed into three categories; abstinence (< 7pg/mg), social or excessive consumption (>7 and <30 pg/mg), and the chronic excessive consumption group (>30 pg/mg) (P. Kintz, 2015).

Statistical analysis

IBM SPSS 24.0 for windows was used for the statistical analysis. Descriptive data were calculated prior to any correlation analysis. Results presented as mean and ± standard deviation (SD) or median and interquartile range (IQR), after investigating normality distribution by Shapiro-Wilk test. Comparisons among the groups were performed using parametric [Analysis of variance (ANOVA) and posthoc test] and non-parametric [Kruskal-Wallis and Mann Whitney] tests depending on data normality. Correlation analyses between maternal NIC and EtG detected levels with the two clinical subgroups of FGR were evaluated using regression models by adjusting for covariates including maternal age, gestational age, maternal BMI, parity, the neonatal sex. A p value < 0.05 was considered statistically significant.

Results

Background data

Background characteristics are presented in Table 1. In general, we did not detect major differences between the three groups AGA, IUGR and SGA regarding the maternal age, gestational age and BMI. We noted that the number of nulliparous and multiparous women in the FGR group were 34 (22%) and 30 (20%), respectively. Among the 153 patients, FGR were 64 (42%). In the FGR group, 42 (66%) of the neonates were males and 22 (34%) were females. While in the AGA group, 41(46%) of the neonates were males and 48 (54%) were females. We detected a significant differences between mean values of the birth weight among the three groups, at the p<.05 level [F (2, 150) = 187.7, p = 0.000]. Post hoc comparisons using the Tukey test indicated that the mean of AGA (M = 3296, SD = 299) was significantly higher than the IUGR (M = 2069, SD = 421) and SGA (M = 2741, SD = 245). Also, IUGR mean values was significantly higher (M = 2069, SD = 421) than SGA group."

Table 1 shows the detailed demographic data of the three groups.

Table 1. Demographic data of the recruited pregnant women. n: number of patients; (%): percentage of the total; SD: standard deviation; AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; Analysis of variance (ANOVA) and post hoc comparisons using the Tukey: *** $p < 0.001$.

Figure 1.

Fig 1. Neonatal birth weights of the three groups. Mean birth weights of the AGA (white), IUGR (grey) and SGA (dashed) groups are presented, significant differences in the birth weight of the AGA compared to the IUGR and SGA ($p<0.001$). Error bars represent standard deviations (SD). Increased mean birth weight of the SGA compared to the IUGR group ($p<0.001$). AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; Analysis of variance (ANOVA) and post hoc comparisons using the Tukey test, *** $p < 0.001$.

Nicotine analysis

The maternal hair detected levels of NIC in the three groups are illustrated in Table 2.

Table 2. Maternal hair levels of NIC detected in the three groups. N: number of samples; NIC: nicotine; AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; SD: standard deviation; IQR: interquartile range. Kruskal-Wallis and Mann Whitney test were used to compare the levels of NIC; ***p<0.001.

A Kruskal-Wallis H test showed that there was a statistically significant difference in the maternal hair levels of NIC between the different groups, χ^2 (2) = 19.59, p<0.001, with a mean rank of 63 for AGA, 85 IUGR and 109 for SGA. Using a Mann-Whitney U test, we detected a significant increase in the maternal hair levels of NIC in all FGR compared to the AGA group (U = 1656, p<0.001). Also, NIC levels in each of FGR subgroups; IUGR (U= 1174, p <0.01) and SGA (U = 482, p<0.001) showed a significant increase compared to AGA (Figure 2). However, the maternal hair NIC levels in the IUGR and the SGA groups did not show any differences.

Figure 2. Box and whisker plot of maternal hair levels of NIC (ng/mg) in the AGA (white box), IUGR (grey box) and SGA (dashed box). Boxes represent the 25th and 75th percentiles. The median is the bold central line, whiskers denote the minimum and maximum values and circles indicate outliers. Error bars represent standard deviations (SD). Significant increases in the levels of NIC in IUGR, and SGA compared to the AGA group were noted. NIC: nicotine; AGA, appropriate for gestational age; IUGR, intrauterine growth restriction; SGA, small for gestational age. Kruskal-Wallis and Mann Whitney test were used to compare the levels of NIC; ***p<0.001.

We, also, calculated the distribution of the mothers and their neonates among the three categories of NIC exposure. Our analysis showed that 24% (37/153) of mothers were smokers, 16 % (25/153) of the smoking mothers were active smokers and 8 % (12/153) were heavy smokers. The percentages of FGR born in the category 1(low exposure), category 2 (active smokers) and category 3 (heavy smokers) were 39, 60 and 91 %, respectively (Figure 3).

Figure 3. Percentages of the neonates in each of the three groups AGA (white), IUGR (grey) and SGA (dashed), distributed among the three categories of NIC levels. AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age.

Our linear regression analysis showed a significant negative correlation between the maternal hair levels of NIC with birth weight (F (1, 151) =11.02, p≤ 0.001), with an R^2 of 0.68. Our results did not change with adjusting for other covariates, however the gestational age (ß=0.5, p<0.001) was the most influential factor in reducing birth weight compared to the others.

In the binary regression analysis of birth weight as outcome (AGA and FGR), and the NIC levels (continuous variable) as exposure, the odds ratio (ORs) of being FGR was 1.4 compared to the AGA group {p<0.05, 95% CI (1.0 -1.9)}. Our multinomial regression analysis showed that the ORs for either being IUGR {p<0.05, 95% CI (1.0 -2.0)} or SGA {p<0.05, 95% CI (1.0 -1.9)} was 1.4 compared to the AGA group among smoking mothers. Upon adjusting for the covariates, the results did not differ however; the male sex was significantly {p<0.05, 95% CI (0.2 -1.0)} influencing the IUGR group.

Binary Regression analysis of birth outcome (AGA and FGR), using the NIC categories as exposure, we noted that ORs of being born with FGR was 2 times {p<0.001, 95% CI (1.0-6.7)} if mothers were active smokers compared to the low exposure group. Also, the ORs of being born with FGR was 20 times {p<0.001, 95% CI (2.60- 167.73)} if mothers were heavy smokers compared to the low exposure group. No changes were detected with adjusting for the covariates.

Using the exposure categories of NIC, in the multinomial regression model we noted that ORs of being IUGR {p<0.01, 95% CI (2.5 - 81.4)} was 21 times while the ORs was 14 times for being SGA {p<0.05, 95% CI (1.4 -45.9)} when mothers were heavy smokers. When mothers were active smokers, the ORs for being born SGA was 3.5 times {p<0.05, 95% CI (1.2 -9.8)} however the results for IUGR did not reach significance [ORs=1.01 {p= 0.09, 95% CI (0.3 - 3.4)}]. These results did not change with adjusting for the other covariates.

Alcohol analysis

The maternal hair detected levels of EtG in the three groups are illustrated in Table 3.

Table 3. Maternal hair levels of EtG detected in the three groups. EtG: ethyl glucuronide; AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; SD: standard deviation; IQR: interquartile range. Kruskal-Wallis and Mann Whitney test were used to compare the levels of EtG; ***p<0.001.

A Kruskal-Wallis H test showed that there was a statistically significant difference in the maternal hair levels of EtG between the different groups, $\chi 2$ (2) = 68.57, p<0.001, with a mean rank of 52 for AGA, 109 IUGR and 115 for SGA. Using a Mann-Whitney U test, we detected a significant increase in the maternal hair levels of EtG in all FGR compared to the AGA group (U = 595, p<0.001). Also, the EtG levels were significantly increased in each of IUGR (U= 411, p<0.001) and SGA (U= 184, p<0.001) compared to the AGA group (Figure 4). However, the maternal hair EtG levels were not different when the IUGR and the SGA groups were compared.

Figure 4. Box and whisker plot of maternal hair levels of EtG (pg/mg) in the AGA (white box), IUGR (grey box) and SGA (dashed box). Boxes represent the 25th and 75th percentiles. The median is the bold central line, whiskers denote the minimum and maximum values and

circles indicate outliers. Error bars represent standard deviations (SD). Significant increases in the levels of EtG in IUGR, and SGA compared to the AGA group were noted. EtG: ethyl glucuronide; AGA, appropriate for gestational age; IUGR, intrauterine growth restriction; SGA, small for gestational age. Kruskal-Wallis and Mann Whitney test were used to compare the levels of EtG; ***p<0.001.

We used the three categories of EtG exposure to stratify the distribution of the participants and their neonates in our cohort. Our analysis showed that 65% (99/153) of the pregnant women belonged to the social and chronic excessive drinking categories, and 35% (54/153) of the pregnant patients were in the abstinent category. The percentages of the neonates in each of the three groups, detected in each category of the EtG levels are shown in Figure 5.

Figure 5. Percentages of the neonates in each of the three groups AGA (white), IUGR (grey) and SGA (dashed), distributed among the three categories of the EtG levels. AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age.

Our linear regression model analysis using birth weight as a numerical outcome and the maternal hair levels of EtG as a predictor, we detected a negative correlation between maternal hair levels of EtG and birth weight ($F(1 , 151)=37.2$, $p\leq0.001$), with an R^2 of 0.2. These results did not change with adjusting with the other covariates; however, the gestational age showed significant influence on birth weight (ß=0.5, $p<0.001$).

Binary regression analysis of birth weight outcome (AGA and FGR) and EtG levels (continuous variable) as exposure showed that the ORs of being born with FGR was 1.3 if mothers consumed alcohol during pregnancy {p<0.001, 95% CI (1.2- 1.4)} compared to the AGA group. Multinomial regression analysis showed the same ORs of 1.3 {p<0.001, 95% CI (1.2- 1.5)} for either being IUGR or SGA when mothers consumed alcohol during pregnancy. Upon adjusting for the covariates, our results showed that the maternal age (p<0.05) and the gestational age (p<0.001) were the only covariates, which showed significance only in the IUGR group.

Binary Regression analysis of birth outcome (AGA and FGR), using the EtG categories as exposure, we noted that ORs of being born with FGR was 28 {p<0.001, 95% CI (8.2- 97.4)} if mothers consumed alcohol socially compared to the abstinent group. In the multinomial regression model, using the EtG categories as exposure, the ORs of being born IUGR was 23 times {p<0.001, 95% CI (5.2- 101.4)} and was 38 times {p<0.001, 95% CI (4.9- 288.4)} for being born SGA when mothers consumed alcohol socially compared to the abstinent category. No changes were detected with adjusting for the covariates.

Cross tabulation showing the distribution of maternal exposure to smoking and alcohol during pregnancy in the form of categories Table 4.

Table 4. Cross tabulation of the maternal exposure to smoking and alcohol during pregnancy. EtG: ethyl glucuronide; NIC: nicotine; Cat: category.

Our analysis showed that 46 % (70/153) pregnant women consumed alcohol but they did not smoke. About 19 % (29/153) smoked and consumed alcohol during pregnancy. Only 30% are exposed to low levels of alcohol and smoking. The percentage of FGR born to mothers who smoked was 37.5 % (24/64) and to those who consumed alcohol was 95% (61/64). Also, we detected a significant correlation between maternal hair levels of NIC and EtG ($r = 0.3$, $p<0.001$). Our regression model to predict the impact of exposure to both toxins, the OR of being born with FGR was 3.6 ($p<0.05$) when mothers smoked and consumed alcohol during pregnancy compared to the AGA group.

In our linear model to investigate the direct influence of maternal alcohol consumption by adjusting for smoking, we detected that the alcohol consumption was the main predictor influencing birth weight {$p<0.001$, 95% CI (1.2-1.5)}. Likewise, in binary regression model, the maternal EtG exposure was significantly correlated to FGR {$p<0.001$, 95% CI (1.2-1.4)} while adjusting for maternal smoking. In the multinomial regression model, maternal alcohol exposure maintained its significance for both clinical forms of FGR, by adjusting for smoking.

Discussion

Our analysis showed that the percentage of women, who smoked were 24 % and consumed alcohol were 65 % during pregnancy. The percentage of patients who actively smoked and consumed alcohol simultaneously were 19 %. Our data showed higher percentage of alcohol consumption among pregnant women compared to the previously published data.

The percentage of FGR born to mothers who smoked was 37.5 % and those who were born to alcohol consuming was 95%. Also, we detected increased levels of both NIC and EtG in the maternal hair of FGR compared to the AGA group. Alongside, there was an increased risk for being an IUGR when mothers were active smokers. In contrast, there was an increased likelihood of being born as SGA when mothers were consuming alcohol socially during gestation.

In general, our study was able to replicate previous findings regarding the negative influence of maternal exposure to these toxins and FGR (Aliyu et al., 2009; O'Leary, Nassar, Kurinczuk, & Bower, 2009; Yang et al., 2001). However, we detected risk differences between the FGR two clinical subtypes with maternal tobacco and alcohol exposure. This finding may reflect different mechanisms affecting birth weight. Yet, the biological mechanism through which maternal smoking and alcohol consumption affect fetal birth weight is to be determined. The most accepted theory is that cigarette smoke contains carbon monoxide, nicotine, and other toxic components, which can be responsible for birthweight

reduction (Wickstrom, 2007). While alcohol crosses freely both the placenta and the fetus' blood–brain barrier (Ross, Graham, Money, & Stanwood, 2015), which may also affect fetal growth. It has been shown that the fetus is exposed longer to the same amount of alcohol than the mother, as the fetus has less alcohol dehydrogenase to metabolize the alcohol than does the mother (Flynn, Marcus, Barry, & Blow, 2003), causing hypoxia, impaired cell proliferation and affect placental development (Abel, 1982) and Fetal Alcohol Spectrum Disorders (FASD). FASD has a major negative impact on fetal neurodevelopment and cognitive functions and fetuses may present with reduced weight (Squeglia, Jacobus, & Tapert, 2009). We reasoned that the dissimilarities in the risk we noted between the FGR clinical subtypes as a result of maternal exposure to smoking and alcohol due to the different toxic components in each of these toxins and their various metabolite pathways in the mother and fetus.

Smoking and alcohol consumption tend to be highly related, and several studies found it challenging to distinguish between the effects of the two toxins on fetal weight. However, it has been suggested that maternal smoking and alcohol intake during pregnancy may have a synergistic effect that may lead to an increased likelihood of FGR (Odendaal, Steyn, Elliott, & Burd, 2009). Our results showed that the reduced birth weight was mainly driven by maternal alcohol consumption and maternal smoking may aggravate the negative influence of maternal alcohol consumption on birth weight.

Despite of the consistent published association between maternal alcohol consumption and FGR, the negative correlation between maternal consumption of moderate amounts of alcohol and FGR has been controversial (Chiaffarino et al., 2006; Day et al., 1989; Passaro, Little, Savitz, & Noss, 1996; Shu, Hatch, Mills, Clemens, & Susser, 1995; Windham, Fenster, Hopkins, & Swan, 1995). From a clinical prospective, women are advised to withhold tobacco and alcohol consumption during pregnancy. They are even encouraged to abstain alcohol intake prior to getting pregnant, as there is no safe drinking level during pregnancy. However, our results confirmed that women residing in Barcelona continued to smoke and consume alcohol during gestation. Even more, the high percentage of women consuming alcohol during pregnancy is an alarming result as it reflects women's behavior during their pregnancy. Spanish society is well known with a long-lasting tradition of alcohol consumption and is labelled as 'wet drinking cultures' which has high tolerance to high levels of alcohol consumption and the consequences of such behavior (Gordon, Heim, & MacAskill, 2012). Public awareness campagnas should be implemented targeting maternal behavior and habits during gestation in such high-risk society and their implication on fetal wellbeing and pregnancy outcomes, in parallel to defining screening tests to detect the high-risk group. Hence, the consistent use of a validated screening test should improve the identification of prenatal alcohol use.

Of note, our study has several limitations. We are aware of the relatively small sample size in in each of the groups, and thus the results warrants further confirmation in larger-scale, well-designed studies and to clearly examine the unique contribution of varying maternal exposures, the magnitude and timing of these exposures on fetal growth deficits. Another

point worth considering is the EtG levels in the maternal hair samples differentiate between moderate social drinking and chronic alcohol abuse. This enables us to correlate the adverse outcomes with the estimated amounts of consumed alcohol. However, this marker lacks the ability to neither confirm absolute abstinence nor estimate previous alcohol consumption (Pascal Kintz et al., 2015), which creates uncertainty about the effects of low levels of alcohol consumption during pregnancy.

To our knowledge, this is the first report investigating the correlation between the levels of NIC and EtG detected in the maternal hair with different clinical small fetuses' subgroups. Our results showed differential correlations between maternal alcohol consumption with the SGA, and selective association between maternal smoking and the IUGR group, which is worth exploring in the future.

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	AGA Group	IUGR Group	SGA Group
n (%)	89 (58%)	36 (24%)	28 (18%)
Maternal Age, Mean (SD)	32 (5)	34 (4)	30 (5)
Gestational Age, Mean (SD)	39 (1.4)	37 (2.1)	39 (1.7)
BMI (kg/m2)	23.2	23.6 (4.8)	23
Parity			
Nullipara, n (%)	45 (29%)	18 (12%)	16 (11%)
Multipara, n (%)	44 (29%)	18 (12%)	12 (8%)
Neonate sex			
Female, n (%)	48 (31%)	11 (7%)	11 (7%)
Male, n (%)	41 (27%)	25 (16%)	17 (11%)
Birth Weight (gm), Mean (SD)	3296 (299)***	2069 (421)***	2741 (245)

Table 1

Table 1. Demographic data of the recruited pregnant women. n: number of patients; (%): percentage of the total; SD: standard deviation; AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; Analysis of variance (ANOVA) and posthoc test: *** $p < 0.001$.

Groups	N	NIC (ng/mg) Mean (SD)	NIC (ng/mg) Median (IQR)	NIC (ng/mg) Geometric Mean
AGA	89	0.51 (1.1)	0.19 (0.4)	0.2
IUGR	36	4.4 (12)	0.4 (1.7)***	0.5
SGA	28	2.2 (4.4)	0.9 (1.5)***	0.9

Table 2

Table 2. Maternal hair levels of NIC detected in the three groups. N: number of samples; NIC: nicotine; AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; SD: standard deviation; IQR: interquartile range. Kruskal-Wallis and Mann Whitney test were used to compare the levels of NIC; ***$p<0.001$.

Table 3

Groups	N	EtG (pg/mg) Mean (SD)	EtG (pg/mg) Median (IQR)	EtG (pg/mg) Geometric Mean
AGA	89	7.6 (5.1)	6.7 (3.5)	6.8
IUGR	36	15.3 (8.4)	14 (11)***	14
SGA	28	14.5 (4.5)	14 (6)***	14

Table 3. Maternal hair levels of EtG detected in the three groups. EtG: ethyl glucuronide; AGA: appropriate for gestational age; IUGR: intrauterine growth restriction; SGA: small for gestational age; SD: standard deviation; IQR: interquartile range. Kruskal-Wallis and Mann Whitney test were used to compare the levels of EtG; ***$p<0.001$.

Table 4

EtG Categories	NIC Categories		
	Cat 1 Low Exposure	Cat 2 Active Smokers	Cat 3 Heavy Smokers
Cat 1 Abstinence	46	8	0
Cat 2 Social or Excessive Consumption	68	17	11
Cat 3 Chronic Excessive Consumption	2	0	1

Table 4. Cross tabulation of the maternal exposure to smoking and alcohol during pregnancy. EtG: ethyl glucuronide; NIC: nicotine; Cat: category.

Figure 2

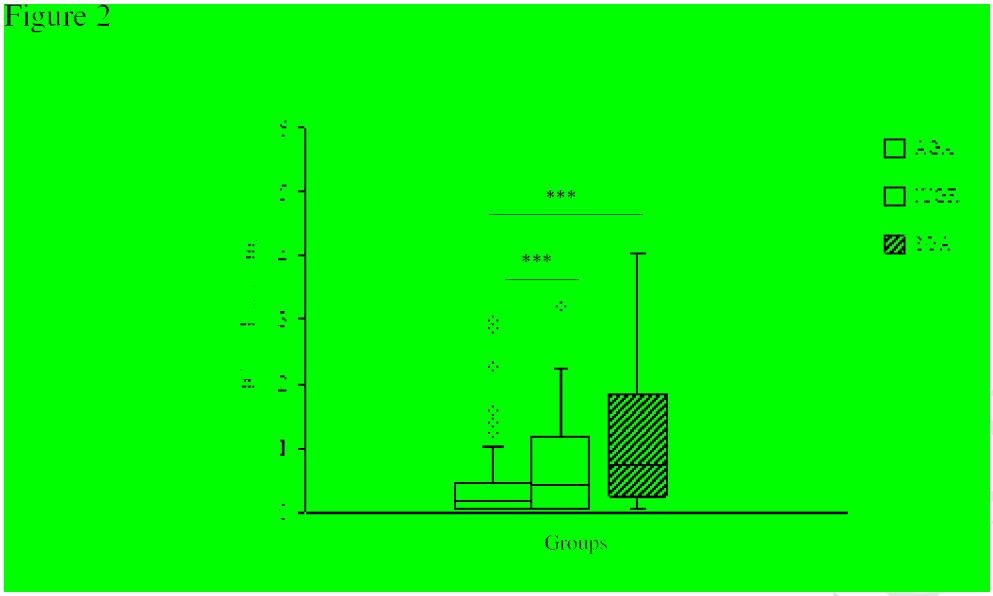


Figure 4

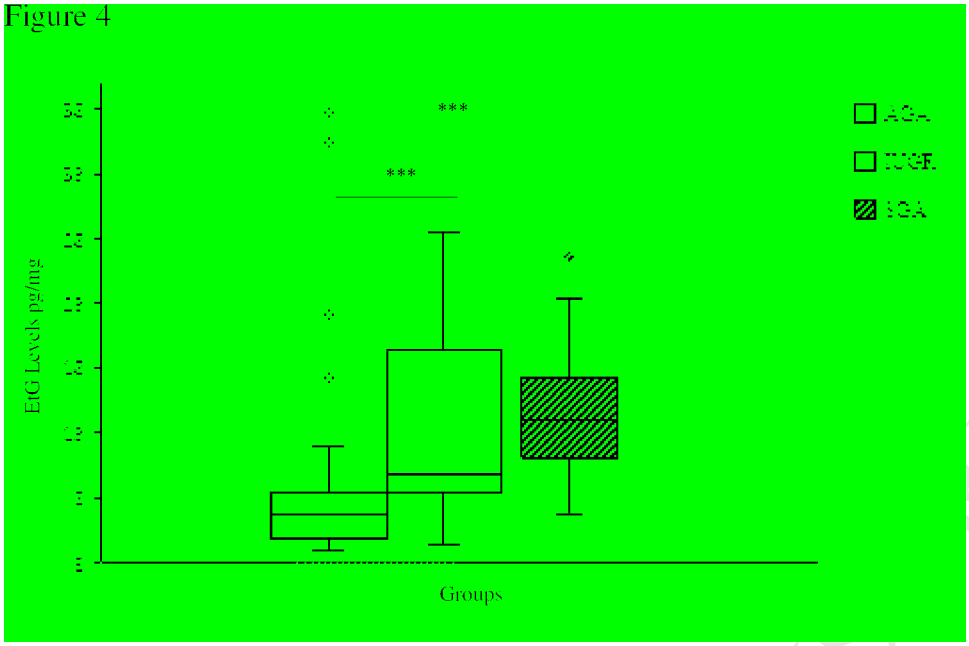


Figure 1.

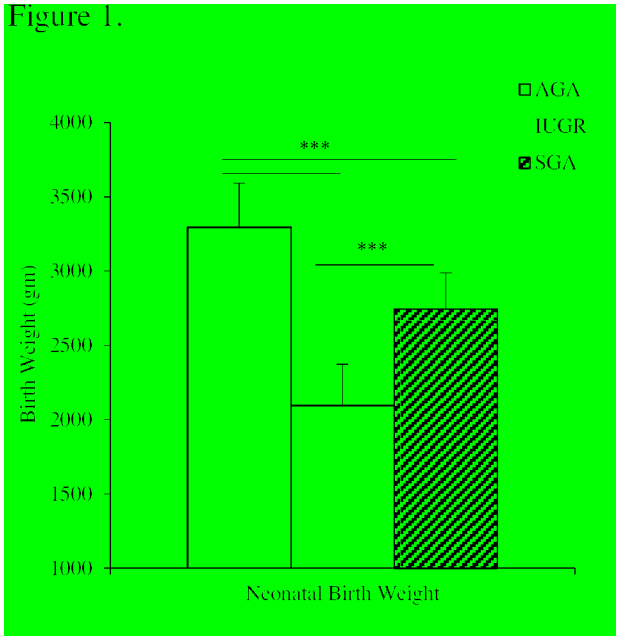


Figure 3

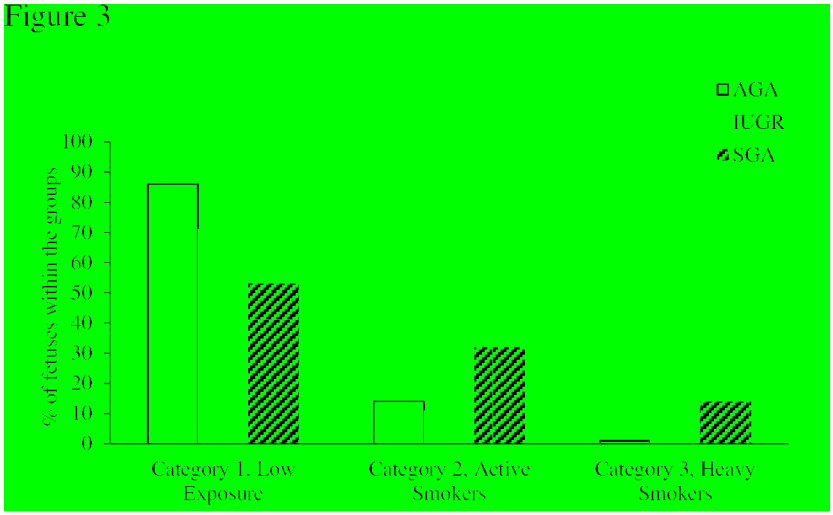
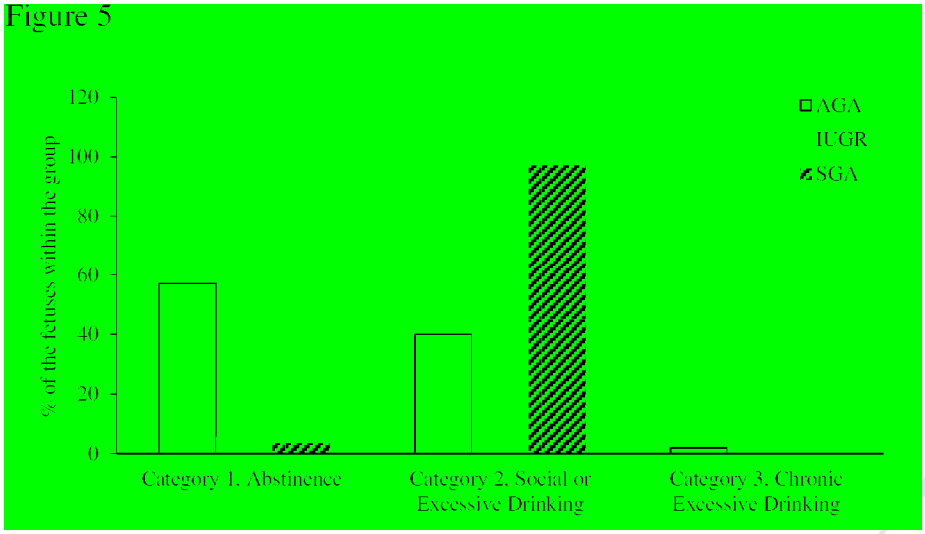


Figure 5



Highlights

- Our results showed selective correlations between maternal hair levels of NIC and EtG with the clinical subtypes of FGR.
- These results suggest different mechanisms affecting fetal weight.
- Alcohol consumption was the main factor affecting fetal birth weight compared to tobacco.
- The increased detected levels of EtG in the maternal hair of pregnant women in Barcelona is alarming as it reflects maternal unawareness of the negative influences of alcohol of fetal wellbeing.