

The Human Serum Metabolome

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Overview

- NMR analysis of the serum metabolome
- GC-MS analysis
- LC-MS (lipidomics)

NMR Analysis Methods

Sample collection

- **69** samples: **15** (healthy) + **54** (from 9 heart transplant patients)

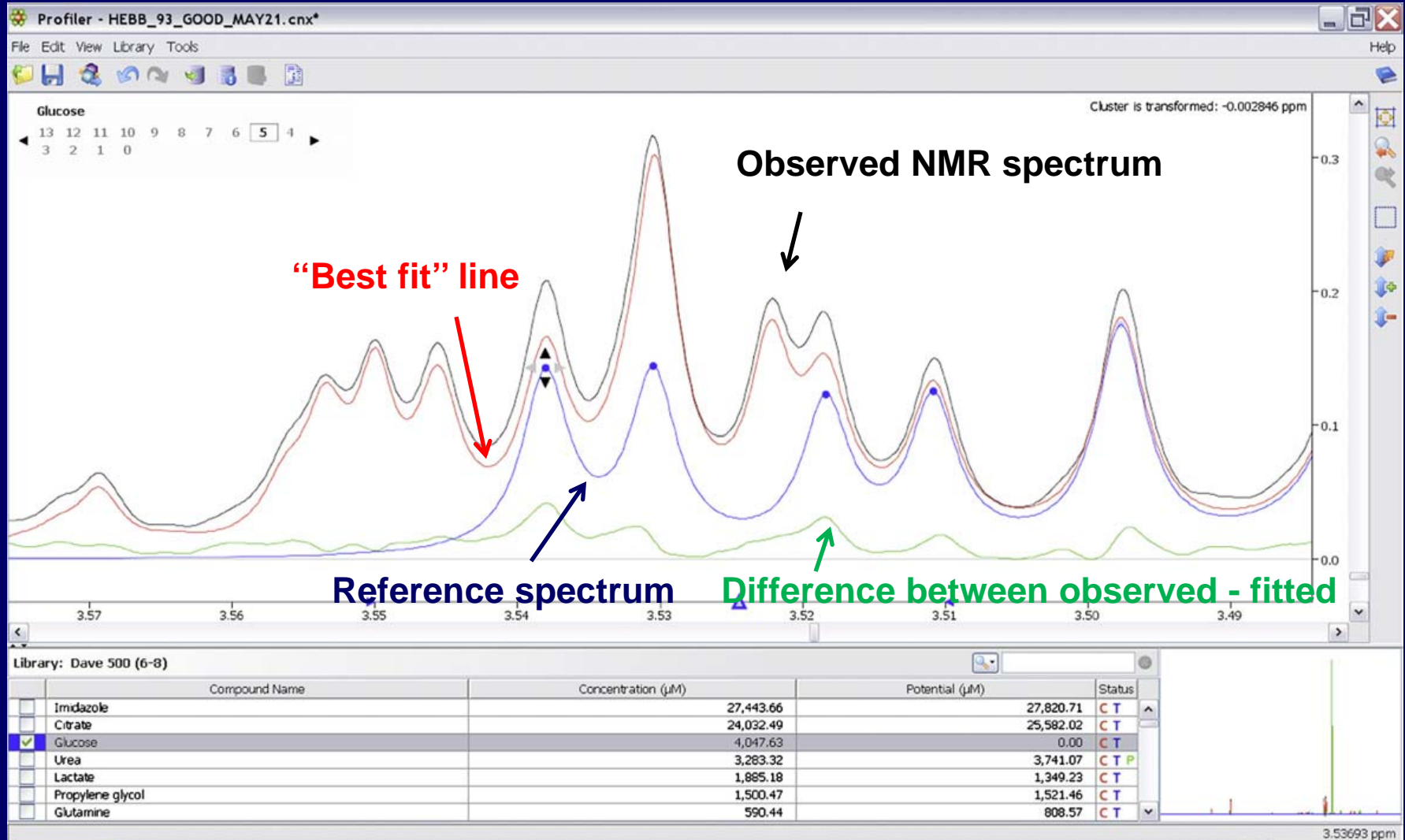
Sample preparation

- Protein removal via ultrafiltration (3kD cut-off filter)

NMR Spectroscopy

- 500 MHz Inova VARIAN (Pulse sequence: tnnoesy)
- DSS (internal standard, quantification)
- Peak confirmation with sample spiking (95% of spectral area covered)

Curve-fitting process (Chenomx NMR Suite 5.0)



37 in healthy subjects (30 ± 2 /spectrum)

44 in heart-transplant patients (32 ± 2 /spectrum)

20 + pH (t-test, $p < 0.05$)

Metabolites

Results

37 metabolites

Average Concentrations and % Occurrence of Serum Metabolites in Healthy Subjects

Compound	Concentration (μM)	SD (μM)	% occurrence (n=15)
2-HB *	20	13	73
3-HB *	37	25	80
Acetate	24	9	100
Acetone	5	2	13
Alanine	251	86	100
Asparagine	56	13	13
Betaine	24	12	100
Carnitine	22	6	100
Choline	7	2	87
Citrate	64	20	100
Creatine	33	25	100
Creatinine	35	11	100
Formate	21	9	80
Glucose	3075	1328	100
Glutamate	140	*	7
Glutamine	299	81	100
Glycerol	384	79	100
Glycine	186	72	100
Histidine	75	21	100

Compound	Concentration (μM)	SD (μM)	% occurrence (n=15)
Isoleucine	37	13	100
Isopropanol	48	76	40
Lactate	973	331	100
Leucine	58	19	100
Lysine	93	26	100
Malonate	56	*	7
Methanol	44	9	100
Methionine	18	8	33
Ornithine	36	8	100
Phenylalanine	44	17	100
Proline	120	56	100
Propylene glycol	67	20	100
Pyruvate	27	9	93
Serine	86	*	7
Threonine	73	23	100
Tyrosine	55	15	100
Urea	3471	1231	100
Valine	111	32	100

* HB: Hydroxybutyrate

Results

44 metabolites

Average Concentrations and % Occurrence of Serum Metabolites in Heart-Transplant Patients

Compound	Concentration (µM)	SD (µM)	% occurrence (n=53)
2-HB *	13.88	8.31	92
2-OV *	6.12	3.16	40
3-HB *	20.07	19.40	96
Acetaminophen	19.15	12.77	8
Acetate	24.09	9.88	100
Acetoacetate	15.62	8.21	25
Acetone	1.85	0.87	4
Alanine	194.26	72.12	100
Asparagine	30.93	12.41	42
Betaine	24.07	11.03	100
Carnitine	23.85	13.63	100
Choline	5.56	2.59	92
Citrate	45.74	25.65	100
Creatine	19.31	21.54	100
Creatinine	49.65	25.45	100
Ethanol	23.00	6.89	13
Formate	11.19	3.86	60
Glucose	2138.84	727.39	100
Glutamate	41.13	21.11	40
Glutamine	215.32	65.29	100
Glycerol	76.54	50.15	100
Glycine	134.23	103.51	100

Compound	Concentration (µM)	SD (µM)	% occurrence (n=53)
Histidine	26.35	10.00	100
Hypoxanthine	29.90	*	2
Isobutyrate	4.82	1.11	11
Isoleucine	25.51	12.29	100
Isopropanol	9.44	12.88	45
Lactate	800.70	395.47	100
Leucine	42.75	19.59	100
Lysine	73.26	31.62	100
Malonate	60.39	54.77	9
Methanol	46.55	31.55	94
Methionine	9.91	5.46	66
Methylmalonate	6.42	*	2
Ornithine	37.37	17.37	100
Phenylalanine	25.59	12.00	94
Proline	91.35	49.33	100
Propylene glycol	20.73	11.38	62
Pyruvate	28.71	22.87	87
Threonine	47.14	27.29	96
Tyrosine	32.67	13.93	100
Urea	1867.76	1057.60	100
Valine	82.38	35.06	100
Xanthine	29.27	*	2

* HB: Hydroxybutyrate

* OV: Oxoisovalerate

Comparison of Average Concentrations of Metabolites found in Healthy Subjects and Heart-Transplant Patients

Metabolites	Concentration (mM)		p
	Controls (n=15)	Heart transplant (n=9)	
3-Hydroxybutyrate	37	20	0.04
Alanine	251	194	0.03
Choline	7	6	0.02
Citrate	64	46	0.01
Creatinine	35	50	0.00
Formate	21	11	0.00
Glucose	3075	2139	0.02
Glutamine	299	215	0.00
Glycerol	384	77	0.00
Glycine	186	134	0.04
Histidine	75	26	0.00
Isoleucine	37	26	0.01
Leucine	58	43	0.01
Lysine	93	73	0.02
Phenylalanine	44	26	0.00
Propylene glycol	67	21	0.00
Threonine	73	47	0.00
Tyrosine	55	33	0.00
Urea	3471	1868	0.00
Valine	111	82	0.01
pH	7.34	7.14	0.00

20 metabolites

Discussion

37 compounds ID (control serum group)

32 concentrations in literature

- 12 – comparable concentrations
 - 14 – significantly lower
 - 3 – significantly lower
 - 3 – ignored
- Some inaccurate literature values (imprecise instrumentation ?)
 - Incomplete serum filtration
 - Inadequate centrifuge speeds
 - Inadequate centrifugation times
 - Blockage of the filter from serum proteins

GC-MS analysis

Pooled blood serum samples were extracted separately for polar and lipid extracts using a 8:1 MeOH:H₂O and a 2:1 CHCl₃:MeOH solution, respectively. The extracted compounds were derivatized using N-methyl-N-trifluoroacetamide with 1% trimethylchlorosilane (TMS)

Extracts were analyzed using an Agilent 7890-5975C GC-MS instrument operating in an electron impact (EI) mode with 1ml/min helium carrier gas flow rate and 310°C for the final oven temperature. The full scan mode of the quadrupole MS was used at a mass range of 50-500 m/z

The AMDIS software (v. 2.62) was used to deconvolute the total ion chromatograms and the EI-MS spectra of the component. The purified MS of each component was subjected to the NIST MS library (2005), using the NIST MS Search software (v. 2.0d). RIs were calculated using an external alkane standard. Metabolites were identified by matching the EI-MS spectral with NIST library and the experimental RI of each metabolite with an in-house compiled RI library for human metabolites

GC-MS analysis

Identification of 70 metabolites

Organic acids	Amino acids	Lipids	Carbohydrates and polyols	Misc
19	18	18	7	8
oxalic acid	threonine	hexanoic acid	erythritol	hydroxyamine
malonic acid	aspartic acid	nonanoic acid	Glycerol	ethanolamine
a-hydroxyvaleric acid	pyroglutamic acid	decanoic acid	ribitol	Creatinine
2-aminobutyric acid	Alanine	n-pentadecanoic acid	myo-inositol	urea
benzoic acid	Glycine	palmitic acid	mannitol	phosphate
succinic acid	Isoleucine	linoleic acid	Glucose	glyceryl phosphate
glyceric acid	Leucine	oleic acid	fructose	myo-inositol phosphate
aminomalonic acid	Lysine	stearic acid		ibuprofen
2,3,4-trihydroxybutyric	Ornithine	arachidonic acid		
2-Hydroxybutyrate	Phenylalanine	cholesterol		
3-Hydroxybutyrate	Proline	octanoic acid		
Citrate	Serine	dodecanoic acid		
Lactate	Tyrosine	tetradecanoic acid		
Pyruvate	Valine	heptadecanoic acid		
adipic acid	Glutamate	oleamide		
cis-aconitic acid	tryptophan	eicosanoic acid		
uric acid	hypoxanthine	tocopherol		
2,4-hydroxybutyric acid	cysteine	palmitelaidic acid		
aminoadipic acid				

GC-MS analysis

Metabolites not quantified by NMR (34)

- **Organic acids:** Oxalic, α-hydroxyvaleric, 2-aminobutyric, benzoic, glyceric, aminomalonic, adipic, 2,3,4-trihydroxybutyric cis-aconitic, 2,4-hydroxybutyric, aminoadipic
- **Amino acids:** Aspartic, pyroglutamic
- **Lipids:** All (18)
- **Misc:** hydroxyamine, ethanolamine, phosphate, glyceryl phosphate, myo-inositol phosphate

Action

- Quantify with GC-MS in normal serum samples
- Try to run a few standards this week (calibration curves)

LC-ESI-MS/MS analysis

Western Human Nutrition Research Center, USDA, Davis CA, USA

- 4 human plasma sample replicates were extracted by Solid Phase Extraction (SPE) and analyzed by LC-ESI-MS/MS for **66** oxidized lipid mediators
- 2 internal standards and 11 deuterated surrogates were used to track instrument drift and extraction efficiency
- Samples were spin filtered at 0.22 micron and analyzed by LC-ESI-MS/MS (Waters UPLC-API 4000 QTRAP) for an expanded list of oxidized lipids (see below). Data was analyzed with Analyst software (Applied Biosystems)

LC-ESI-MS/MS analysis

TrueMass® platform by Lipomics Technologies, Inc (West Sacramento, CA)

- 2 serum samples were analyzed using a Phenomenex Luna C18 RP column (150×2.1mm) connected to a Waters Quattro Premier triple quadrupole MS. The analytes were ionized via negative electrospray in the tandem MS mode and the data was analyzed with the MassLynx (V4.0 SP4 2004, Waters Corporation) software.
- With this method, **18** additional oxylipids were identified

LC-GC-FID analysis

TrueMass® platform by Lipomics Technologies, Inc (West Sacramento, CA)

- 3 human plasma samples were used for the extraction of lipids in the presence of authentic internal standards using CHCl₃:MeOH (2:1, v/v)
- Individual lipid classes within each extract were separated by preparative Thin-Layer Chromatography (TLC). Each lipid fraction was scraped from the TLC plate and trans-esterified in 3 N methanolic-HCl in a sealed vial under a N₂ atmosphere. The resulting fatty acid (FA) methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and prepared for GC by sealing the hexane extracts under N₂
- FA methyl esters were separated and quantified by capillary GC (6890 Hewlett-Packard, Wilmington, DE) equipped with a 60-m DB-23 capillary column (J&W Scientific, Folsom, CA), a Flame Ionization Detector (FID), and Hewlett-Packard ChemStation software.

LC-GC-FID analysis

TrueMass® platform by Lipomics Technologies, Inc (West Sacramento, CA)

- **38** unique FA constituents were identified for **7** different lipid classes
- **25** cholesterol esters (CEs)
- **27** free fatty acids (FFAs)
- **30** lysophosphatidylcholines (LysoPCs)
- The exact positional distribution of each of the **118** quantified FAs in the remaining 4 lipid classes was very challenging!
 - **26** in diacylglycerols (DAGs)
 - **31** in phosphatidylcholines (PCs)
 - **32** in phosphatidylethanol-amines (PEs)
 - **29** in triacylglycerols (TAGs)
- Upper limit and most probable concentration data (μM)

LC-GC-FID analysis

TrueMass® platform by Lipomics Technologies, Inc (West Sacramento, CA)

In total

- **841** DGs
- **1089** PCs
- **1089** PEs
- **273** TGs (out of 3004 possible combinations)

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Thank you ...