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Maternal smoking dysregulates protein expression in second trimester human fetal livers in a sex-specific manner.

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Corresponding Author:	Panagiotis Filis, PhD, MSc, BSc University of Aberdeen Aberdeen, Scotland UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Aberdeen
Corresponding Author's Secondary Institution:	
First Author:	Panagiotis Filis, PhD, MSc, BSc
First Author Secondary Information:	
Order of Authors:	Panagiotis Filis, PhD, MSc, BSc Nalin Nagraath, MSc Margaret J Fraser, HND David C Hay, PhD John P Iredale, PhD Peter O'Shaughnessy, PhD Paul A Fowler, PhD
Order of Authors Secondary Information:	
Abstract:	<p>Context: Maternal smoking during pregnancy has adverse effects on the offspring (e.g. increased likelihood of metabolic syndrome and infertility), which may involve alterations in fetal liver function.</p> <p>Objective: Our aim was to analyze, for the first time, the human fetal liver proteome in order to identify pathways affected by maternal smoking.</p> <p>Design: Fetal liver proteins extracted from elective second trimester pregnancy terminations (12-16 weeks of gestation) were divided in four balanced groups based on sex and maternal smoking.</p> <p>Setting: University of Aberdeen</p> <p>Patients/Participants: Livers were collected from 24 morphologically normal fetuses undergoing termination for non-medical reasons.</p> <p>Intervention: Maternal smoking during pregnancy.</p> <p>Main Outcome Measures: Protein extracts were resolved by 2D-PAGE and analyzed with SameSpots software. Ingenuity Pathway Analysis was used to investigate likely roles of dysregulated proteins identified by tandem liquid chromatography/mass spectrometry.</p> <p>Results: Significant expression differences between one or more groups (fetal sex and/or maternal smoking) were found in 22 protein spots. Maternal smoking affected proteins with roles in post-translational protein processing and secretion (ERP29, PDIA3), stress responses and detoxification (HSP90AA1, HSBP1, ALDH7A1, CAT) and homeostasis (FLT, ECHS1, GLUD1, AFP, SDHA). While proteins involved in necrosis, and cancer development were affected in both sexes, pathways affecting</p>

	<p>cellular homeostasis, inflammation, proliferation and apoptosis were affected in males and pathways affecting glucose metabolism were affected in females.</p> <p>Conclusions: The fetal liver exhibits marked sex differences at the protein level, and these are disturbed by maternal smoking. The foundations for smoke-induced post-natal diseases are likely to be due to sex-specific effects on diverse pathways.</p>
Additional Information:	
Question	Response
<p>STEROID HORMONE ASSAYS:</p> <p>Does your submission include steroid hormone assays? (If you have questions, please contact the editorial staff at jcem@endocrine.org)</p>	No
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55 INTRODUCTION

56

57 The liver is essential for detoxification and homeostasis by metabolizing and/or assisting in the excretion of a wide
58 range of xenotoxicants, including alcohol, drugs, and tobacco smoke constituents. In the human the fetal liver is
59 active and because 70% of its blood supply is directly from the fetomaternal interface, is directly exposed to
60 potentially harmful agents from the maternal circulation. Tobacco smoke contains a mixture of ~5,000 chemicals
61 and the human fetal liver responds to maternal smoking by up-regulating Phase I and Phase II enzyme transcripts
62 (1). Strikingly, the human fetal liver also expresses transcripts and proteins associated with altering steroid
63 hormone function/activity in the mother and fetus. These include CYP19A1 (2), CYP3A7 (2, 3) and SULT
64 enzymes (2, 4). The fetal liver is the main hematopoietic organ during the second trimester in humans (5) and also
65 secretes high levels of α -fetoprotein (AFP), sex-hormone binding globulin (SHBG) (6), IGF-1 and IGF-2 (7). It is
66 clear, therefore, that the human fetal liver functions in the development and protection of the fetus and in the
67 regulation of steroid hormone levels/actions during pregnancy.

68

69 Maternal smoking is associated with diverse negative outcomes for the health of the neonate [including reduced
70 birth weight, premature delivery and stillbirth (8, 9)] and predisposition of the offspring to long-term health risks
71 [including metabolic syndrome (10, 11), reduced fertility (12, 13) and psychosomatic problems (15)]. Around 30%
72 of women continue to smoke once pregnant (16) and it remains one of the most important modifiable risk factors
73 during pregnancy. Maternal smoking restricts the fetal oxygen supply and increases carbon monoxide burden to the
74 conceptus but also disrupt fetal development through other mechanisms; previous studies from this group have
75 identified some of these mechanisms such as endocrine signaling (1, 17) but a greater understanding of how
76 maternal smoking links to disease in the offspring is required. Given the importance of the fetal liver to human fetal
77 development and its susceptibility to maternal smoking we have, for the first time, used a proteomics approach to
78 identify proteins and pathways that are dysregulated in the human fetal liver by maternal smoking.

79

80 MATERIALS AND METHODS

81

82 **Study design**

83

84 Our cohort of 55 human fetal liver extracts from second trimester (12-17 weeks [wk.]) elective pregnancy
85 terminations was retrospectively divided into four groups according to sex and validated smoke exposure (reflected
86 by threshold plasma cotinine levels) ($n=14$ control and 13 smoke exposed females; 15 control and 13 smoke-
87 exposed females) as described previously (1). From this 55-sample cohort, 24 (i.e. $n=6$ from each group) balanced
88 by age fetal liver protein extracts were chosen for proteomics so that each group contained 1 x 12wk, 2 x 13wk, 1 x
89 14wk, 1 x 15 wk and 1 x 16/17wk fetal liver protein extracts.

90

91 **Sample collection and processing**

92

93 Women undergoing elective medical terminations of normally-progressing pregnancies gave full written informed
94 consent for the use of their fetal material to independent nurses at the Aberdeen Pregnancy Counselling Service as
95 previously described (18,19). Blocks of 30 mg from the central lobe of the liver were quickly dissected, snap frozen
96 on dry ice and stored at -80°C until further use. Protein extracts were prepared from frozen liver pieces from 30 mg
97 blocks dissected from the outer side of the right lobe of the liver using AllPrep kits (#80004, Qiagen, Manchester,
98 UK) according to the manufacturer's instructions. Total RNA was extracted using TRIzol (Life Technologies,
99 Paisley, UK) according to the manufacturer's guidelines as previously described (1).

100

101 **Proteomics**

102

103 Within each group, equal amounts (100 μg) of protein extracts from each fetal liver were combined. The four
104 protein pools we treated with ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) to
105 remove salt and other contaminants according to the manufacturer's instructions. The soluble protein fractions were
106 separated using 2D gel electrophoresis in quadruplicate ($n=4$) as described previously (20) using a pH 3 – 10

107 immobilized pH gradient gel (GE Healthcare, Uppsala, Sweden) for the first dimension and a 10–15% gradient
108 polyacrylamide gel for the second dimension. Proteins were detected using Colloidal Coomassie Blue G250 and
109 scanned using an Ettan DIGE Imager (GE Healthcare) and stained gels were analyzed using Progenesis SameSpots
110 software V6.01 (Nonlinear Dynamics, Newcastle, UK) (21). Individual spot volumes were expressed as normalized
111 volumes relative to the total detected spot volume for each gel to minimize potential analytical artefacts from
112 protein loading variations and migration. Progenesis SameSpots was used to combine the gel quadruplicates and
113 calculate fold-changes and significance (by ANOVA of log-normalized values). Molecular mass and pI of spots
114 was estimated from separate gels electrophoresed with pH and MW markers. Proteins in the gel pieces were
115 digested with trypsin and peptides identified using LC-MS/MS as previously described [see (20) and Suppl.
116 Materials and Methods]. Statistically significant Mascot scores and good sequence coverage were considered to be
117 positive identifications.

118

119 **Real-Time PCR**

120

121 Real-time PCR was carried out in fetal liver cDNAs from our larger 55-sample cohort (14 control and 13 smoke
122 exposed females; 15 control and 13 smoke-exposed females) and the same methods as described previously with
123 values normalized against the housekeeping genes *B2M*, *TMM1* and *TBP* (1,2, 22). Primers for query genes were
124 designed using Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to span exon junction and to have an
125 annealing temperature of 65°C. Test real-time PCR runs were performed to ensure that primer pairs do not amplify
126 genomic DNA and amplification efficiency was determined using serial dilution of human fetal liver cDNA as
127 template. Gene primer sequences are shown in Suppl. Table S1.

128

129 **Western Blots**

130

131 Individual human fetal liver protein extracts from the same preparation used for the proteomics were separated (30
132 µg/lane) in 26-well 4-12% Bis-Tris gradient precast gels (Invitrogen Ltd, Paisley, UK) under reducing conditions
133 according to the manufacturer's specifications (20). Gels were blotted on Immobilon-FL membrane (Millipore Ltd,

134 Watford, UK), blocked and probed with antibodies as previously described (20). Antibodies and dilutions used are
135 detailed in Suppl. Table S2. Protein bands were visualized using Odyssey infrared fluorescent imager and images
136 were analyzed using Phoretix 1D Advanced software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) as
137 detailed in (20). Membranes were reused without stripping and were probed with more than one antibody (typically
138 two to three antibodies) when target protein sizes were considerably different. ACTB probe served as a loading
139 control and following the validation of the use of ACTB for this purpose in the fetal liver, the volume of each
140 protein band in each lane was normalized against respective ACTB band volumes for the same lanes.

141

142 **Statistical and Ingenuity Pathway Analysis**

143

144 Statistical analysis was performed using the JMP statistical Software (<http://www.jmp.com/software/>). For each set
145 of normalized values a Normality test was performed followed by either One-way ANOVA (for normally-
146 distributed values) or Wilcoxon-Mann-Whitney test (for non-normally distributed values) to calculate statistically
147 significant differences among means. Proteins exhibiting treatment-specific alterations in expression were analyzed
148 using IPA V9.0 (Ingenuity Systems, <http://www.ingenuity.com>), including canonical pathway analysis, functional
149 network analysis.

150

151 RESULTS

152

153 **Identification of fetal liver proteins affected by maternal smoking and/or fetal sex**

154

155 Overall, 494 fetal liver protein spots showed reproducibility across replicates of Coomassie-stained 2D PAGE gels
156 following analysis by Samespots software. Twenty two of these protein spots showed statistically significant spot
157 volume differences between at least two of the four groups (male/female, smoking/non-smoking, $P < 0.05$) and were
158 suitable for LC-MS/MS identification (Fig. 1). For 3 of these spots, peptide fragments were unambiguously
159 assigned to a single protein. For 14 spots, peptide fragments were identified which belonged to more than one
160 protein and the primary protein in the spot was identified based on (i) high Mascot score, (ii) agreement between
161 estimated (i.e. from electrophoretic gel mobility) and calculated molecular weight and isoelectric point, and (iii)
162 peptide coverage. For 5 spots, peptide fragments could be assigned to two proteins with similar likelihood (Table
163 1). The 25 differentially expressed proteins are involved in processes including post-translational protein
164 processing and secretion (ERP29, PDIA3), stress responses and detoxification (HSP90AA1, HSBP1, ALDH7A1,
165 CAT) and homeostasis (FLT, ECHS1, GLUD1, SERPINA1, AFP, SDHA) (Suppl. Table S4). Spot 1090 (CCT6A)
166 was more abundant in control males than females and spot 1673 (ERP29) was more abundant in smoke-exposed
167 females than smoke-exposed males. ERP29 was also very likely to be the primary protein in spots 1684 and 1678,
168 which were affected by maternal smoking (Table 1). Detailed information of all the proteins identified, spot
169 characteristics and protein functions are provided in Suppl. Tables S3 and S4). Sex-specific spot volume
170 differences (female controls vs male controls) were identified for six spots (990, AFP; 1090, CCT6A; 1678,
171 ERP29; 1221, ALDH7A1; 1501, CRYL1; 1006, SDHA: Table 1). Maternal smoking induced sex differences in
172 spot volumes for 14 spots and reversed sex-differences in 3 spot volumes (Table 1). Accounting for the potential
173 presence of more than one protein in a spot, spot volumes were altered in 24 identified proteins among the four
174 groups overall.

175

176 **Validation of proteomic hits by transcript and/or protein measurements**

177

178 Transcript and/or protein levels of the identified proteins were quantified in individual samples in all groups using
179 real-time PCR and 1D-Western blot respectively (Fig. 2-3; Suppl. Fig. S1-2). There was a modest degree of
180 agreement between proteomic predictions and transcript levels, both with regard to the effect of smoking and/or sex
181 differences. *FLT* and *ALDH7A1* mirrored mean spot volume differences (spots 1786 and 1221 respectively) (Fig.
182 2A-B). In contrast, while there were differences in *PNP* and *HSP901AA1* transcript levels among the four groups,
183 these did not closely mirror protein spot volume differences (Fig. 2C-D). Similarly, *ECHS1*, *GLUD1*, *SPRYD4*,
184 *USP5*, *PGK1*, *CRYL1* and *ERP29* transcript levels transcript levels poorly correlated with spot volume differences
185 (Suppl. Fig. S1). PDIA3, AFP, CALR, SERPINA1, HSPB1 and YWHAE protein levels measured by Western blot
186 related poorly to spot volume differences with only CAT protein levels consistent with proteomics data (Fig. 3;
187 Suppl. Fig S2).

188

189 To investigate the apparent discrepancy between Western blot protein quantitation and proteomic 2D PAGE data,
190 2D-Western blots using the fetal liver protein pools employed for the proteomics were probed with the same
191 antibodies employed for 1D-Western blots. The resulting blots were superimposed on the Coomassie-stained gel
192 images to identify which antibody-stained spots showed volume differences by Samespots analysis. For AFP,
193 PDIA3, HSPB1 and CAT the single band observed in 1D Western blots resolved to two or more spots of similar
194 molecular weight but different pI, only one of which was found to have a statistically significant volume difference
195 among groups (Fig. 3). This demonstrated that total protein level quantification (as in conventional 1D-Western
196 blots) will not always match proteomic predictions and provided an explanation for the discrepancy between 1D-
197 Western blot protein levels and spot volumes measurements. Antibodies against SERPINA1, CALR and YWHAE
198 identified two bands of close molecular weight for each protein in 1D-Western blots. Protein bands for SERPINA1,
199 CALR and YWHAE, resolved to a single spot in 2D-Western blots and indicated that quantification of 1D-Western
200 blot bands should mirror spot volume measurements (Suppl. Fig. S2); since that was not the case, SERPINA1,
201 CALR and YWHAE proteins were considered as false positives.

202

203 **Sex-specific liver proteome alterations by maternal smoking**

204

205 From the fetal liver proteins affected by maternal smoking, only ECHS1, ALDH7A1, TPI1, KRT8 and ERP29
206 were affected in both sexes. AFP, PGK1, KRT8, GLUD1, CAT, CRYL1, USP5, HSP90AA1, CAT, SDHA were
207 primarily affected in females while SPRYD4, FLT1, PNP, PDIA3, HSPB1 and EEF1B2 were primarily affected in
208 males (Table 1). The fetal liver proteins affected by maternal smoking in either sex were functionally analyzed and
209 related to physiological and disease pathways using IPA software (Table 2). The majority of proteins belonging to
210 pathways relating to cancers, cancer-development and necrosis were dysregulated in both sexes. Inflammation,
211 cellular homeostasis, proliferation and apoptotic pathways were preferentially dysregulated in males and glucose
212 metabolism disorder pathway was preferentially dysregulated in females (Table 2). Protein levels of the mitotic
213 marker phospho-serine10 histone H3 were measured in protein extracts and the absence of any statistically
214 significant differences indicates that proliferation was not affected by fetal sex or maternal smoking (Suppl. Fig
215 S3).

216

217 DISCUSSION

218

219 We took a global approach not previously used for the human fetal liver and compared the proteomes of male and
220 female control and smoke-exposed second trimester fetuses. This led to the identification of proteins primarily
221 involved in stress responses, homeostasis, metabolism, post-translational protein processing and secretion,
222 paralleling the known effects of cigarette smoking in adults (23, 24, 25). Even though maternal smoking modestly
223 altered the levels of affected proteins (alterations ranged from 15-40% compared to Controls, Table 1), small
224 changes in the levels of multiple proteins and/or protein post-translational modifications can lead to significant
225 phenotypic alterations in organ function (26). Given that the affected proteins are involved in both pathological and
226 physiological processes, smoking-induced alterations in their levels in the fetal liver may be linked to potentially
227 adverse health outcomes for the offspring. In some instances, *in utero* smoke exposure has been associated with
228 differential health outcomes in prepubertal boys and girls; boys are at a higher risk of conduct disorder (15) while
229 girls are more prone to drug dependence and increased body weight (15, 27). The differential responses between
230 male and female fetal livers described here suggests that sex-specific health outcomes might be related to important
231 differential *in utero* molecular responses, indicating that sex differences in fetal liver responses to maternal
232 smoking may contribute to subsequent disease predisposition.

233

234 The approach used in this study relied on the 2D separation of proteomes by size and pI, which allowed us to
235 simultaneously compare protein levels in the observable proteomes among the four groups. Subsequent LC-MS/MS
236 was used to identify the proteins whose volumes showed significant changes and 2D-WBs were used to provide
237 information about the putative post-translational modification(s) of the identified proteins. In contrast to the
238 conventional, hypothesis-driven 1D WBs, our approach aimed to provide a top-down view of the proteomes
239 examined. However, the liver proteome contains over 6,800 individual proteins (28) and the 494 good quality
240 Coomassie-stained spots included in our analysis inevitably represent the most abundant proteins, which is a
241 significant disadvantage compared to conventional 1D-WB approaches. Proteins can resolve to multiple spots of
242 different MW and/or pI depending on isoform expression and/or post-translational modifications and obtaining 24
243 different proteins (Table 1) from 22 differentially expressed protein spots is by no means surprising. 2D gel

244 separation-based proteomic approaches as described here can identify volume differences in a single spot that
245 may not correspond to the total protein levels but rather specific forms/isoforms of a given protein. This is the most
246 likely explanation for most cases where poor correlations between transcript/protein levels and spot volumes were
247 observed. Group differences in individual fetal PDIA3, AFP and HSBP1 total protein levels (quantified using 1D-
248 Western blots) were likely not matched to proteomic spot volume differences because significant volume changes
249 occurring in a single spot containing a given protein, as opposed to total spot volume of all spots containing that
250 protein will not necessarily reflect total protein levels (Fig. 3). In the case of SERPINA1, CALR and YWHAE, a
251 single band by 1D-Western blot reflected a single spot in 2D-Western blots (Suppl. Fig. S2). Therefore, a lack of
252 correlation between spot volume differences and protein level quantification strongly implies that SERPINA1,
253 CALR and YWHAE were either false positives or the spot volume differences reflected the presence of other
254 unresolved proteins in our proteomic analysis.

255 256 **Endoplasmic reticulum changes induced by maternal smoking**

257
258 Changes in PDIA3, AFP, HSBP1 and CAT proteins suggest that maternal smoking has subtle effects on the fetal
259 liver proteome. Single bands on 1D-Western blot for these proteins resolved as two or more bands on 2D gels,
260 indicating alterations in protein conjugation or phosphorylation. The chaperone protein PDIA3 has disulfide
261 isomerase activity and is involved in the folding of endoplasmic reticulum proteins. PDIA3 is frequently
262 encountered in proteomic analyses of liver function (29) and its different forms represent different phosphoforms
263 (29, 30, 31). PDIA3 phosphoform changes occur during sperm capacitation (31), in hyperoxic lung epithelial cells
264 (30) and in rat livers after fasting or leptin administration (29). Our results suggest that the phosphorylation status
265 of PDIA3 is affected in fetal human livers by maternal smoking. Interestingly, hyperoxia in lung epithelial cells and
266 fasting or leptin administration in the rat liver primarily alters the spot volume of the second most-acidic PDIA3
267 phosphoform, which may modulate signal transducer and activator of transcription 3 (STAT3) signaling (29, 30,
268 32). This probably corresponds to the same phosphoform increased in male fetal livers in our analysis (Fig. 3Aiii).
269 ERP29 is another, widely-distributed, endoplasmic reticulum protein involved in processing secreted proteins.
270 Isoelectric focusing resolves ERP29 to a major spot and several more acidic forms, likely the result of selective

271 deamidations (33). Our results show that both native and deamidated forms were altered in response to maternal
272 smoking. Taken together with the change in PDIA3 phosphoforms, maternal smoking clearly has the potential, via
273 modifying protein components of the endoplasmic reticulum, to affect the processing and probably functionality
274 and half-lives of fetal liver secreted proteins. In support of the latter, cigarette smoke disrupts endoplasmic
275 reticulum components, increases protein disulphide isomerase activity and the formation of aberrant multi-protein
276 complexes in mice (24) and is associated with elevated protein secretory responses in humans (25).

277

278 AFP, a major plasma protein secreted by the fetal liver, binds fatty acids (34) and modulates estrogenic responses
279 (35). In adults increased serum AFP levels are associated with liver cirrhosis, hepatic carcinomas or non-
280 seminomatous germ cell tumors (36). Here the reduction in a single AFP spot volume in female fetuses in response
281 to maternal smoking (Fig. 3Biii) may be the result of altered ERP29, which is also dysregulated in females.

282

283 **Stress responses in the human fetal liver**

284

285 Consistent with previous measurements of human fetal liver transcripts (1, 2), the expression of fetal liver proteins
286 involved in stress responses and detoxification was altered in response to maternal smoking, including the
287 ubiquitous molecular chaperone HSP90AA1 (up-regulated in female smoke-exposed livers; Fig. 2C). Since
288 HSP90AA1 is essential for the function of the xenotoxicant sensor, Aryl Hydrocarbon Receptor (AHR) (37),
289 increased HSP90AA1 levels in female smoke-exposed livers may reflect increased AHR levels and/or signaling,
290 via elevated smoking-delivered polycyclic aromatic hydrocarbon levels in female fetuses (19). Levels of CAT
291 (which inactivates reactive oxygen species) were also increased in smoke-exposed females, possibly counteracting
292 the catalase-inhibiting activity of tobacco smoke (38), and/or the increase of by-product reactive oxygen species
293 from tobacco smoke by Phase I enzymes (39). In male fetal livers maternal smoking was associated with changes
294 in the volume of a single spot identified as HSBP1 (Fig. 3D). HSBP1 resolves to several isoforms of similar MW
295 but different pI, corresponding to native (basic) and phosphorylated protein (more acidic) forms (40). Consistent
296 with the increased phosphorylation and shift in pI of HSBP1 in response to stressors (40), the phosphorylated
297 HSBP1 (more acidic) form was up-regulated in smoke-exposed male fetuses (Fig. 3Diii). ALDH7A1 plays a role in

298 detoxification by reducing aldehydes (41) and both ALDH7A1 protein and transcript levels fell in females but
299 increased in males (Fig. 2B). Such sex-specific responses in stress- and detoxification- related protein expression
300 reported here have also been observed in zebrafish livers in response to xenotoxicants (42).

301

302 **Sex-specific pathway alterations in the fetal liver**

303

304 Even though maternal smoking affected pathways relating to cancer in both sexes, there were sex-differences in the
305 pathways involved (Table 2). Cellular homeostasis, inflammation and proliferation/apoptosis pathways are linked
306 to liver fibrosis and cirrhosis (43) and were preferentially affected in male livers. In contrast, the glucose
307 metabolism disorder pathway, which is also linked to cirrhosis (44), was preferentially affected in females. This
308 finding is supported by animal studies where exposure of male and female mice *in utero* to tobacco smoke can
309 contribute to liver fibrosis in adulthood (45) suggesting that while different pathways may be affected by maternal
310 smoking, there can be a convergence in disease outcomes later in life. Sexually dimorphic responses can manifest
311 in other types of stress including differential rodent liver responses to chronic hydrocarbon exposure (46) as well as
312 high fat diet-reduced insulin sensitivity (47). Sex-specific gene expression differences in the human fetal liver (2),
313 as well as other organs including the placenta (48) underline that it is not surprising to find sexually dimorphic
314 responses to stress. Our findings are, therefore, in line with similar studies demonstrating sex-specific responses.
315 This suggests that sex-specific ameliorative and/or preventive treatments might be applicable for diseases
316 developing in *in utero* smoke-exposed individuals.

317

318 Overall, in this study we have shown that maternal smoking disrupts diverse pathways in the fetal liver which
319 suggests that for a set of childhood and/or adulthood diseases commonly linked to maternal smoking, disruption of
320 fetal liver physiology may be one of the bridges connecting developmental perturbations to disease later in life. Our
321 proteomic screen and IPA analysis has also highlighted the sex-specific manner in which fetal livers are affected by
322 maternal smoking suggesting that males and females may be predisposed to different diseases as a result of smoke
323 exposure.

324

325

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327

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330

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495

496

497 FIGURE LEGENDS

498

499 **Figure 1: Summary of altered spot analysis, group distribution and spot position on 2D-PAGE.** The
500 distribution of spots altered by maternal smoking (blue circle), by sex irrespective of maternal smoking (pink
501 circle) and of those whose sex-differences were affected by maternal smoking (yellow circle) are shown in the
502 Venn diagram.

503

504 **Figure 2: Comparison between spot volume differences and transcript levels of identified proteins.** Panels (A-
505 D) show (i) spot volume and (ii) transcript of all data points with means represented by a green line. Significant
506 differences (One-way ANOVA or Wilcoxon test) between groups are indicated by letters in the boxes above each
507 graph. Within each spot volume (i) or transcript (ii), groups that do not share a letter are significantly ($P < 0.05$)
508 different, **i.e. where the superscript letters are shared between groups, there is not statistically significant difference.**
509 “C”: control; “SE” smoke exposed.

510

511 **Figure 3: Comparison between spot volume differences and total protein levels of identified proteins.** Panels
512 (A-D) show (i) spot volume, (ii) protein levels with means represented by a green line and (iii) representative 1D
513 **(each lane corresponding to a protein extract from each of the four groups)** and 2D Western blots. Significant
514 differences (One-way ANOVA or Wilcoxon test) between groups are indicated by letters in the boxes above each
515 graph. Groups that do not share a letter are significantly ($P < 0.05$) different, **i.e. where the superscript letters are**
516 **shared between groups, there is not statistically significant difference.** Black arrowheads in (iii) indicate the protein
517 band in question (1D) and the spot whose volume was altered (2D). X-ed arrowheads represent bands from
518 immunoblotting of the membrane with other antibodies. “C”: Control; “SE” smoke exposed.

519

Table 1: Protein candidates from LC-MS/MS spot analysis. Spot numbers with an asterisk indicate detection of a single candidate. Only fold-changes that achieved significance ($p < 0.05$) are shown. **ISD**, induction of sex-difference; **RSD**, reversal of sex-difference; **ASD**, abolishment of sex-difference.

Spot #	Gene symbol	Protein Name	Fold Change by Maternal Smoking	Fold Change by Sex in Controls	Smoke effect on Sex Differences (fold change)
743	<i>USP5</i>	Ubiquitin carboxyl-terminal hydrolase 5	+1.22 (♀)	No	No
	<i>HSP90AA1</i>	Heat shock protein 90 isoform 2			
990	<i>AFP</i>	Alpha fetoprotein	-1.31 (♀)	+1.15 (♀)	RSD;+1.20 (♂)
1006	<i>SDHA</i>	Succinate dehydrogenase	-1.23 (♀)	+1.26 (♀)	No
1090 *	<i>CCT6A</i>	T-complex protein 1 subunit zeta isoform a	No	+1.15 (♂)	No
1123	<i>CAT</i>	Catalase	+1.14 (♀)	No	IDS;+1.13 (♀)
1129	<i>GLUD1</i>	Glutamate dehydrogenase 1, mitochondrial precursor	+1.18 (f)	No	IDS;+1.23 (♀)
	<i>CAT</i>	Catalase			
1149	<i>PDIA3</i>	Protein disulphide isomerase A3	+1.21 (♂)	No	IDS;+1.21 (♂)
1150 *	<i>SERPINA1</i>	alpha-1-antitrypsin precursor	+1.23 (♂)	No	IDS;+1.28 (♂)
1172	<i>CALR</i>	Calreticulin precursor	+1.30 (♀)	No	IDS;+1.14 (♀)
1190	<i>KRT8</i>	Keratin type II Cytoskeletal 8 isoform 2	+1.30 (♀)	No	IDS;+1.16 (♀)
1205	<i>KRT8</i>	Keratin type II Cytoskeletal 8 isoform 2	+1.13 (♂)	No	IDS;+1.24 (♂)
1221 *	<i>ALDH7A1</i>	alpha-aminoadipic semialdehyde dehydrogenase isoform 2	-1.22 (♀); +1.24 (♂)	+1.27 (♀)	RDS;+1.26 (♂)
1328	<i>PGK1</i>	Phosphoglycerate kinase 1	+1.27 (♀)	No	IDS;+1.40 (♀)
1501 *	<i>CRYL1</i>	Lambda crystallin homolog	+1.25 (♀)	+1.16 (♂)	No
1589	<i>ECHS1</i>	Enoyl Coenzyme Hydratase 1	+1.28 (♀) -1.33 (♂)	No	IDS; +1.42 (♀)
1591	<i>PNP</i>	Purine nucleoside phosphorylase	+1.24 (m)	No	IDS;+1.13 (♂)
1645	<i>EEF1B2</i>	Elongation factor 1 beta	+1.20 (♂)	No	IDS;+1.16 (♂)
	<i>YWHAE</i>	14-3-3 protein epsilon			
1673	<i>ERp29</i>	Endoplasmic reticulum resident protein 29 isoform 1 precursor	No	No	IDS;+1.13 (♀)
1678	<i>ERp29</i>	Endoplasmic reticulum resident protein 29 isoform 1 precursor	+1.16 (♀); -1.18 (♂)	+1.13 (♂)	RDS;+1.18 (♀)
	<i>TPI1</i>	Triose phosphate isomerase isoform 1			
1679	<i>HSPB1</i>	Heat shock protein beta 1	+1.18 (♂)	No	No
1684	<i>ERp29</i>	Endoplasmic reticulum resident protein 29 isoform 1 precursor	+1.14 (♀)	No	IDS;+1.16 (♀)
1786	<i>SPRYD4</i>	SPRY domain-containing protein 4	+1.35 (♂)	No	IDS;+1.22 (♂)
	<i>FLTI</i>	Ferritin Light Chain			

1 **Table 2: Pathways affected in the human fetal liver by maternal smoking.** Disease pathways formatted in
 2 **bold** were dysregulated in both sexes. Diseases Pathways are presented by descending *P*-value.

3

	Diseases	Molecules	<i>P</i>-value	
Males	abdominal cancer	ALDH7A1, ECHS1, EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP, TPI1	3.29E-02	
	apoptosis	FLT1, HSPB1, KRT8, PDIA3, PNP	2.13E-02	
	cellular homeostasis	FLT1, HSPB1, KRT8, PNP	1.38E-02	
	genital tract cancer	ALDH7A1, EEF1B2, FLT1, HSPB1, KRT8, PDIA3	1.55E-02	
	inflammation of organ	FLT1, KRT8, PDIA3, TPI1	5.44E-04	
	lung cancer	EEF1B2, FLT1, KRT8, TPI1	2.00E-03	
	malignant neoplasm of pelvis	ECHS1, EEF1B2, FLT1, HSPB1, KRT8, PDIA3	1.18E-02	
	necrosis	FLT1, HSPB1, KRT8, PDIA3, PNP	1.94E-02	
	pelvic cancer	EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP	2.35E-02	
	proliferation of cells	EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP	1.94E-02	
	quantity of cells	FLT1,HSPB1,KRT8,PNP	1.73E-02	
	Females	abdominal cancer	AFP, ALDH7A1, CAT, CRYL1, ECHS1, GLUD1, HSP90AA1, KRT8, PGK1, SDHA, TPI1, USP5	7.88E-03
		breast and colorectal cancer	AFP, CAT, CRYL1, ECHS1, HSP90AA1, KRT8, PGK1, TPI1	3.38E-02
digestive tract cancer		AFP, ALDH7A1, CAT, CRYL1, ECHS1, GLUD1, HSP90AA1, KRT8, PGK1, SDHA, TPI1	8.67E-03	
epithelial cancer		AFP, ALDH7A1, CAT, ECHS1, GLUD1, HSP90AA1, KRT8, PGK1, SDHA, TPI1, USP5	4.16E-02	
genital tract cancer		AFP, ALDH7A1, HSP90AA1, KRT8, PGK1, SDHA, USP5	1.87E-02	
glucose metabolism disorder		CAT, ECHS1, GLUD1, KRT8	1.74E-02	
metastasis		AFP, CAT, HSP90AA1, KRT8	2.27E-03	
necrosis		AFP, CAT, GLUD1, HSP90AA1, KRT8, SDHA	1.64E-02	
proliferation of tumor cell lines		AFP, CAT, HSP90AA1, KRT8	3.07E-02	

Figure 1

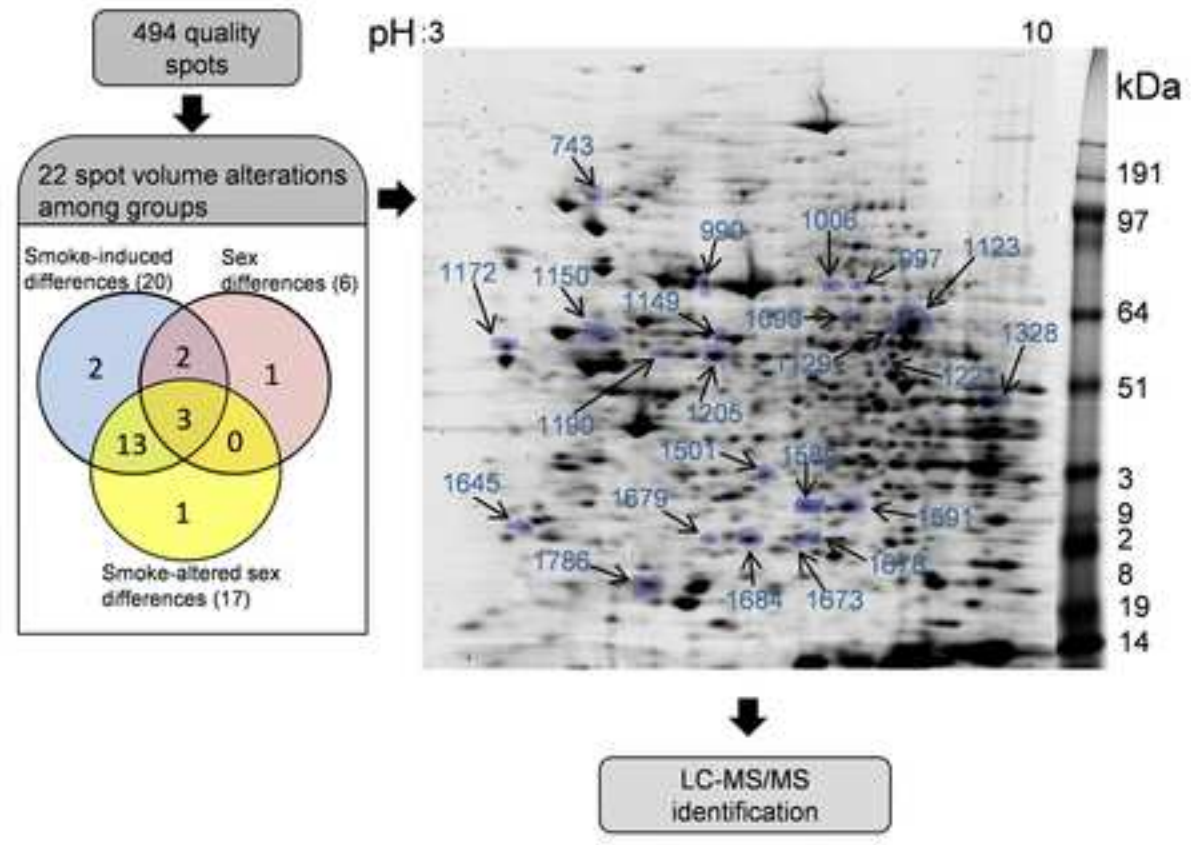
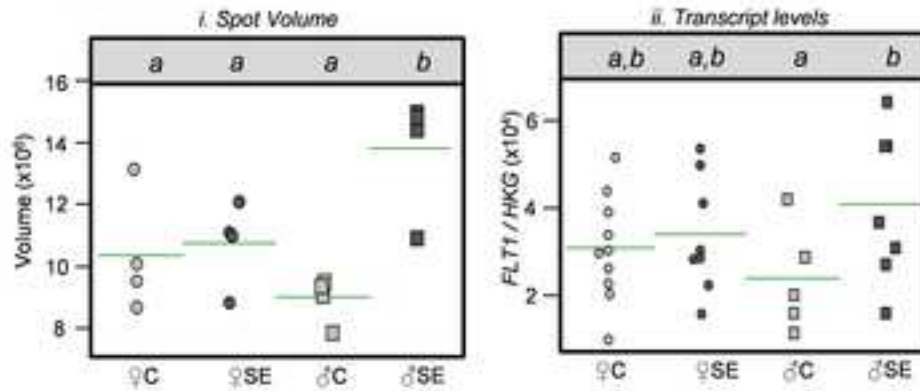
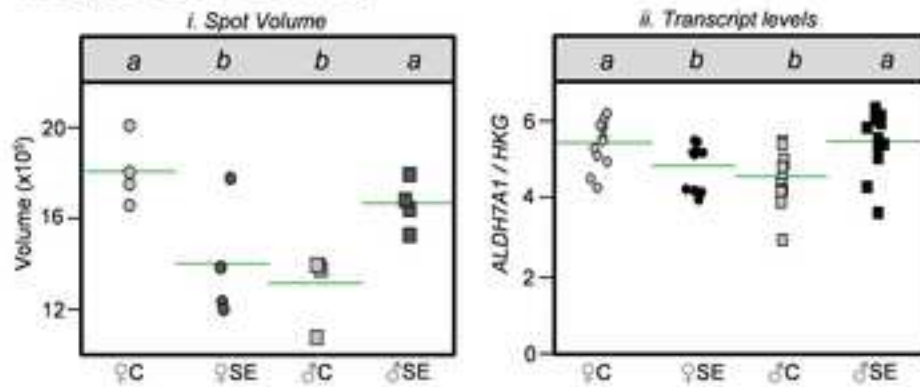


Figure 2

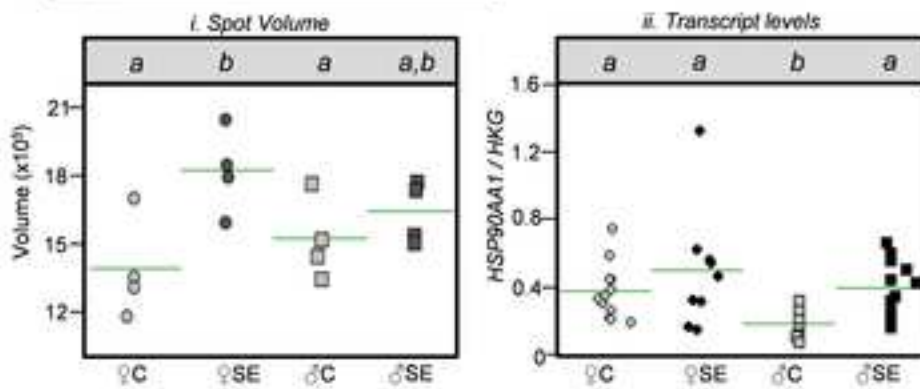
A Spot 1786; *FLT1*



B Spot 1221; *ALDH7A1*



C Spot 743; *HSP90AA1 isoform 2*



D Spot 1591; *PNP*

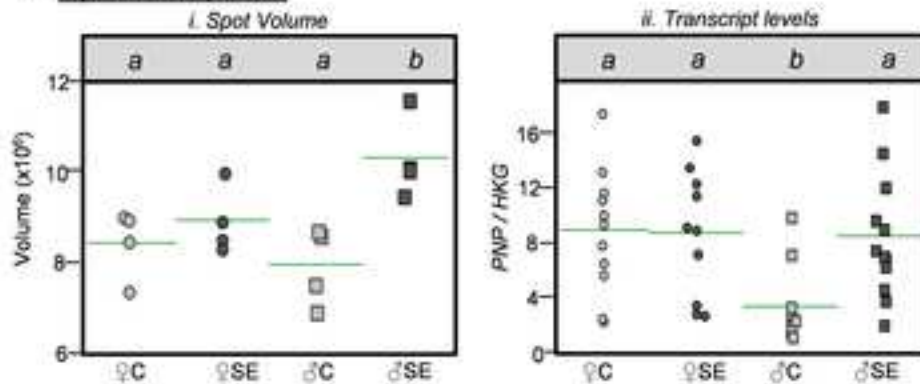
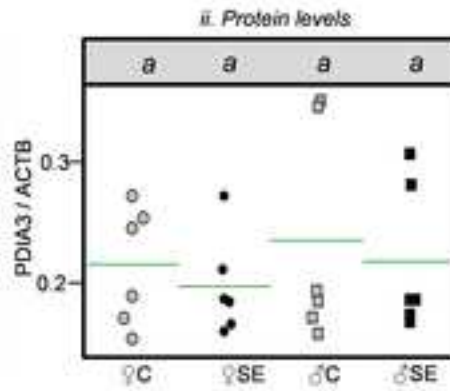
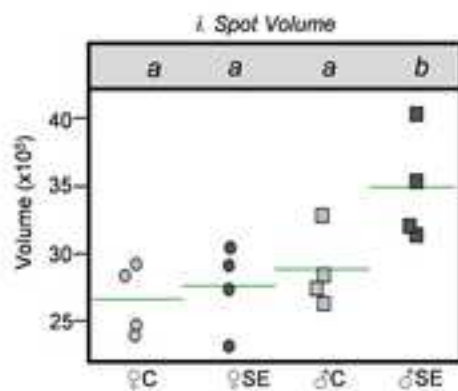
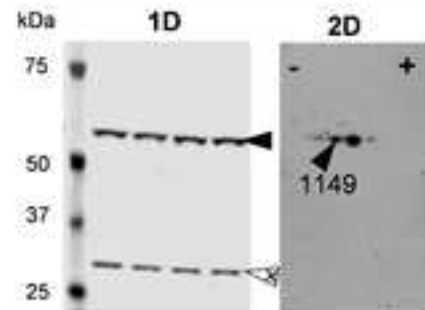


Figure 3

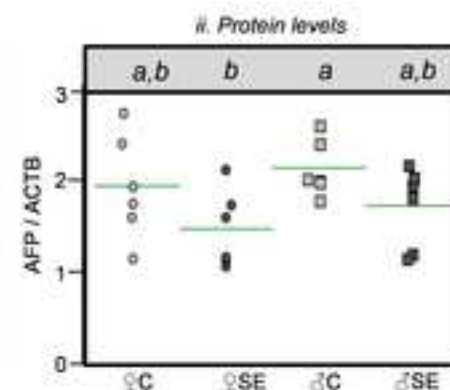
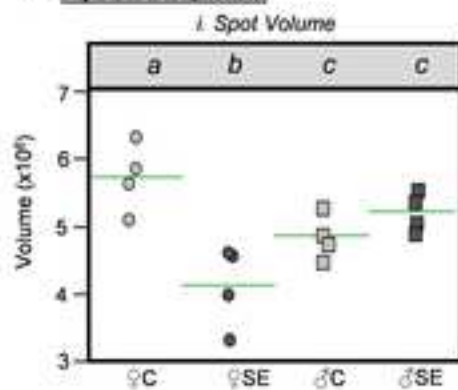
A Spot 1149; PDIA3



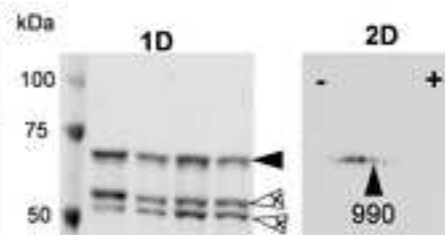
iii. Representative 1D and 2D Western Blots



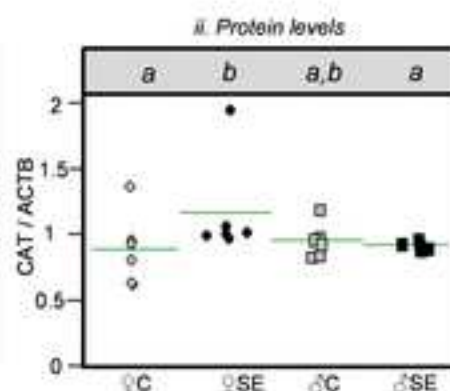
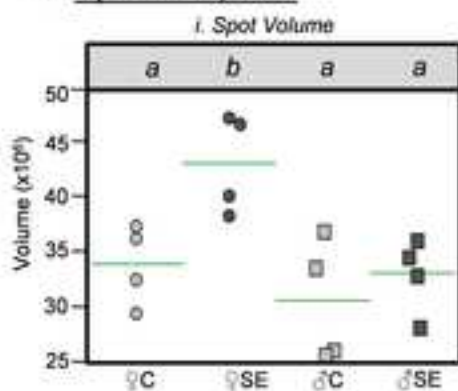
B Spot 990; AFP



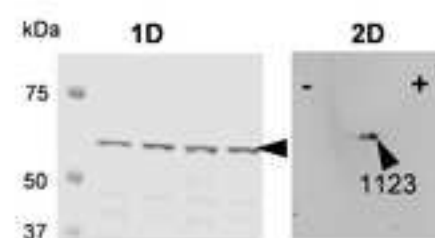
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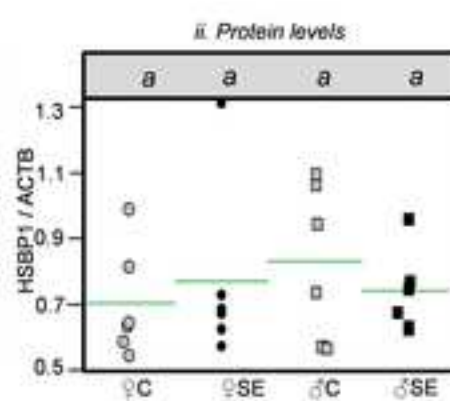
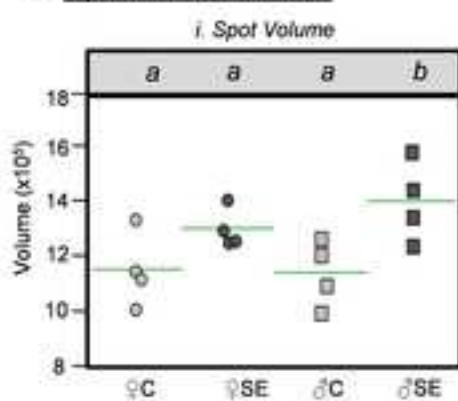
C Spot 1123; CAT



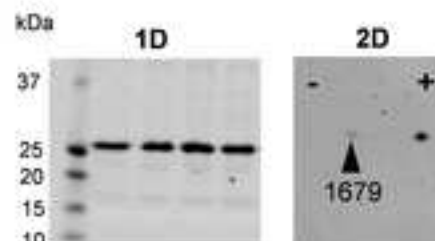
iii. Representative 1D and 2D Western Blots



D Spot 1679; HSPB1



iii. Representative 1D and 2D Western Blots



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