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

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

---Manuscript Draft---

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

**Short Title:** Maternal smoking and the fetal liver proteome

**Article Type:** Original Article

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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.

Objective: Our aim was to analyze, for the first time, the human fetal liver proteome in order to identify pathways affected by maternal smoking.

Design: Fetal liver proteins extracted from elective second trimester pregnancy terminations (12-16 weeks of gestation) were divided in four balanced groups based on fetal sex and maternal smoking.

Setting: University of Aberdeen

Patients/Participants: Livers were collected from 24 morphologically normal fetuses undergoing termination for non-medical reasons.

Intervention: Maternal smoking during pregnancy.

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.

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...
cellular homeostasis, inflammation, proliferation and apoptosis were affected in males and pathways affecting glucose metabolism were affected in females. 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.

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INTRODUCTION

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

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

Study design

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

Sample collection and processing

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

Proteomics

Within each group, equal amounts (100 μg) of protein extracts from each fetal liver were combined. The four protein pools we treated with ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) to remove salt and other contaminants according to the manufacturer’s instructions. The soluble protein fractions were separated using 2D gel electrophoresis in quadruplicate (n=4) as described previously (20) using a pH 3 – 10
immobilized pH gradient gel (GE Healthcare, Uppsala, Sweden) for the first dimension and a 10–15% gradient
polyacrylamide gel for the second dimension. Proteins were detected using Colloidal Coomassie Blue G250 and
scanned using an Ettan Dige Imager (GE Healthcare) and stained gels were analyzed using Progenesis SameSpots
software V6.01 (Nonlinear Dynamics, Newcastle, UK) (21). Individual spot volumes were expressed as normalized
volumes relative to the total detected spot volume for each gel to minimize potential analytical artefacts from
protein loading variations and migration. Progenesis SameSpots was used to combine the gel quadruplicates and
calculate fold-changes and significance (by ANOVA of log-normalized values). Molecular mass and pI of spots
was estimated from separate gels electrophoresed with pH and MW markers. Proteins in the gel pieces were
digested with trypsin and peptides identified using LC-MS/MS as previously described [see (20) and Suppl.
Materials and Methods]. Statistically significant Mascot scores and good sequence coverage were considered to be
positive identifications.

Real-Time PCR

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

Western Blots

Individual human fetal liver protein extracts from the same preparation used for the proteomics were separated (30
μg/lane) in 26-well 4-12% Bis-Tris gradient precast gels (Invitrogen Ltd, Paisley, UK) under reducing conditions
according to the manufacturer’s specifications (20). Gels were blotted on Immobilon-FL membrane (Millipore Ltd,
Watford, UK), blocked and probed with antibodies as previously described (20). Antibodies and dilutions used are detailed in Suppl. Table S2. Protein bands were visualized using Odyssey infrared fluorescent imager and images were analyzed using Phoretix 1D Advanced software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) as detailed in (20). Membranes were reused without stripping and were probed with more than one antibody (typically two to three antibodies) when target protein sizes were considerably different. ACTB probe served as a loading control and following the validation of the use of ACTB for this purpose in the fetal liver, the volume of each protein band in each lane was normalized against respective ACTB band volumes for the same lanes.

**Statistical and Ingenuity Pathway Analysis**

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

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

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

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

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

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

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

The approach used in this study relied on the 2D separation of proteomes by size and pI, which allowed us to simultaneously compare protein levels in the observable proteomes among the four groups. Subsequent LC-MS/MS was used to identify the proteins whose volumes showed significant changes and 2D-WBs were used to provide information about the putative post-translational modification(s) of the identified proteins. In contrast to the conventional, hypothesis-driven 1D WBs, our approach aimed to provide a top-down view of the proteomes examined. However, the liver proteome contains over 6,800 individual proteins (28) and the 494 good quality Coomassie-stained spots included in our analysis inevitably represent the most abundant proteins, which is a significant disadvantage compared to conventional 1D-WB approaches. Proteins can resolve to multiple spots of different MW and/or pI depending on isoform expression and/or post-translational modifications and obtaining 24 different proteins (Table 1) from 22 differentially expressed protein spots is by no means surprising. 2D gel
separation-based proteomic approaches as described here can identify volume differences in a single spot that may not correspond to the total protein levels but rather specific forms/isoforms of a given protein. This is the most likely explanation for most cases where poor correlations between transcript/protein levels and spot volumes were observed. Group differences in individual fetal PDIA3, AFP and HSBP1 total protein levels (quantified using 1D-Western blots) were likely not matched to proteomic spot volume differences because significant volume changes occurring in a single spot containing a given protein, as opposed to total spot volume of all spots containing that protein will not necessarily reflect total protein levels (Fig. 3). In the case of SERPINA1, CALR and YWHAE, a single band by 1D-Western blot reflected a single spot in 2D-Western blots (Suppl. Fig. S2). Therefore, a lack of correlation between spot volume differences and protein level quantification strongly implies that SERPINA1, CALR and YWHAE were either false positives or the spot volume differences reflected the presence of other unresolved proteins in our proteomic analysis.

Endoplasmic reticulum changes induced by maternal smoking

Changes in PDIA3, AFP, HSBP1 and CAT proteins suggest that maternal smoking has subtle effects on the fetal liver proteome. Single bands on 1D-Western blot for these proteins resolved as two or more bands on 2D gels, indicating alterations in protein conjugation or phosphorylation. The chaperone protein PDIA3 has disulfide isomerase activity and is involved in the folding of endoplasmic reticulum proteins. PDIA3 is frequently encountered in proteomic analyses of liver function (29) and its different forms represent different phosphoforms (29, 30, 31). PDIA3 phosphoform changes occur during sperm capacitation (31), in hyperoxic lung epithelial cells (30) and in rat livers after fasting or leptin administration (29). Our results suggest that the phosphorylation status of PDIA3 is affected in fetal human livers by maternal smoking. Interestingly, hyperoxia in lung epithelial cells and fasting or leptin administration in the rat liver primarily alters the spot volume of the second most-acidic PDIA3 phosphoform, which may modulate signal transducer and activator of transcription 3 (STAT3) signaling (29, 30, 32). This probably corresponds to the same phosphoform increased in male fetal livers in our analysis (Fig. 3Aiii). ERP29 is another, widely-distributed, endoplasmic reticulum protein involved in processing secreted proteins. Isoelectric focusing resolves ERP29 to a major spot and several more acidic forms, likely the result of selective
deamidations (33). Our results show that both native and deamidated forms were altered in response to maternal smoking. Taken together with the change in PDIA3 phosphoforms, maternal smoking clearly has the potential, via modifying protein components of the endoplasmic reticulum, to affect the processing and probably functionality and half-lives of fetal liver secreted proteins. In support of the latter, cigarette smoke disrupts endoplasmic reticulum components, increases protein disulphide isomerase activity and the formation of aberrant multi-protein complexes in mice (24) and is associated with elevated protein secretory responses in humans (25).

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

**Stress responses in the human fetal liver**

Consistent with previous measurements of human fetal liver transcripts (1, 2), the expression of fetal liver proteins involved in stress responses and detoxification was altered in response to maternal smoking, including the ubiquitous molecular chaperone HSP90AA1 (up-regulated in female smoke-exposed livers; Fig. 2C). Since HSP90AA1 is essential for the function of the xenotoxicant sensor, Aryl Hydrocarbon Receptor (AHR) (37), increased HSP90AA1 levels in female smoke-exposed livers may reflect increased AHR levels and/or signaling, via elevated smoking-delivered polycyclic aromatic hydrocarbon levels in female fetuses (19). Levels of CAT (which inactivates reactive oxygen species) were also increased in smoke-exposed females, possibly counteracting the catalase-inhibiting activity of tobacco smoke (38), and/or the increase of by-product reactive oxygen species from tobacco smoke by Phase I enzymes (39). In male fetal livers maternal smoking was associated with changes in the volume of a single spot identified as HSBP1 (Fig. 3D). HSBP1 resolves to several isoforms of similar MW but different pI, corresponding to native (basic) and phosphorylated protein (more acidic) forms (40). Consistent with the increased phosphorylation and shift in pI of HSBP1 in response to stressors (40), the phosphorylated HSBP1 (more acidic) form was up-regulated in smoke-exposed male fetuses (Fig. 3Diii). ALDH7A1 plays a role in
detoxification by reducing aldehydes (41) and both ALDH7A1 protein and transcript levels fell in females but increased in males (Fig. 2B). Such sex-specific responses in stress- and detoxification- related protein expression reported here have also been observed in zebrafish livers in response to xenotoxicants (42).

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

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

Overall, in this study we have shown that maternal smoking disrupts diverse pathways in the fetal liver which suggests that for a set of childhood and/or adulthood diseases commonly linked to maternal smoking, disruption of fetal liver physiology may be one of the bridges connecting developmental perturbations to disease later in life. Our proteomic screen and IPA analysis has also highlighted the sex-specific manner in which fetal livers are affected by maternal smoking suggesting that males and females may be predisposed to different diseases as a result of smoke exposure.
The staff at Grampian NHS Pregnancy Counseling Service were essential for collecting fetuses. We thank the Aberdeen Proteomics Core Facility (University of Aberdeen) for their expert assistance.
REFERENCES


(21) Silva E, O'Gorman M, Becker S, Auer G, Eklund A, Grunewald J, Wheelock AM. In the eye of the beholder: does the master see the SameSpots as the novice? J Proteome Res 2010; 9:1522-32.


**FIGURE LEGENDS**

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

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

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

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<td>ECHS1</td>
<td>Enoyl-Coenzyme Hydratase 1</td>
<td>+1.28 (♀);-1.33(♂)</td>
<td>No</td>
<td>IDS:+1.42 (♀)</td>
</tr>
<tr>
<td>1591</td>
<td>PNP</td>
<td>Purine nucleoside phosphorylase</td>
<td>+1.24 (m)</td>
<td>No</td>
<td>IDS:+1.13 (♀)</td>
</tr>
<tr>
<td>1645</td>
<td>EEF1B2</td>
<td>Elongation factor 1 beta</td>
<td>+1.20 (♀)</td>
<td>No</td>
<td>IDS:+1.16 (♀)</td>
</tr>
<tr>
<td>1673</td>
<td>YWHAE</td>
<td>14-3-3 protein epsilon</td>
<td>+1.14 (♀); -1.18(♂)</td>
<td>+1.13 (♀)</td>
<td>RDS:+1.18 (♀)</td>
</tr>
<tr>
<td>1678</td>
<td>ERp29</td>
<td>Endoplasmic reticulum resident protein 29 isoform 1 precursor</td>
<td>+1.14 (♀); -1.18(♂)</td>
<td>+1.13 (♀)</td>
<td>RDS:+1.18 (♀)</td>
</tr>
<tr>
<td>1679</td>
<td>HSPB1</td>
<td>Heat shock protein beta 1</td>
<td>+1.18 (♀)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1684</td>
<td>ERp29</td>
<td>Endoplasmic reticulum resident protein 29 isoform 1 precursor</td>
<td>+1.14 (♀)</td>
<td>No</td>
<td>IDS:+1.16 (♀)</td>
</tr>
<tr>
<td>1786</td>
<td>SPRYD4</td>
<td>SPRY domain-containing protein 4</td>
<td>+1.35 (♀)</td>
<td>No</td>
<td>IDS:+1.22 (♀)</td>
</tr>
<tr>
<td>1845</td>
<td>FLT1</td>
<td>Ferritin Light Chain</td>
<td>+1.22 (♀)</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
**Table 2: Pathways affected in the human fetal liver by maternal smoking.** Disease pathways formatted in **bold** were dysregulated in both sexes. Diseases Pathways are presented by descending *P*-value.

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Molecules</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>abdominal cancer</td>
<td>ALDH7A1, ECHS1, EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP, TP II</td>
<td>3.29E-02</td>
</tr>
<tr>
<td>apoptosis</td>
<td>FLT1, HSPB1, KRT8, PDIA3, PNP</td>
<td>2.13E-02</td>
</tr>
<tr>
<td>cellular homeostasis</td>
<td>FLT1, HSPB1, KRT8, PDIA3, PNP</td>
<td>1.38E-02</td>
</tr>
<tr>
<td>genital tract cancer</td>
<td>ALDH7A1, EEF1B2, FLT1, HSPB1, KRT8, PDIA3</td>
<td>1.55E-02</td>
</tr>
<tr>
<td>inflammation of organ</td>
<td>FLT1, KRT8, PDIA3, TP II</td>
<td>5.44E-04</td>
</tr>
<tr>
<td>lung cancer</td>
<td>EEF1B2, FLT1, KRT8, TP II</td>
<td>2.00E-03</td>
</tr>
<tr>
<td>malignant neoplasm of pelvis</td>
<td>ECHS1, EEF1B2, FLT1, HSPB1, KRT8, PDIA3</td>
<td>1.18E-02</td>
</tr>
<tr>
<td>necrosis</td>
<td>EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP</td>
<td>1.94E-02</td>
</tr>
<tr>
<td>pelvic cancer</td>
<td>EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP</td>
<td>2.35E-02</td>
</tr>
<tr>
<td>proliferation of cells</td>
<td>EEF1B2, FLT1, HSPB1, KRT8, PDIA3, PNP</td>
<td>1.94E-02</td>
</tr>
<tr>
<td>quantity of cells</td>
<td>FLT1, HSPB1, KRT8, PNP</td>
<td>1.73E-02</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>abdominal cancer</td>
<td>AFP, ALDH7A1, CAT, CRYL1, ECHS1, GLUD1, HSP90AA1, KRT8, PGK1, SDHA, TP II</td>
<td>7.88E-03</td>
</tr>
<tr>
<td>breast and colorectal cancer</td>
<td>AFP, CAT, CRYL1, ECHS1, HSP90AA1, KRT8, PGK1, TPII</td>
<td>3.38E-02</td>
</tr>
<tr>
<td>digestive tract cancer</td>
<td>AFP, ALDH7A1, CAT, CRYL1, ECHS1, GLUD1, HSP90AA1, KRT8, PGK1, SDHA, TPI</td>
<td>8.67E-03</td>
</tr>
<tr>
<td>epithelial cancer</td>
<td>AFP, ALDH7A1, CAT, ECHS1, GLUD1, HSP90AA1, KRT8, PGK1, SDHA, TPII</td>
<td>4.16E-02</td>
</tr>
<tr>
<td>genitalic tract cancer</td>
<td>AFP, ALDH7A1, HSP90AA1, KRT8, PGK1, SDHA, USP5</td>
<td>1.87E-02</td>
</tr>
<tr>
<td>glucose metabolism disorder</td>
<td>CAT, ECHS1, GLUD1, KRT8</td>
<td>1.74E-02</td>
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<tr>
<td>metastasis</td>
<td>AFP, CAT, HSP90AA1, KRT8</td>
<td>2.27E-03</td>
</tr>
<tr>
<td>necrosis</td>
<td>AFP, CAT, GLUD1, HSP90AA1, KRT8, SDHA</td>
<td>1.64E-02</td>
</tr>
<tr>
<td>proliferation of tumor cell lines</td>
<td>AFP, CAT, HSP90AA1, KRT8</td>
<td>3.07E-02</td>
</tr>
</tbody>
</table>
Figure 1

The image shows a Venn diagram illustrating the distribution of 494 quality spots among groups with 22 spot volume alterations. The diagram categorizes spots into groups: smoke-induced differences (20), sex differences (6), and smoke-altered sex differences (17). The spots are visualized under pH 3 conditions, with molecular weight (kDa) on the right side. Each spot is annotated with a number, indicating its molecular weight in the range of 3 to 191 kDa, and 14 to 19 kDa. The diagram also indicates that the spots are identified using LC-MS/MS.
Figure 3

A. Spot 1149; PDIA3
   i. Spot Volume
   - a, a, a, b
   - Volume (x10^6)
   - C, SE, C, SE, C
   
   ii. Protein levels
   - PDIA3/ACTB
   - a, a, a, a
   - C, SE, C, SE
   
   iii. Representative 1D and 2D Western Blots
   - 1D
   - 2D
   
B. Spot 990; AFP
   i. Spot Volume
   - a, b, c, c
   - Volume (x10^6)
   - C, SE, C, SE, C
   
   ii. Protein levels
   - AFP/ACTB
   - a, b, b, a, a
   - C, SE, C, SE
   
   iii. Representative 1D and 2D Western Blots
   - 1D
   - 2D
   
C. Spot 1123; CAT
   i. Spot Volume
   - a, b, a, a
   - Volume (x10^6)
   - C, SE, C, SE, C
   
   ii. Protein levels
   - CAT/ACTB
   - a, b, a, b, a
   - C, SE, C, SE
   
   iii. Representative 1D and 2D Western Blots
   - 1D
   - 2D
   
D. Spot 1679; HSPB1
   i. Spot Volume
   - a, a, a, b
   - Volume (x10^6)
   - C, SE, C, SE, C
   
   ii. Protein levels
   - HSPB1/ACTB
   - a, a, a, a
   - C, SE, C, SE
   
   iii. Representative 1D and 2D Western Blots
   - 1D
   - 2D
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