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1 **Quantification of ethyl glucuronide, ethyl sulfate, nicotine and its metabolites in human**
2 **fetal liver and placenta**

3
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28 **Abstract**

29 *Purpose* Tobacco and alcohol use during pregnancy are serious public health concerns and result
30 in adverse developmental outcomes. Identifying in utero exposure is often achieved through
31 meconium analysis or via maternal self-report. In this study, we analyzed fetal liver and placenta
32 to examine second trimester alcohol and smoking exposure.

33 *Methods* A validated liquid chromatography-tandem mass spectrometry method for simultaneous
34 analysis of nicotine and its metabolites and alcohol markers (ethyl glucuronide: EtG and ethyl
35 sulfate: EtS) was employed to analyze 193 fetal liver and 48 placenta ($n=47$ paired) samples
36 from electively-terminated pregnancies.

37 *Results* EtG, EtS and nicotine markers' limits of detection were 0.7-20 ng/g in fetal samples.
38 Ninety-eight fetal liver and 23 placenta samples were EtG/EtS-positive, while 137 liver and 25
39 placenta samples were positive for tobacco exposure. When both alcohol markers were present in
40 samples, EtG/EtS ratios were 1.6-11.1 in 17 livers and 2.5-31.1 in 10 placentas. Median [range]
41 summed tobacco marker concentrations were 422 [1.0-2776] and 154 [1.6-1621] ng/g in livers
42 and placentas. Median EtG and nicotine marker concentrations were higher in liver than placenta
43 in paired samples. Strong evidence of exposure occurred in 11 and 22 pairs, respectively, when
44 both samples were positive for alcohol and/or tobacco markers.

45 *Conclusions* These paired fetal liver and placenta alcohol and tobacco data provided a unique
46 means for examining the effects of in utero exposure, a critical first step in selecting fetal
47 samples for proteomic and RNA-sequencing studies that could provide mechanisms for adverse
48 developmental outcomes.

49

50 **Key words** Ethyl glucuronide, Nicotine, Fetal liver, Placenta, Pregnancy, Prenatal exposure

51 **Introduction**

52 Tobacco and alcohol use during pregnancy are serious public health concerns, resulting in
53 adverse obstetrical and neonatal outcomes including miscarriage, preeclampsia, low birth weight,
54 preterm delivery, fetal growth restriction, decreased head circumference, placenta abruption, and
55 facial abnormalities [1-4]. Fetal alcohol spectrum disorders (FASD) encompass growth
56 retardation, cognitive impairments, and craniofacial dysmorphology associated with prenatal
57 alcohol exposure [5-6]. Prenatal tobacco exposure also can have a profound impact on infant
58 brain structure and function, infant irritability, and risk for child behavioral and attention
59 disorders [7-8]. Despite these documented health risks, in the 2015 US National Survey of Drug
60 Use and Health (NSDUH), 13.9% of pregnant respondents reported current tobacco use and
61 9.3% current alcohol use [9].

62 Identifying prenatal alcohol and tobacco exposure is often achieved through meconium
63 drug quantification and/or maternal self-reported drug use during pregnancy. Meconium is a
64 good matrix for in utero drug exposure assessment in pregnancies carried to term. Although
65 meconium formation begins as early as 13 weeks, other methods must assess earlier exposure. In
66 this study, we examined whether analysis of fetal liver and placenta from electively-terminated
67 pregnancies could determine exposure patterns during the second trimester.

68 In adults, nicotine is oxidized to cotinine and *trans*-3'-hydroxycotinine (OHCOT)
69 primarily by CYP2A13 and CYP2A6 [10], with additional phase II conjugation to nicotine-*N*-
70 glucuronide (Nic-G), cotinine-*N*-glucuronide (Cot-G), and OHCOT-*O*-glucuronide (OHCOT-G)
71 [10]. Nicotine and these 5 metabolites account for 73-96% of the nicotine dose excreted in adult
72 smokers' urine [10]. Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are chemical markers
73 indicating ethanol ingestion with longer detection times than ethanol. UDP-

74 glucuronosyltransferase catalyzes ethanol conjugation with glucuronic acid to form EtG [11],
75 while sulfotransferases conjugate ethanol and activated sulfate to form EtS [12]. Fetal liver and
76 placental EtG is likely of maternal origin as EtG readily crosses the placenta and fetal
77 glucuronidation capacity is limited [13-14]. Little is known about placental EtS transfer;
78 however, fetal liver and placental EtS concentrations may result from maternal and/or fetal
79 contributions, as variable yet significant fetal sulfotransferase activity was observed [15-16].
80 Fetal liver and placental EtG and EtS levels were investigated previously in a small cohort with
81 few positives [17], while there are no available data on human fetal liver nicotine and its
82 metabolites' concentrations.

83 Adult health is partly programmed during fetal development [18-21]. Gestational
84 exposure to tobacco, alcohol, and other drugs alters normal hormone regulation early in fetal
85 development [22-23]. Mechanisms through which fetal drug exposures result in reduced adult
86 health are poorly understood. We present a validated analytical method for quantifying EtG/EtS
87 and nicotine markers in fetal liver and placenta samples and describe how this method assessed
88 in utero alcohol and tobacco exposure in fetuses from electively-terminated pregnancies. The
89 goal was to select the most appropriate fetal samples to conduct fetal endocrine disruption and
90 DNA/RNA damage assessments.

91

92 **Materials and methods**

93 **Tissue samples**

94 The drug- and alcohol-negative matrix for human fetal liver analyses was a frozen calf liver.
95 Human placentas from healthy pregnancies were donated by volunteers and the Johns Hopkins
96 Bayview Medical Center Pathology Department. Blank matrices were confirmed negative at our

107 limits of quantification (LOQ) prior to calibrator and quality control (QC) preparation. Samples
108 (193 fetal livers and 48 placentas including 47 paired samples) obtained from women who
109 voluntarily terminated their pregnancies were analyzed. Fetus collection in Aberdeen was
110 approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21). Women
111 seeking elective terminations of pregnancy were recruited with full written, informed consent by
112 nurses working independently of the study at Aberdeen Pregnancy Counseling Service. Maternal
113 data, medications used, and self-reported number of cigarettes smoked per day were recorded.
114 Only fetuses from normally-progressing pregnancies (determined by ultrasound scan), from
115 women >16 years old and between 8-20 weeks of gestation, were collected following termination
116 as detailed previously [24]. Fetuses were transported to the laboratory within 30 min of delivery,
117 weighed, crown-rump length recorded, and sexed. Collection of human fetal liver and placenta
118 pairs occurred at the University of Newcastle upon Tyne (England) through the Human
119 Developmental Biology Resource at the Institute of Human Genetics under an Institutional
120 Review Board-approved study.

111

112 **Reagents**

113 *S*(-)-Nicotine, (\pm)-nicotine-*d*₄, (-)-cotinine, (\pm)-cotinine-*d*₃, (*R,S*)-norcotinine, EtG, EtG-*d*₅, EtS,
114 and EtS-*d*₅ were purchased from Cerilliant Corporation (Round Rock, TX, USA); nicotine-*N*- β -
115 D-glucuronide, cotinine-*N*- β -D-glucuronide, cotinine-*d*₃-*N*- β -D-glucuronide, OHCOT, OHCOT-
116 *d*₃, OHCOT-*O*- β -D-glucuronide, and (*S*)-cotinine-*N*-oxide as powders from Toronto Research
117 Chemicals Inc. (Toronto, Canada); LCMS grade methanol, ammonium acetate, formic acid,
118 HPLC grade acetonitrile, dichloromethane, isopropanol, ACS grade hydrochloric acid and
119 ammonium hydroxide from Fisher Scientific (Fair Lawn, NJ, USA); fritted filters (10 μ m, 15

120 mL) from United Chemical Technologies Inc. (Bristol, PA, USA); Isolute supported liquid
121 extraction (SLE) columns (1 mg/6 mL) and Evolute-AX anion exchange solid-phase extraction
122 (SPE) cartridges (100 mg/3 mL) from Biotage (Charlotte, NC, USA).

123

124 **Instrumentation**

125 Tobacco and alcohol markers were quantified on a SCIEX 5500 Qtrap® mass spectrometer with
126 a TurboV electrospray ionization (ESI) source (SCIEX, Foster City, CA, USA), connected to a
127 Shimadzu UFLCXR system with two LC-20ADXR pumps, a CTO-20 AC column oven, and a
128 SIL-20ACXR autosampler (Shimadzu Corporation, Columbia, MD, USA). Data were acquired
129 and processed with Analyst 1.5.1 (SCIEX). A Mini-BeadBeater-8 (BioSpec Products,
130 Bartlesville, OK, USA) pulverized tissue. A CEREX-48 positive-pressure manifold was utilized
131 for SPE (SPEware Corporation, Baldwin Park, CA, USA). Evaporation under nitrogen was
132 conducted in a TurboVap LV evaporator (Zymark, Hopkinton, MA).

133

134 **Standard Solution Preparation**

135 Individual methanolic standard solutions were diluted to 100 mg/L in methanol. Powdered
136 standards were reconstituted in the manufacturer's recommended solvent and diluted to 100
137 mg/L in methanol, except for OHCOT, which was maintained at 1 g/L. Methanolic dilutions
138 yielded mixed working calibrator solutions of 0.01, 0.02, 0.05, 0.1, 0.25, 0.5, 1.5 and 3 mg/L for
139 cotinine, and 2.5, 5, 10 and 20 times more concentrated for Nic-G, OHCOT; nicotine, EtS, Cot-
140 G; OHCOT-G; and EtG, respectively. QC solutions were prepared from different stocks than
141 calibrators. Low, medium, and high QCs were prepared across the linear dynamic range for each

142 analyte. A mixed working internal standard methanolic stock was prepared at 0.1 (cotinine-*d*₃),
143 0.25 (EtS-*d*₅, OHCOT-*d*₃, Nic-G-*d*₃), 0.5 (nicotine-*d*₄, Cot-G-*d*₃), and 1 mg/L (EtG-*d*₅).

144

145 **Procedures**

146 Blank liver or placenta (0.25 g) was fortified with 25 μ L calibrator or QC solution or 25 μ L
147 methanol for authentic samples, and 25 μ L internal standard solution was added. Six to eight 3.2-
148 mm chrome-steel beads were added to each sample, followed by 0.95 mL 0.01% formic acid in
149 methanol and pulverized for 2 s. The methanol was filtered and collected in polypropylene tubes.
150 The original samples were rinsed with an additional 1 mL 0.01% formic acid in methanol,
151 vigorously vortexed for 1 min, and filtered into the same tubes. Two 450- μ L aliquots were
152 transferred to separate tubes for SLE of nicotine and its metabolites and for anion-exchange SPE
153 of EtG and EtS.

154 For nicotine and metabolite extraction, 525 μ L 0.25% ammonium hydroxide in methanol
155 was added to the nicotine aliquot yielding pH >10. Samples were vortexed and poured onto 1 mL
156 SLE beds. After 5 min equilibration, analytes were eluted with 2 x 2.5 mL 95:5
157 dichloromethane/isopropanol into glass centrifuge tubes. Positive pressure was gradually applied
158 up to 2.4 L/min to complete elution. A 50- μ L volume of 1% HCl in methanol was added to the
159 eluents before evaporation under nitrogen at 35°C. Reconstitution occurred in 200 μ L of 10 mM
160 ammonium acetate in water with 5 μ L methanol added to aid pellet formation. Samples were
161 vortexed and centrifuged at 1800g at 4°C for 5 min. A 150- μ L volume was transferred to a
162 microcentrifuge tube and centrifuged at 20,800g for 5 min at 4°C. A 125- μ L volume was
163 transferred to autosampler vials and 5 μ L injected. For EtG and EtS extraction, 2 mL acetonitrile

164 and 10 μ L 236 mM ammonium hydroxide were added to the 450 μ L tissue supernatant, and the
165 SPE followed our published meconium assay for these markers [25].

166

167 **Liquid chromatography-tandem mass spectrometry**

168 Nicotine and its metabolites were chromatographically separated on a Poroshell 120 EC-C8
169 column (150 x 2.1 mm i.d., 2.7 μ m particle size), fitted with a matching guard column (Agilent
170 Technologies, Santa Clara, CA, USA) similarly to a previous publication [26]. Mobile phases A
171 and B were 10 mM ammonium acetate in water and methanol, respectively. At 0.3 mL/min flow,
172 the gradient started at 0% B, increased to 60% B over 6 min, increased to 100% B in 0.1 min,
173 held at 100% B for 2.4 min, decreased to 0% B in 0.1 min, and held for 2.9 min; total run time
174 was 11.5 min. Liquid chromatograph (LC) eluent was diverted to waste for the first min and the
175 final 5.5 min.

176 Mass spectrometric data for nicotine and metabolites were acquired via positive ESI.
177 Compound-specific tandem mass spectrometry (MS/MS) parameters were optimized by infusing
178 10 μ g/L reference solutions at 10 μ L/min dissolved in A/B (50:50, v/v) mobile phase (Table S1).
179 Optimized source parameters were gas 1 60 psi, gas 2 40 psi, curtain gas 45 psi, source
180 temperature 700°C, and ion spray voltage 5500 V. Three periods were utilized to acquire
181 multiple reaction monitoring (MRM) mode data with period 1 (2.5 min, 100 ms dwell) consisting
182 of Cot-G and Cot-G- d_3 ; period 2 (1.7 min, 50 ms dwell) Nic-G, OHCOT-G, and Nic-G- d_3 ; and
183 period 3 (7.3 min, 30 ms dwell) OHCOT, cotinine, nicotine, OHCOT- d_3 , cotinine- d_3 , and
184 nicotine- d_4 . Additional MRMs for two isobaric compounds were not quantified; cotinine-*N*-
185 oxide (m/z 193.0>96.0, 193.0>78.8) and norcotinine (m/z 163.0>80.0, 163.0>118.1) were only
186 monitored in periods 2 and 3, respectively.

187 LC and MS parameters for EtG and EtS were adapted from our previously published
188 meconium method [25]. However, the quantifying EtG transition, m/z 221>75, was replaced by
189 m/z 221>85 m/z , due to matrix interferences at low concentrations (Table S1).

190

191 **Validation**

192 Sensitivity, linearity, specificity, accuracy, imprecision, extraction efficiency, matrix effect,
193 dilution integrity, carryover, and stability were evaluated according to Scientific Working Group
194 for Forensic Toxicology (SWGTOX) guidelines [27] as summarized in Table S2.

195

196 **Results**

197 This method is the first to offer simultaneous extraction of EtG, EtS, nicotine, cotinine, OHCOT,
198 and 3 prominent nicotine glucuronide metabolites from a human fetal liver or placenta sample.

199 Although initial tissue extraction was simultaneous, marked physiochemical differences between
200 acidic EtG and EtS and basic nicotine and metabolites required different SPE and LC
201 approaches.

202 Limits of detection (LODs), LOQs, linear ranges, and calibration curves for both matrices
203 are presented in Table 1. Accuracy and imprecision results for liver and placenta are presented in
204 Table 2. Extraction recoveries and matrix effects for liver and placenta are given in Table 3.
205 Stability results for all conditions and all samples are summarized in Table 4. All validation
206 experiments met acceptability criteria (Table S2).

207 The validated methods were used for the analysis of 193 fetal livers and 48 placentas (47
208 paired) samples. Summarized concentration data for all analytes and matrices are found in Table
209 5. When both alcohol markers were present in a sample (17 livers and 10 placenta samples), an

210 EtG/EtS ratio was calculated and ranged from 1.6-11.1 in liver and 2.5-31.1 in placenta. The six
211 tobacco marker concentrations in each individual sample were summed. Median [range]
212 summation concentrations were 422 [1.0-2776] and 154 [1.6-1621] ng/g in liver and placenta,
213 respectively.

214 We identified in utero alcohol and tobacco exposures based on EtG/EtS and nicotine
215 marker concentrations in liver and placenta. Currently, there are not sufficient pre-existing fetal
216 data in the literature to guide interpretation. Our criteria for determining evidence of alcohol and
217 tobacco exposure are described in Table 6, based on presence of and concentrations of multiple
218 analytes. For each matrix, numbers of samples in each predicted exposure category are also
219 tabulated. Results from this study are utilized for selecting fetuses for later DNA and RNA
220 analyses that will examine exposure and possible damage at the genomic level.

221 When both alcohol markers were detected together, EtG concentrations were 27.0-2050
222 ng/g and 34.3-1168 ng/g, and EtS concentrations were 7.3-423 ng/g and 7.9-214 ng/g in 17 fetal
223 livers and 10 placentas, respectively. Only one fetal liver had detectable EtG (31.9 ng/g) without
224 EtS with no paired placenta for comparison, while 6 placenta samples were positive for EtG only
225 (35.1-628 ng/g). EtS was often detected alone in 81/193 fetal liver (5.1-42.0 ng/g) and 7/48
226 placenta (6.9-35.6 ng/g) samples. Median EtG concentrations were higher in liver than placenta
227 (Table 5). Of the 11 paired samples indicating strong evidence of recent alcohol exposure, 3
228 pairs contained both markers in both matrices, while 3 pairs were negative in liver but positive
229 for EtG and EtS in placenta. Overall, 95/193 fetal liver and 25/48 placenta samples, including 21
230 paired samples, were negative for EtG and EtS (Table 6).

231 One or more nicotine markers were detected in 137/193 liver and 25/48 placenta samples.
232 Median nicotine and metabolite concentrations were higher in liver than in placenta, including

233 median concentration sums (Table 5). Low tobacco marker concentrations or presence of few
234 analytes were difficult to interpret and were classified as weak or moderate evidence of recent
235 exposure, but did not preclude previous maternal use if there was a period of abstinence prior to
236 pregnancy termination. Summed nicotine marker concentrations weakly indicative of recent
237 tobacco exposure were 1.0-13.0 ng/g and 1.6-10.8 ng/g in fetal liver and placenta, respectively,
238 while moderate evidence encompassed summed concentrations of 8.3-421 ng/g and 43.7-154
239 ng/g in fetal liver and placenta, respectively. When strong evidence of tobacco exposure was
240 predicted, summed concentrations were 290-2776 ng/g and 148-1621 ng/g in fetal liver and
241 placenta, respectively (Table 5). Of the paired samples, 6 indicated strong evidence of both
242 alcohol and tobacco exposure. No tobacco analytes were detected above the LOQ in 56/193 fetal
243 livers and 23/48 placentas, including 20 paired samples (Table 6).

244

245 **Discussion**

246 This novel, comprehensive method was fully validated according to SWGTOX guidelines [27]
247 for EtG, EtS, nicotine and phase I and phase II metabolites in a fetal liver or placenta sample.
248 The methodology was successfully applied to the analysis of 193 fetal liver and 48 placenta
249 samples from electively terminated pregnancies for the determination of in utero exposure to
250 alcohol and tobacco.

251 Our investigation is only the second to look at fetal liver and placental EtG and EtS. A
252 previous study by Morini et al. [17] tested 35 matched liver-placenta pairs from women who
253 terminated their pregnancy at week 12. In this previous study, four fetal livers were EtG-positive
254 (33-391 ng/g) with three also positive for EtS (15-51 ng/g). In placenta, four were EtG- (122-
255 1307 ng/g) and EtS- (10-126 ng/g) positive and were paired with the four fetal livers with

256 detectable EtG. Two additional placenta samples were positive only for EtS (29 and 175 ng/g).
257 EtG/EtS ratios in the three dual positive liver samples were 3.9, 5.3, and 7.3; while EtG/EtS
258 ratios in the four dual positive placentas were 12.7, 12.7, 4.8, and 10.4. EtG and EtS were always
259 higher in placenta compared to those of liver [17]. Our EtS LOQ is the same as that of Morini et
260 al. (5 ng/g) [17], but our EtG LOQ was 20 ng/g. We fully homogenized the 250 mg liver by
261 bead-beating, enabling a more efficient and reproducible release of drugs as compared to an
262 acetonitrile wash reported by them, but produced additional matrix effects, requiring us to raise
263 our LOQ. However, our LOQ was below the concentrations in all samples identified and
264 reported by them.

265 In order to select samples for further testing, we examined alcohol and tobacco markers
266 in fetal, infant, and adult studies. EtG readily crosses the placenta [20]; therefore, fetal liver EtG
267 is likely to be primarily of maternal origin, as fetal glucuronidation capacity is limited [13]. No
268 placental diffusion EtS studies exist. However, fetal liver sulfotransferases showed variable yet
269 significant activities [15]. It is possible that the fetal liver EtS is of fetal origin, or of both the
270 fetal and maternal origin.

271 When considering adult alcohol markers, if EtG and EtS are present, there is a strong
272 indication of alcohol ingestion [28]. EtG and EtS are only produced by antemortem drinking,
273 enabling differentiation of postmortem ethanol formation from antemortem drinking. EtG was
274 reported to occasionally degrade in postmortem samples [28-29], suggesting superiority of EtS
275 analysis. In addition, EtS is not formed in the presence of putrefaction or with lack of
276 preservatives [28,30]. For our fetal samples, presence of EtS or EtG only as well as that of both
277 compounds can be considered alcohol-exposed.

278 In meconium, we previously reported that COT, NIC, or OHCOT concentrations ≥ 10
279 ng/g suggested active maternal tobacco exposure during pregnancy [31], although subsequent
280 work by our group found lower cutoffs, equivalent to analytical LOQs (1 ng/g COT, 2.5 ng/g
281 NIC, and 5 ng/g OHCOT), and better identified active maternal smoking [3]. This suggests that
282 detecting any marker in meconium indicates maternal smoking during pregnancy, and
283 environmental or passive exposure would produce undetectable tobacco marker concentrations in
284 meconium. In serum, COT ≤ 3 ng/mL differentiated passive from active maternal exposure
285 [10,31]. In support of our previous 10 ng/g cutoff, Braun et al. [32] showed that in infants of
286 tobacco-smoking mothers with a mean serum COT >3 ng/mL, the lower limit of the 95%
287 confidence interval for meconium COT was 10 ng/g. Additionally, among these infants with
288 exposure to maternal active tobacco smoking, multiple meconium tobacco markers were detected
289 [31]. For our fetal samples, presence of multiple markers, especially above the median, was
290 considered the criteria for tobacco-exposed.

291 To our knowledge, these are the first fetal liver tobacco marker concentrations, although
292 there are other placenta and fetal human brain concentration data. The transfer rate for nicotine
293 across the placenta was about 90% [33]. In 12th week-terminated pregnancies, evidence of fetal
294 tobacco exposure was observed, with placenta median (range) COT and NIC concentrations of
295 80 (25-190) ng/g and 61 (33-120) ng/g, respectively [34]. In a single human postmortem fetal
296 brain sample, 40 ng/g COT, 65 ng/g OHCOT, and no NIC were detected [35], although the
297 timing of tobacco exposure was unavailable. In adult postmortem liver, COT was always higher
298 (4-25 times) than NIC and both analytes were detected at higher concentrations than in blood;
299 COT and NIC concentrations in adult liver were 260-1586 ng/g and 14-325 ng/g, respectively
300 [36].

301 We predicted alcohol and tobacco exposure based on analyte presence and their
302 concentrations, although there are few data to guide interpretation. It is important to publish
303 quantitative data for future investigations. Molecular analyses in prenatal alcohol and tobacco-
304 exposed fetuses may eventually suggest mechanisms for adverse fetal outcomes and perhaps
305 correlations between fetal liver or placenta concentrations and outcomes. Relevantly, a balanced
306 population of the 80 fetal livers and the 145 Aberdeen fetal livers that we report here is currently
307 being analyzed by shotgun proteomics and RNA-sequencing. No recent alcohol exposure was
308 demonstrated by absence of EtG and EtS, as absence of these markers may only indicate
309 maternal abstinence for a few days prior to termination. It is possible that these samples could
310 provide a wider window of alcohol or tobacco detection, but we have no data on timing or
311 magnitude of exposure. The fetal liver EtG detection window following acute or chronic
312 maternal alcohol consumption is unknown, although EtG and EtS are detected in adult urine for
313 40-130 h after the end of drinking in most cases [37-38].

314 Fetuses exhibiting strong evidence of recent alcohol exposure were selected primarily
315 from samples positive for both EtG and EtS ($n = 17$ for liver, $n = 10$ for placenta), with an
316 additional 2 placenta identified when EtG concentration \geq median concentration. Moderate
317 evidence of recent alcohol exposure identified 45 liver and 4 placenta samples with EtS detected
318 \geq median concentration despite no detectable EtG. Given potential EtG instability and higher
319 EtG LOQ, EtS-only positive cases could indicate exposure. In addition, differential metabolic
320 pathway rates in mothers or fetuses could result in more EtS than EtG as EtS is stable [28], and
321 there are no published cases of artificial EtS formation. However, in adults, urine EtS detection
322 could occur after extreme non-traditional drinking behaviors, including non-alcoholic beer [39],
323 sauerkraut [39], ripened bananas [39], grape juice [39], and non-alcoholic wine [29]. In the

324 present study, three placentas were EtG and EtS positive, although paired liver samples were
325 negative. Additionally, three liver samples were positive for EtS-only (8.6, 9.9, and 17.0 ng/g)
326 with no analytes detected in placenta, while nine EtS-only placenta (6.9-35.6 ng/g) samples had
327 paired liver samples negative for both alcohol markers. Therefore, alcohol exposure was
328 suggested in this study, but cannot be definitively determined.

329 No recent tobacco exposure was identified in fetal liver and placenta samples that were
330 negative for all six tobacco markers. Tobacco marker absence may indicate recent maternal
331 abstinence but not necessarily throughout pregnancy. Detection windows for tobacco markers in
332 fetal liver and placenta are unknown. In cases where only a few markers were detected,
333 interpretation was difficult and low concentrations could possibly be attributed to minimal
334 maternal smoking, abstinence for a few days prior to termination or passive maternal tobacco
335 smoke exposure. Large population sampling studies showed positive, but low, mean serum
336 cotinine concentrations among self-reported nonsmokers and passively exposed individuals [10].
337 For this reason, samples positive for 5/6 or 6/6 analytes with high summed concentrations were
338 considered to exhibit strong evidence of recent tobacco exposure, especially due to the presence
339 of phase I and phase II nicotine metabolites.

340

341 **Conclusions**

342 While timing of gestational alcohol or tobacco use is unknown, we were able to identify
343 multiple, specific alcohol and tobacco markers for selecting samples for proteomic and RNA-
344 sequencing studies. Our validated analytical method successfully quantified alcohol (EtG and
345 EtS) and tobacco markers (nicotine and phase I and phase II metabolites) and sought to predict
346 alcohol and tobacco exposure. After consulting the literature for fetal studies and examining

347 analyte concentrations in other matrices, we stratified our fetal liver and placenta data to
348 categorize strength of evidence of recent maternal alcohol or tobacco consumption, exposing
349 pre-term fetuses to potentially harmful chemicals at a young gestational age. For the first time, a
350 large cohort of paired, exposed fetal liver and placenta samples were analyzed. Publishing these
351 data is crucial to inform future research, as this is the first comprehensive analysis of paired fetal
352 liver and placenta samples for evidence of in utero alcohol and tobacco exposure.

353

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360

361 **Compliance with Ethical Standards**

362 **Conflict of interest** The authors declare that they have no conflicts of interest.

363 **Ethical approval** All procedures performed in studies involving human participants were in
364 accordance with the ethical standards of all involved institutions and with the 1964 Helsinki
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369 No identifying information is included in this article.

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Table 1 Linearity, and limit of detection (LOD) and limit of quantification (LOQ) results

Analyte	LOD (ng/g)	LOQ (ng/g)	Linear range (ng/g)	Liver			Placenta		
				y-Intercept (mean±SD, n=5)	Slope (mean±SD, n=5)	R ² (range, n=5)	y-Intercept (mean±SD, n=5)	Slope (mean±SD, n=5)	R ² (range, n=5)
Cotinine- <i>N</i> -glucuronide	5	5	5-1500	0.011 ± 0.065	1.223 ± 0.128	0.997 - 0.999	-0.024 ± 0.018	1.252 ± 0.054	0.993 - 0.999
Nicotine- <i>N</i> -glucuronide	1.75	2.5	2.5-750	-0.025 ± 0.005	0.812 ± 0.060	0.993 - 0.998	-0.029 ± 0.004	0.832 ± 0.025	0.994 - 0.998
OHCOT- <i>O</i> -glucuronide	7	10	10-1500	0.000 ± 0.000	0.017 ± 0.001	0.995 - 1.000	-0.001 ± 0.001	0.034 ± 0.007	0.992 - 1.000
OHCOT	1.75	2.5	2.5-750	-0.005 ± 0.011	1.172 ± 0.027	0.997 - 0.999	-0.003 ± 0.006	1.134 ± 0.018	0.998 - 0.999
Cotinine	0.7	1	1-300	0.040 ± 0.019	0.982 ± 0.039	0.993 - 0.998	0.054 ± 0.016	1.007 ± 0.065	0.996 - 0.999
Nicotine	3.5	5	5-1500	0.066 ± 0.044	1.058 ± 0.024	0.995 - 0.998	0.085 ± 0.047	1.172 ± 0.218	0.993 - 0.998
Ethyl sulfate (EtS)	5	5	5-1500	0.014 ± 0.031	1.200 ± 0.010	0.997 - 0.999	0.000 ± 0.000	1.100 ± 0.032	0.996 - 0.999
Ethyl glucuronide (EtG)	20	20	20-3000	-0.050 ± 0.111	7.182 ± 0.270	0.989 - 0.998	0.000 ± 0.000	5.036 ± 0.282	0.996 - 1.000

SD standard deviation, *OHCOT trans-3'*-hydroxycotinine

Table 2 Accuracy (bias) and imprecision results for tobacco and alcohol markers in fetal liver and placenta samples

Analyte	Concentration (ng/g)	Liver				Placenta			
		Mean between-run bias (% , n=20)	Within-run bias range (% , n=5)	Between-run imprecision (%CV, n=20)	Maximum-within run imprecision (%CV, n=5)	Mean between-run bias (% , n=20)	Within-run bias range (% , n=5)	Between-run imprecision (%CV, n=20)	Maximum-within run imprecision (%CV, n=5)
Cotinine- <i>N</i> -glucuronide	15	99.1	88.7 - 109	5.2	9.9	96.6	84.0 - 117	11.9	13.2
	300	104	91.7 - 114	5.4	6.1	102	91.7 - 113	5.7	5.9
	1200	101	90.8 - 114	5.5	5.9	108	92.5 - 116	5.6	7.8
Nicotine- <i>N</i> -glucuronide	7.5	106	94.8 - 113	4.2	4.6	99.7	94.5 - 112	4.5	5.9
	150	97.6	87.3 - 112	6.6	6.7	94.9	84.7 - 108	5.4	9.1
	600	92.4	84.5 - 103	6.9	6.7	99.5	87.2 - 112	7.5	7.4
OHCOT- <i>O</i> -glucuronide	30	106	94.0 - 115	5.8	6.4	105	89.0 - 119	8.5	6.9
	600	104	92.3 - 115	6.7	7.8	104	92.3 - 119	8.6	10.6
	1200	108	97.5 - 115	5.5	5.8	107	90.8 - 116	7.4	10.1
OHCOT	7.5	110	102 - 115	3.3	6.0	113	105 - 117	2.4	4.7
	150	110	106 - 115	2.4	3.5	113	103 - 117	3.6	6.9
	600	107	94.5 - 115	4.7	4.0	110	99.7 - 115	3.6	6.0
Cotinine	3	102	92.3 - 113	5.9	7.1	106	97.7 - 114	4.1	3.5
	60	104	94.8 - 113	5.3	6.6	107	92.2 - 115	5.0	8.6
	240	95.5	87.9 - 113	8.3	7.7	94.9	85.0 - 107	6.2	8.2
Nicotine	15	107	88.7 - 115	6.9	12.1	107	90.7 - 117	7.3	10.6
	300	107	99.3 - 115	4.7	4.5	108	95.0 - 115	5.0	7.8
	1200	111	99.2 - 115	3.5	4.9	108	80.7 - 116	10.6	13.0
Ethyl sulfate (EtS)	15	105	95.3 - 115	5.9	5.8	108	98.7 - 117	4.7	6.9
	300	103	95.7 - 115	5.0	4.7	107	92.3 - 116	5.2	8.3
	1200	106	98.3 - 114	4.0	3.6	106	93.3 - 115	5.2	4.8
Ethyl glucuronide (EtG)	60	98.3	86.8 - 113	8.6	10.4	98.4	85.3 - 107	4.7	8.5
	1200	93.9	84.2 - 107	8.8	10.3	97.5	85.0 - 108	7.3	8.2
	2400	105	87.9 - 115	8.4	9.6	110	90.8 - 118	5.6	8.8

CV coefficient of variation

Table 3 Extraction efficiencies and matrix effects for tobacco and alcohol markers in liver and placenta samples

Analyte	Liver				Placenta			
	Extraction efficiency (%, <i>n</i> =5)		Matrix effect (%, <i>n</i> =5)		Extraction efficiency (%, <i>n</i> =5)		Matrix effect (%, <i>n</i> =5)	
	Low ^a	High ^b	Low	High	Low	High	Low	High
Cotinine- <i>N</i> -glucuronide	54.4	53.0	12.3	11.0	31.6	31.3	-16.6	-32.4
Nicotine- <i>N</i> -glucuronide	64.4	70.5	-40.0	-25.2	48.8	56.6	-2.5	-16.3
OHCOT- <i>O</i> -glucuronide	60.4	57.9	-57.3	-50.3	55.9	60.9	-15.6	-35.1
OHCOT	92.6	93.6	-44.3	-32.4	88.5	93.9	-11.2	-20.8
Cotinine	92.3	92.2	-42.2	-32.8	107	103	-22.7	-23.9
Nicotine	90.9	80.3	-33.2	-33.3	82.8	92.2	8.0	-21.3
Ethyl sulfate (EtS)	76.1	71.9	-30.6	-17.5	73.1	68.1	-24.5	-10.5
Ethyl glucuronide (EtG)	86.8	86.0	-50.6	-51.4	94.1	91.5	-27.3	-24.5
Cot-G- <i>d</i> ₃	49.2	45.8	17.3	14.8	27.6	32.4	-10.0	-27.1
Nic-G- <i>d</i> ₃	57.9	59.7	-38.2	-25.7	51.4	58.2	-4.4	-8.3
OHCOT- <i>d</i> ₃	85.7	82.7	-43.4	-28.6	91.3	89.7	-18.2	-14.2
Cotinine- <i>d</i> ₃	89.1	83.6	-45.7	-32.1	102	101	-35.8	-23.7
Nicotine- <i>d</i> ₄	80.4	73.9	-42.5	-27.9	93.4	92.1	-18.8	-16.3
EtS- <i>d</i> ₅	63.4	72.6	-34.9	-35.9	68.0	74.0	-28.6	-38.3
EtG- <i>d</i> ₅	78.1	96.5	-44.1	-50.5	88.3	97.1	0.2	-11.4

Cot-G cotinine-*N*-glucuronide, *Nic-G* nicotine-*N*-glucuronide

^aLow quality control concentrations were 3 ng/g cotinine, 7.5 ng/g nicotine-*N*-glucuronide and OHCOT, 15 ng/g nicotine, cotinine-*N*-glucuronide, and EtS, 30 ng/g OHCOT-*O*-glucuronide and 60 ng/g EtG

^bHigh quality control concentrations were 240 ng/g cotinine, 600 ng/g nicotine-*N*-glucuronide and OHCOT, 1200 ng/g nicotine, cotinine-*N*-glucuronide, EtS and OHCOT-*O*-glucuronide, and 4800 ng/g EtG

Table 4 Stability results (% , *n* = 4) of fortified liver and placenta samples in various storage conditions prior to and following extraction

Analyte	12h Room temperature		72h 4°C		3 Freeze/thaw cycles		72h Autosampler (4°C)		5 Days supernatant ^c (4°C)	
	Low ^a	High ^b	Low	High	Low	High	Low	High	Low	High
Liver										
Cotinine- <i>N</i> -glucuronide	84.4	83.3	87.6	101	96.9	96.7	98.8	99.6	96.4	92.6
Nicotine- <i>N</i> -glucuronide	86.1	83.0	95.8	93.8	89.2	84.6	97.4	85.6	90.8	81.4
OHCOT- <i>O</i> -glucuronide	89.1	101	109	117	94.4	111	111	106	108	104
OHCOT	114	109	110	116	110	110	111	101	107	94.3
Cotinine	112	115	113	108	113	98.5	104	94.9	98.6	85.6
Nicotine	110	113	112	113	115	108	112	99.3	104	92.3
Ethyl sulfate (EtS)	106	103	101	105	103	105	102	111	104	107
Ethyl glucuronide (EtG)	91.1	106	98.1	99.7	102	104	97.0	109	95.2	98.2
Placenta										
Cotinine- <i>N</i> -glucuronide	106	108	91.3	99.1	101	107	88.3	116	98.7	113
Nicotine- <i>N</i> -glucuronide	97.3	103	91.8	87.0	103	90.6	97.4	104	98.5	93.2
OHCOT- <i>O</i> -glucuronide	115	116	96.3	92.2	99.5	96.0	109	114	97.0	112
OHCOT	115	108	110	102	116	111	115	112	115	111
Cotinine	88.3	81.7	110	96.2	111	98.4	108	101	104	94.7
Nicotine	81.9	86.4	111	106	109	112	99.1	90.4	109	117
Ethyl sulfate (EtS)	107	97.3	99.9	103	103	113	108	104	107	113
Ethyl glucuronide (EtG)	96.5	104	92.6	95.1	101	113	113	116	102	113

^aLow quality control concentrations were 3 ng/g cotinine, 7.5 ng/g nicotine-*N*-glucuronide and OHCOT, 15 ng/g nicotine, cotinine-*N*-glucuronide, and EtS, 30 ng/g OHCOT-*O*-glucuronide and 60 ng/g EtG

^bHigh quality control concentrations were 240 ng/g cotinine, 600 ng/g nicotine-*N*-glucuronide and OHCOT, 1200 ng/g nicotine, cotinine-*N*-glucuronide, EtS and OHCOT-*O*-glucuronide, and 4800 ng/g EtG

^cAcidified, methanolic tissue supernatant resulting from filtration, prior to extraction

Table 5 Summary concentration data for alcohol and nicotine markers in fetal liver ($n=193$) and placenta ($n=48$) among positive samples, including minimum, mean, median, and maximum concentrations (ng/g).

	LOQ (ng/g)	EtG	EtS	<i>EtG/EtS ratio</i>	CotG	NicG	OHCotG	OHCOT	COT	NIC	<i>Individual summations of tobacco markers</i>
		20	5		5	2.5	10	2.5	1	5	
Liver (n=193)	Total positive (n)	18	97	<i>17</i>	111	117	51	113	136	105	<i>137</i>
	Minimum positive (ng/g)	27.0	5.1	<i>1.6</i>	5.2	2.9	10.0	2.5	1.0	5.1	<i>1.0</i>
	Mean (ng/g)	303	26.7	<i>3.9</i>	192	74.3	25.2	82.0	279	25.1	<i>592</i>
	Median (ng/g)	122	13.0	<i>3.5</i>	153	54.0	16.0	70.0	126	22.5	<i>422</i>
	Maximum (ng/g)	2050	423	<i>11.1</i>	745	437	375	313	1332	108	<i>2776</i>
Placenta (n=48)	Total positive (n)	16	17	<i>10</i>	20	18	10	22	24	22	<i>25</i>
	Minimum positive (ng/g)	34.3	6.9	<i>2.5</i>	6.4	2.8	15.1	4.4	1.6	5.5	<i>1.6</i>
	Mean (ng/g)	253	36.6	<i>8.7</i>	80.4	42.2	44.7	97.9	67.6	45.6	<i>304</i>
	Median (ng/g)	86.0	17.0	<i>6.7</i>	21.6	5.5	28.7	59.5	48.8	22.7	<i>154</i>
	Maximum (ng/g)	1168	214	<i>31.1</i>	455	467	118	455	221	460	<i>1621</i>

Ratio of EtG to EtS is presented for samples with both analytes present. Summations of tobacco markers were also calculated for individual samples.

CotG cotinine-*N*-glucuronide, *NicG* nicotine-*N*-glucuronide, *OHCotG* hydroxycotinine-*N*-glucuronide, *COT* cotinine, *NIC* nicotine

Table 6 Summary of criteria used to stratify evidence of alcohol and tobacco exposure for fetal liver and placenta and number of samples in each category of predicted exposure

	Liver (<i>n</i> = 193)				Placenta (<i>n</i> = 48)				Liver & placenta pairs (<i>n</i> = 47)		
	No evidence	Weak evidence	Moderate evidence	Strong evidence	No evidence	Weak evidence	Moderate evidence	Strong evidence	No evidence	Weak evidence	Strong evidence
Alcohol	EtG & EtS not detected > LOQ	EtG or EtS < median concentration	Absence of EtG, + EtS ≥ median concentration	EtG & EtS detected > LOQ; or EtG ≥ median	EtG & EtS not detected > LOQ	EtG or EtS < median concentration	Absence of EtG, + EtS ≥ median concentration	EtG & EtS detected > LOQ; or EtG ≥ median	Both matrices negative for EtG & EtS	One matrix positive for EtG or EtS	Either matrix positive for EtG & EtS; or both matrices positive for EtG or EtS
	<i>n</i> = 95	<i>n</i> = 36	<i>n</i> = 45	<i>n</i> = 17	<i>n</i> = 25	<i>n</i> = 7	<i>n</i> = 4	<i>n</i> = 12	<i>n</i> = 21	<i>n</i> = 15	<i>n</i> = 11
Tobacco	No analytes detected > LOQ	1-2 Analytes present < median concentration	≥ 3 Analytes detected but sum < median	All analytes detected; or 5 analytes with sum > median	No analytes detected > LOQ	1-2 Analytes present < median concentration	≥ 3 Analytes detected but sum < median	All analytes detected; or 5 analytes with sum > median	Both matrices negative for all analytes	1-2 Analytes present in liver +/- placenta	≥ 3 Analytes in both matrices; or moderate & strong evidence between the two matrices
	<i>n</i> = 56	<i>n</i> = 23	<i>n</i> = 44	<i>n</i> = 70	<i>n</i> = 23	<i>n</i> = 4	<i>n</i> = 9	<i>n</i> = 12	<i>n</i> = 20	<i>n</i> = 5	<i>n</i> = 22