Figure 1. Extracted ion chromatogram of a plasma sample.

GC/Q-TOF Applied to Explore the Process of Energy Generation in Pathophysiology by Targeted Metabolomics

Marta Riera-Borrull, Esther Rodríguez-Gallego, Pol Herrero, Anna Hernández-Aguilera, Fedra Luciano, Rosa Ras, Elisabet Cuyàs, Jordi Camps, Antonio Segura-Carretero, Javier A. Menendez, Jorge Joven, Salvador Fernández-Arroyo

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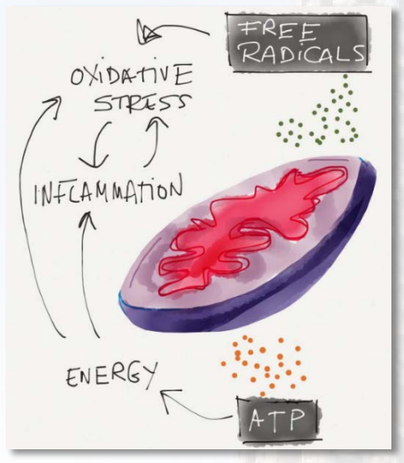
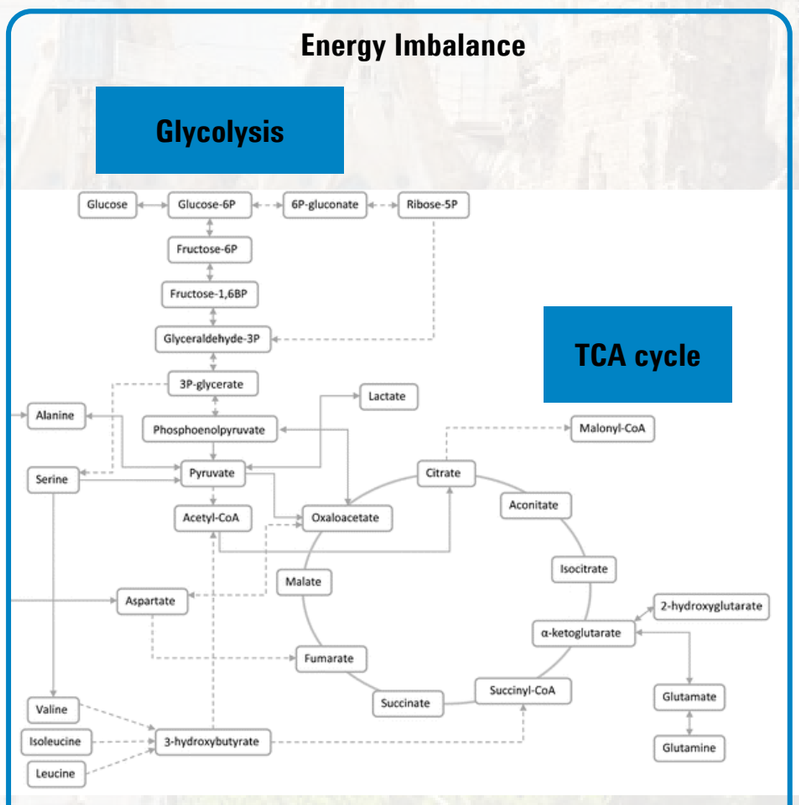
Introduction

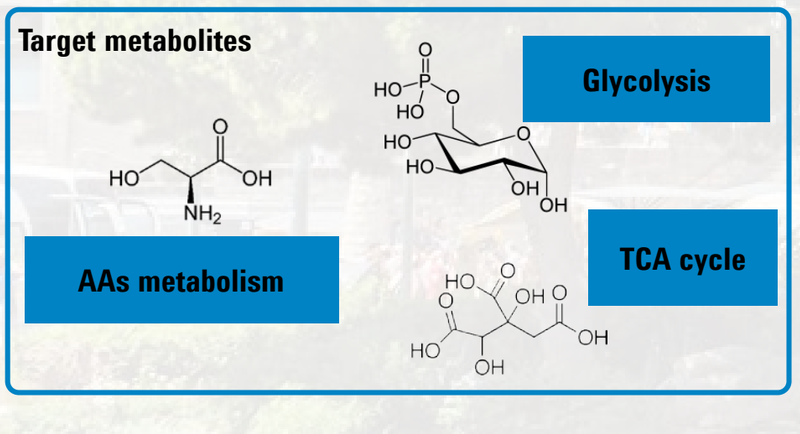
Abnormalities in mitochondrial metabolism and regulation of energy balance contribute to human diseases. The consequences of high fat and other nutrient intake, and the resulting acquired mitochondrial dysfunction, are essential to fully understand common disorders, including obesity, cancer, and atherosclerosis.

To simultaneously and noninvasively measure and quantify indirect markers of mitocondrial function, we have developed a method based on gas chromatography coupled to quadrupole time of flight mass spectrometry and an electron ionization interface.



Method was validated using plasma from patients with peripheral artery disease, human cancer cells, and mouse tissues. This approach was used to increase sensibility in the measurement of a wide dynamic range and chemical diversity of multiple intermediate metabolites used in energy metabolism. We demonstrate that our targeted metabolomics method allows for quick and accurate identification and quantification of molecules, including the measurement of small yet significant biological changes in experimental samples. The apparently low process variability required for its performance in plasma, cell lysates, and tissues allowed a rapid identification of correlations between interconnected pathways. Our results suggest that delineating the process of energy generation by targeted metabolomics can be a valid surrogate for predicting mitochondrial dysfunction in biological samples. Importantly, when used in plasma, targeted metabolomics should be viewed as a robust and noninvasive source of biomarkers in specific pathophysiological scenarios.





Experimental

Instrumental conditions

Instrument:	7890A GC - 7200 GC-qTOF
Column:	J&W HP5-MS (30m x 0.25mm, 0.25 µm)
Injection mode:	Split 20:1
Injection volume:	1 µL
Carrier gas:	Helium
Flow rate:	1.5 mL/min
Oven program:	70-190ºC at 12ºC/min; 190-325ºC at 20 ºC/min
Ionization:	EI
Source Temp:	230ºC
Mass range:	150-320 m/z
Scan rate	5 scan/sec

Sample preparation

A volume of 100µ of plasma samples, $2x10^6$ cells and 100mg of liver and adipose tissue were extracted with 900µl, 500µl and 1000µl, respectively, of MeOH/water 4:1 (v/v) containing 1µg/ml of succinic acid-d4 as internal standard.

Cells were lysed with three cycles of freezing and thawing by LN_2 and ultrasonicated with three cycles of 30s. Liver and adipose tissue were homogenized using a Precellys 24 system (Izasa).

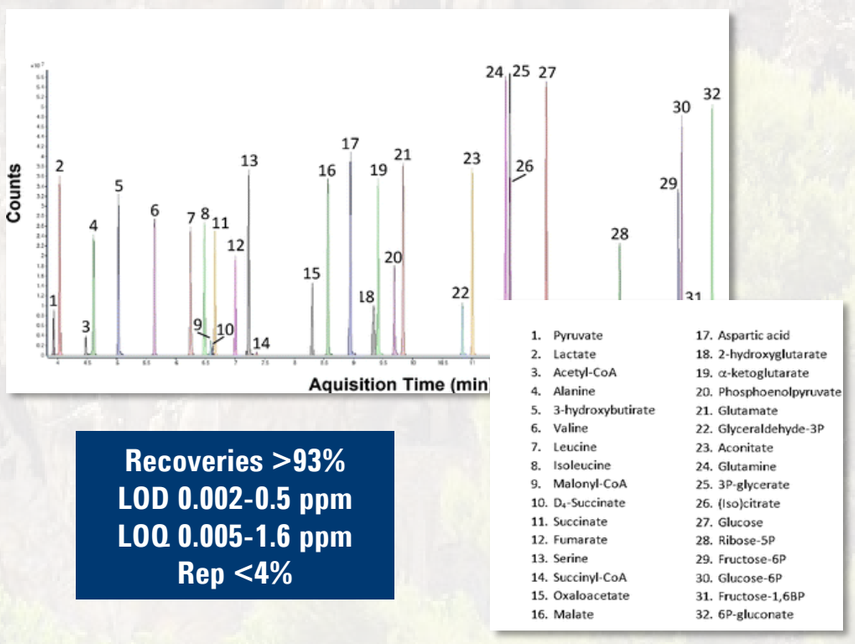
After incubation for 2h at -20ºC for protein precipitation samples were centrifuged at 14.000rpm for 10min at 4ºC. Supernatant was collected and filtered using 0.22µm filters and dried under N_2 flow.

Metabolite derivatization

A volume of 30µl of 40mg/ml methoxyamine hydrochloride in pyridine was added to dried extract samples, and after incubation at 37ºC during 1.5h with agitation, a volume of 45µl of N-(trimethyl silyl)trifluoroacetamide with *1%* trimethyl chlorosilane (MSTFA + 1% TMCS) were added. After 1h at dark and room temperature, derivatized extracts were analysed by GCMS within 24 hours.

Results and Discussion

GC/(EI)-QTOF results and method validation



Recoveries >93%
LOD 0.002-0.5 ppm
LOQ 0.005-1.6 ppm
Rep <4%

Application to real biological samples

Human plasma from healthy donors (n=50) and Peripheral Artery Disease (PAD) patients (n=50).

MCF10A cells infected whit a retroviral $KRAS^{V12}$, (CTRL n=12, INF n=12).

Liver (n=10) and adipose tissue (n=10) from LDL receptor-deficient (Ldlr-/-) mice with simulates human metabolic syndrome.

J. Am. Soc. Mass Sectrom. (2016) 27, 168-177

Human plasma from healthy donors and morbidly obese patients for Non-alcoholic Fatty Liver Disease (NAFLD) for biomarkers discovery.

Int. J. Obesity (2015) 39, 279-287

Results and Discussion

Results of plasma samples analysis from healthy and PAD donors

J. Am. Soc. Mass Sectrom. (2016) 27, 168-177

Metabolite	CTRL (n = 50)	PAD (n = 50)	FC	P value
2-Hydroxyglutarate	9.2 ± 0.4	9.0 ± 0.3	–1.02	N.S.
3-Hydroxybutirate	0.13 ± 0.01	0.31 ± 0.07	2.38	<0.0001
3-Phosphoglycerate	-	-	-	-
6-Phosphogluconate	-	-	-	-
α-Ketoglutarate	3.3 ± 0.2	4.6 ± 0.4	1.39	0.0091
Aconitate	0.52 ± 0.01	4.4 ± 0.9	8.46	<0.0001
Alanine	211 ± 11	203 ± 27	–1.04	N.S.
Aspartic acid	133 ± 3	199 ± 11	1.50	<0.0001
(Iso)citrate	279 ± 13	706 ± 22	2.53	<0.0001
Fructose-1,6-BP	-	-	-	-
Fructose-6-P	-	-	-	-
Fumarate	0.33 ± 0.02	0.26 ± 0.03	–1.27	<0.0001
Glucose	4856 ± 305	5044 ± 346	1.04	N.S.
Glucose-6-P	N.Q.	N.Q.	-	-
Glutamate	462 ± 366	5197 ± 317	11.25	<0.0001
Glutamine	1115 ± 206	3691 ± 237	3.31	<0.0001
Glyceraldehyde-3-P	-	-	-	-
Isoleucine	49 ± 1	63 ± 2	1.29	<0.0001
Lactate	395 ± 8	359 ± 13	–1.10	0.0323
Leucine	73 ± 2	90 ± 3	1.23	0.0032
Malate	1.57 ± 0.08	3.0 ± 0.3	1.91	<0.0001
Malonyl-CoA	N.Q.	N.Q.	-	-
Oxaloacetate	54 ± 5	N.Q.	-	-
Phosphoenolpyruvate	-	-	-	-
Pyruvate	11 ± 1	10 ± 1	–1.1	0.0270
Ribose-5-P	-	-	-	-
Serine	104 ± 2	145 ± 4	1.39	<0.0001
Succinate	10.7 ± 0.1	12.2 ± 0.4	1.14	N.S.
Succinyl-CoA	6.6 ± 0.9	11.9 ± 0.9	1.80	0.0014
Valine	88 ± 2	105 ± 3	1.19	N.S.

Results of α-ketoglutarate as predictor of morbid obesity-associated to NAFLD.

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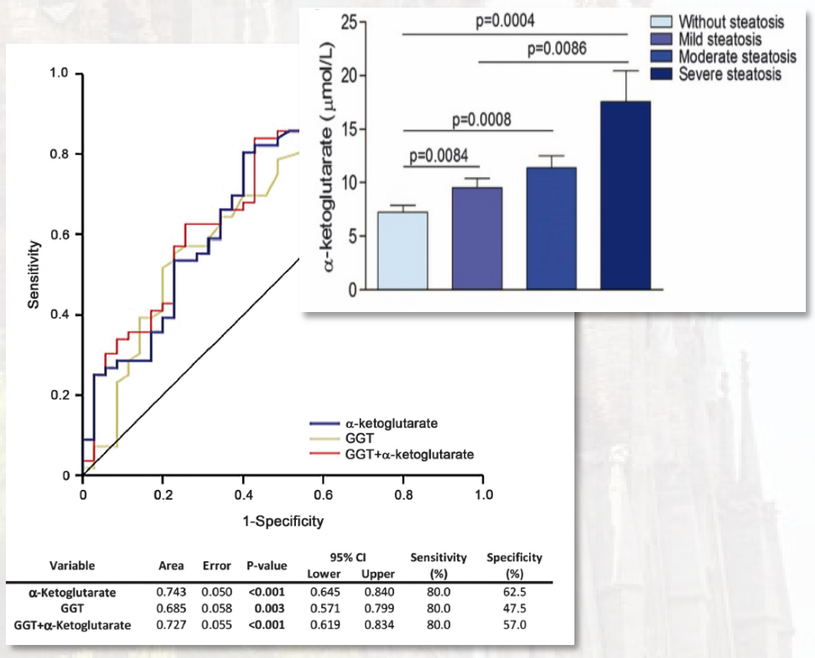

Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
α-Ketoglutarate	0.743	0.050	<0.001	0.645	0.840	80.0	62.5
GGT	0.685	0.058	0.003	0.571	0.799	80.0	47.5
GGT+α-Ketoglutarate	0.727	0.055	<0.001	0.619	0.834	80.0	57.0

Conclusions

- The developed GC-(EI)qTOF method allows to separate, detect, and quantify numerous metabolites involved in energy generation metabolism, including glycolysis, TCA cycle and aminoacid, lipid and pentose phosphate metabolism.
- The method delivers an overall assessment of metabolism in biological samples as, cell-culture lysates, tissues and patient plasma.
- The measurement of indirect intermediates involved in mitochondrial metabolism in plasma should be considered as an alternative to assess the mitochondrial dysfunction in vivo for the clinical management of common metabolic diseases.
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