

## Biochemical Mapping of Metabolic Alterations in Lungs of Rat Embryos

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### Introduction

GC-TOF or LC-MS unbiased surveys of biological samples yield hundreds of resolved peaks per chromatogram. Statistical significant differences between semi-quantitative peak intensities can be routinely assigned to the classes of study designs. Metabolite peaks are then often called 'putative biomarkers' which must be validated in subsequent studies and confirmed to be specific for a diagnostic case. Consequently, the biomarker peaks must be unambiguously re-detectable over months in subsequent studies. This can best be performed by establishing standardized mass spectrometric metabolome databases. Secondly, valid biomarkers require a clear route to annotation of novel compounds to be implemented in routine clinical screens. Thirdly, interpretation of differential regulation of the identified metabolites should be guided by biochemical mapping to be of biomedical relevance.

### Methods

PBS-perfused rat lungs were fresh frozen prior to homogenization and extraction. GC-TOF mass spectrometry (Leco Pegasus IV) was performed using Gerstel automatic liner exchange and cold injection conditions. GC-TOF spectra were filtered by the in-house BinBase mass spectral database. Metabolites were identified by a retention index/MS library of 713 authentic standards including PubChem and KEGG identifiers. Quantitative results were statistically evaluated, and significant differences were mapped to biochemical and chemical databases by open-access tools. Unknown metabolites were annotated using chemical ionization GC-TOF MS (Waters GC-T) and database queries according to accurate mass and accurate isotope data. Lipid fingerprints complemented the survey using nanoelectrospray (Advion) coupled to FT-ICR MS (ThermoFisher LTQFT). Data were aligned by the Expressionist software (Genedata).

### Results

Timed pregnant rats were subjected to environmental tobacco smoke daily at 1 mg/m<sup>3</sup> for 6 hours each day in controlled chambers from gestation day 5 to gestation day 20 (term is 21 days). After sample preparation, lung tissue GC-TOF chromatograms showed on average 852 deconvoluted peaks. However, many of these peaks were not consistently detectable in subsequent analysis of biological replicate samples. Consequently, this number of peaks was reduced to a much cleaner data set of 305 peaks using the in-house database BinBase by employing mass spectral metadata (peak purity, unique masses, s/n, apex masses). 155 of these metabolites were unambiguously identified by both retention index and mass spectral match criteria using a recently released mass spectral library and step-wise increase of similarity thresholds based on peak purity and peak abundance. Missing data were subsequently replaced from ion traces of unprocessed netCDF files, yielding a coherent result data sheet of 31,570 metabolic values (Fig. 1). Raw and processed data are publicly available for downloads from [http://fiehnlab.ucdavis.edu:8080/m1/main\\_public.jsp](http://fiehnlab.ucdavis.edu:8080/m1/main_public.jsp).

Multivariate statistics clearly proved that metabolic phenotypes in developing lungs were altered by cigarette smoke. 46 metabolites were differentially regulated in fetal lungs which were exposed perinatally to environmental tobacco smoke, indicating changes in carbohydrate and lipid metabolism. Only six

BinBase name	Ret. Index	quant mas:	BinBase id	mass spec	PubChem	KEGG id	treatment												
							FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	FA (HEPA)	
vaniline	702331	353	203224	85.13610.81	1189	C00385	27338	58961	28330	37398	28634	33383	52115	30604	3950	5189	8518	5171	4:
valine	313224	144	193605	85.48.0.88	6287	C00183	152413	257428	160304	185722	187421	156342	165267	157329	629404	573179	452127	625416	5:
uridine minor	856953	258	213127	85.2472.0.6	8929	C00299	1258	1498	1556	2249	1083	3711	1878	2827	4314	4596	2735	4:	
urea	328046	171	193594	85.882.0.86	1176	C00086	80341	5932	210030	282352	311079	261545	138509	176663	584754	525827	591917	475702	5:
uracil	385903	99	193600	85.620.0.86	1174	C00106	539503	137952	482950	54421	53949	57857	96628	43992	25085	37712	64472	37729	4:
tgrosine minor	653438	179	193901	85.75.0.86:	1153	C00082	8940	12770	8646	8432	8742	7443	21626	10974	11789	11401	7850	29881	4:
tgrosine	671085	218	193781	86.146.0.89	6057	C00082	62801	134605	54910	77688	45446	52888	56217	60801	212623	264878	321733	2:	
trgptophan	779834	202	193776	86.27.0.87:	6305	C00078	26440	50527	26503	32583	28805	26817	34027	23508	286	172	801	46416	1:
tris(ethylenglycol) NIST	471814	117	203267	86.40.0.87:			11423	23011	10833	7954	1122	11918	13536	18003	6311	6274	1587	7954	8:
trehalose	947837	191	193289	86.85.0.89:	7427	C00183	613	6734	532	736	988	1038	5915	537	6815	2899	414	1135	3:
tocopherol	186719	237	193211	85.104.0.87	14985	C02477	1009	150	2278	2596	2827	1632	709	3620	511	512	499	362	5:
threonine 2TMS	369345	117	193780	85.463.0.86	6288	C00188	32237	25509	10759	5712	2119	12070	31898	12363	42292	62277	56377	52402	6:
threonine	409403	117	193626	85.164.0.86	6288	C00188	409104	460286	447215	402532	378812	374659	41891	244501	425224	378499	343521	426315	4:
threonic acid	497167	292	193262	86.2.0.89:	5282333	n/a	110634	106135	64698	88516	96179	72818	87164	79457	221943	208849	145393	229444	1:

Fig. 1. Screenshot of the result data sheet of GC-TOF mass spectrometry data processed by BinBase. Each deconvoluted peak is detailed by name, retention index, quantification mass, string-encoded mass spectra and database outlinks to PubChem and KEGG for identified compounds.

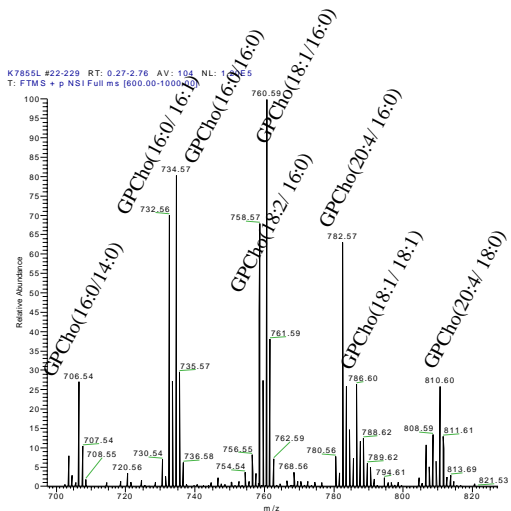


Fig. 2 Identified Phospholipids in rat lungs by nanoESI-LTQ-FTICR MS using accurate mass MS and MS/MS lookup tables from LipidMaps.

statistically significant differences were annotated as unknown biomarkers. These peaks were subjected to identification by GC-TOF under chemical ionization and altered derivatization schemes. Additionally relative changes of polar membrane lipids were investigated by direct infusion nano-electrospray LTQ-FTICR mass spectrometry, and identification was achieved by using LipidMaps as template to construct accurate mass and MS/MS lookup tables (fig. 2). Analysis of quantitative changes in ratios of polar lipids in lungs showed a tendency of up-regulation of lipids with higher degree of unsaturation and longer acyl carbon chain lengths under chronic exposure to environmental tobacco smoke.

Metabolic differences for small metabolites (<500 Da) for lungs of dams and fetuses were eventually mapped onto chemical similarity networks (Tanimoto distances) to enable straightforward interpretations of major metabolic disruptions (Fig. 3). Notably, fetus lungs were only indirectly smoke exposed through the dam's blood streams.

In contrast to lungs of dams, fetus lungs showed major down regulation (blue nodes) for free fatty acids which might lead to impairment of lung function and asthmatic conditions at later stages of life. Interestingly, a similarity of metabolic changes in lungs of fetuses and dams was observed for down regulation of purines such as adenosine and guanosine which may indicate lower rates of metabolic turnover in smoke-impaired lungs. Only few up-regulations of metabolites (red nodes) were observed such as for key energy metabolites like ketone bodies and sugar phosphates. Metabolites that were not altered at statistically significance levels of  $p < 0.05$  are displayed as small yellow nodes. Peaks that were not identified by the Fiehn mass spectral libraries are not displayed in these Cytoscape network graphs.

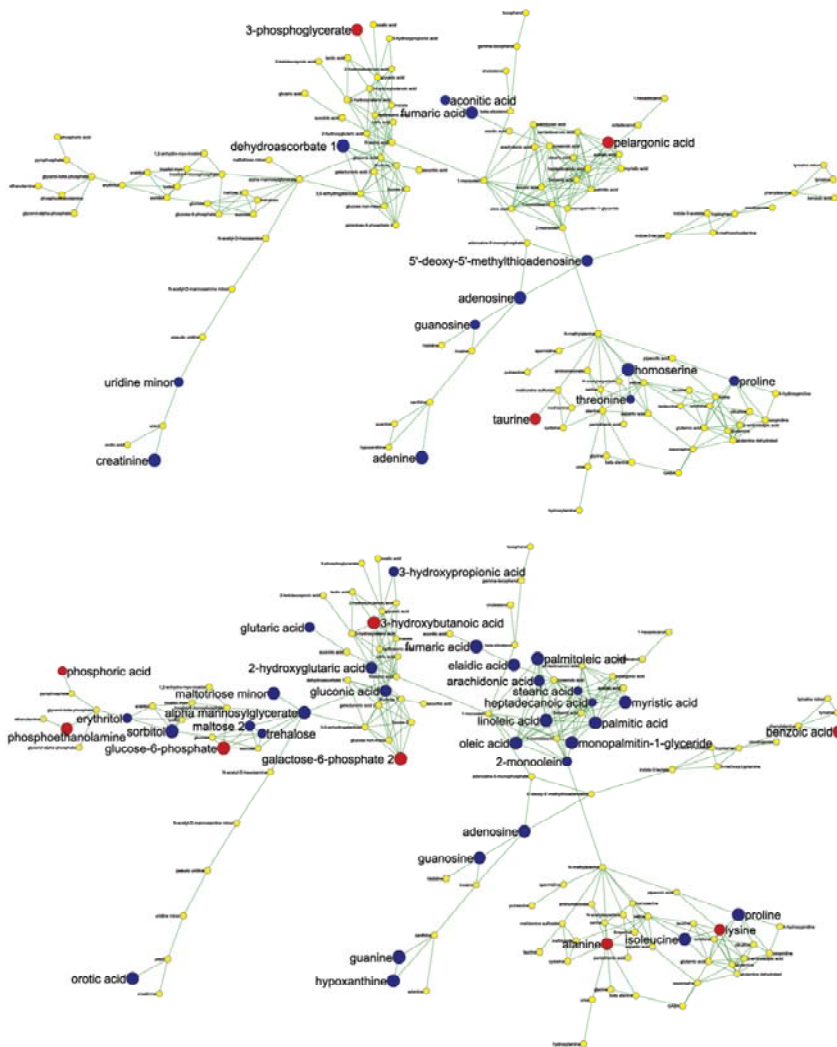


Fig. 3. Tanimoto chemical similarity maps of metabolic changes in rat lungs after 20 d exposure to environmental tobacco smoke during gestation. Upper panel: changes in dam lungs. Lower panel: changes in fetal lungs.

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