Application of metabolomics: Focus on the quantification of organic acids in healthy adults

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Abstract. Metabolomics, a 'budding' discipline, may accurately reflect a specific phenotype which is sensitive to genetic and epigenetic interactions. This rapidly evolving field in science has been proposed as a tool for the evaluation of the effects of epigenetic factors, such as nutrition, environment, drug and lifestyle on phenotype. Urine, being sterile, is easy to obtain and as it contains metabolized or non-metabolized products, is a favored study material in the field of metabolomics. Urine organic acids (OAs) reflect the activity of main metabolic pathways and have been used to assess health status, nutritional status, vitamin deficiencies and response to xenobiotics. To date, a limited number of studies have been performed which actually define reference OA values in a healthy population and as reference range for epigenetic influences, and not as a reference to congenital metabolic diseases. The aim of the present study was thus the determination of reference values (RVs) for urine OA in a healthy adult population. Targeted metabolomics analysis of 22 OAs in the urine of 122 healthy adults by gas chromatography-mass spectrometry, was conducted. Percentile distributions of the OA concentrations in urine, as a base for determining the RVs in the respective population sample, were used. No significant differences were detected between female and male individuals. These findings can facilitate the more sensitive determination of OAs in pathological conditions. Therefore, the findings of this study may contribute or add to the information already available on urine metabolite databases, and may thus promote the use of targeted metabolomics for the evaluation of OAs in a clinical setting and for pathophysiological evaluation. However, further studies with well-defined patients groups exhibiting specific symptoms or diseases are warranted in order to discern between normal and pathological values.

Introduction

Metabolomics is the youngest of the emerging 'omics' sciences and along with 'sister' genomics, transcriptomics and proteomics, comprises a Systems Biology approach with a potential significant role in personalized medicine (1,2) While genomics relates to the genotype and metabolomics reflects the phenotype, recent studies have demonstrated that the association of genetic polymorphisms and consequent metabolite changes may provide unique individual genetic and phenotype information towards personalized medicine, and may reflect individual responses to lifestyle and epigenetic factors (3,4). Organic acids (OAs) are a particular group of metabolites, which have gained increasing scientific interest. Indeed, these organic compounds are intermediate metabolites of critical metabolic pathways, such as the Krebs cycle, carbohydrate metabolism, ketone body metabolism, fatty acid β-oxidation, neurotransmitters turnover and protein metabolism. The determination of OAs in urine has been utilized, from the early study by Tanaka and Isselbacher, who documented the first organic aciduria in 1970 (5). This breakthrough finding was revolutionarily utilized to

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detect congenital errors of metabolism (3,4). The underlying concept in diagnosing congenital metabolic diseases is that the quantification of OAs and other metabolites can pinpoint dysfunction or even the absence of an enzyme due to a genetic defect (Fig. 1). Accordingly, the dysfunctioning or missing enzyme may cause metabolite accumulation in the related pathway (6,7). In the early seventies, Horning and Horning, first described the term metabolic profile (8). Moreover, the research efforts of Horning's team (8) in parallel with those of Pauling *et al* led to the use of gas chromatography-mass spectrometry (GC-MS) for the quantification of metabolites in urine (9). This was the beginning of the use of metabolomics as a personalized approach in individuals not suffering from congenital metabolic diseases (10). Further developments in the field allowed for the quantification of OAs to become a fast-growing systems biology approach. Thus, in 2004, the human metabolome project was launched to identify all human metabolites, and its first version was released in 2007 (11). The metabolome is not only a simpler and more reflective index of health status, but it also incorporates environmental influences, for example, from exercise and diet, according to a Harvard Magazine article (7).

Urine OAs may reflect the activity of main metabolic pathways, and their quantification can be used to assess health status, nutritional status, vitamin deficiencies and response to pharmaceutical interventions, and may even be applied in toxi-cology and poisoning (1,7,12). For instance, urine fumarate and urine malate levels have been found to be associated with mito-chondrial disease with a 5% false positive rate (13). Given the fact that metabolomics reflects the phenotype and is sensitive to genetic, epigenetic and environmental factors, such as diet, age, lifestyle and xenobiotic exposure, it has been proposed as a useful tool for the identification of specific pathological conditions by assessing their influence on phenotype. The concentration of OAs in urine may vary in different populations due to different interactions among genetic and phenotypic factors (14). OAs have been widely used to screen inborn errors of metabolism, where the alterations in metabolite levels may be a hundred- or a thousand-fold increased in comparison to those of healthy subjects and this is reflected on the precision of reference values (RVs) currently used in laboratories (14,15).

To date, a limited number of studies have focused on the generation of sensitive RVs for a healthy population. Thus, the aim of the present study was the evaluation of RVs in urine OAs in a well-defined healthy adult population.

Subjects and methods

Subjects. In this study, 122 Caucasian adults (51 males and 71 females) from Greece were included. The mean age of all the participants was 42.0±9.6 years with a range of 21-81 years. Sampling procedures for the patients and healthy controls were defined according to the ethical guidelines of the Ethics Committee of the University of Crete (approval no. 1019/18/07_12_2016). The participants did not exhibit any symptoms of disease, showed normal results on clinical laboratory tests (hemogram and chemical constituents) and were not on a restricted diet. At the time of the analysis, no supplements or medication were taken. Early morning urine samples were collected and stored at -80°C until analysis.

Table I. Urine OA metabolites and their validated analytical parameters.

Analyte	Precision (cv% duplicates)	Linearity	Recovery
2-OH Glutaric acid	4.4	1.0	101
3-Methylglutaric acid	9.7	1.0	112
3-OH-3 methylglutaric acid	11.2	1.0	55
3-OH Isobutyric	14.8	0.99	78
3-OH Isovaleric	64.1	0.84	102
4-OH Butyric	12.0	1.0	63
Adipic acid	6.4	1.0	114
Glyceric acid	9.7	1.0	79
Ethylmalonic acid	5.1	1.0	100
Fumaric acid	9.2	1.0	87
Glutaric acid	1.2	1.0	101
Glycolic acid	13.7	0.99	86
Hexanoylglycine	16.5	0.98	109
2-Ketoglutaric acid	15.5	1.0	84
Malic acid	30.4	0.99	76
Methylmalonic acid	5.0	1.0	104
Mevalonic acid	57.6	0.92	49
Pyroglutamic acid	4.5	1.0	82
Sebacic acid	7.3	1.0	105
Suberic acid	6.5	1.0	101
Tiglylglycine	5.8	1.0	92
Vanilactic acid	10.3	0.99	88

OA, organic acid.

GC-MS, as previously described by Tanaka *et al* (16), was used to identify 22 different OAs (Table I). In brief, OAs were extracted from urine by liquid-liquid extraction after mixing the specimen with an internal standard solution. The oxidation of 2-keto acids with hydroxylamine hydrochloride was performed. OAs were converted to their corresponding trimethylsilyl (TMS) ethers with *N,O*,-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (both from Supelco, Bellefonte, PA, USA). The derivatization imparts volatility to the OAs, which is required for GC-MS analysis. The OA-TMS ethers were separated in a capillary gas chromatography column containing an immobilized, non-polar stationary phase. Following chromatographic separation, OAs were routinely detected by electron impact mass spectrometry performed in the scan mode with a mass range between m/z 50 and 550. Identification was achieved by comparison to published spectra compounds and quantification by comparison to the calibration of pure standard compounds in ratio to an internal standard.

Statistical analysis. A list of descriptive statistics for tendency (means, median, min and max) and dispersion (standard devia-tion, standard error of mean, percentiles and interquartile range) were used for describing the levels of OAs. The physiological values of OAs were set using 95% confidence intervals of the

Table II. The concentration percentiles for specific metabolites.

Urine OA metabolites	Detected (>LOD)	Mean	SD	Min-Max	Estimated 95% CI	Known 95% CI	No of cases outside 95% CI[a]
5-HIAA	92.6%	4.9	9.3	1.0-61.8	0.0-23.4	0-7.0	4 (13)
3-OH Pr	96.7%	8.0	8.0	1.0-54.4	0.0-23.9		
Pim	42.6%	1.8	0.8	1.0-4.0	0.1-3.4		
EMA	97.5%	2.2	1.7	1.0-10.1	0.0-5.5	0.4-4.2	9 (13)
3-OH Glut	51.6%	1.1	0.3	1.0-2.6	0.5-1.6	1.0-10.0	3 (0)
HVA	92.6%	2.2	1.4	1.0-8.7	0.0-4.9		
Glyc	77.0%	2.7	6.2	1.0-44.6	0.0-14.9	0.0-3.0	2 (2)
Pyrog	100.0%	27.8	15.5	1.7-103.4	0.0-58.6		
3-OH Isob	99.2%	10.3	8.6	2.0-78.0	0.0-27.4		
2-OH Glut	96.7%	3.4	2.3	1.0-18.0	0.0-7.9		
ICit	98.4%	5.2	5.9	1.3-61.4	0.0-16.9	16.0-99.0	2 (0)
Cit	100.0%	88.7	75.9	2.0-509.5	0.0-239.0	0.0-656.0	3 (0)
Mal	82.0%	1.1	0.3	1.0-2.5	0.6-1.6	0.0-5.3	5 (0)
Suc	89.3%	4.8	7.1	1.0-64.0	0.0-19.0	29.0-87.0	3 (0)
2-ketog	100.0%	18.9	19.2	1.0-145.8	0.0-57.0	41.0-82.0	5 (112)
Pyr	100.0%	9.6	6.9	2.0-55.2	0.0-23.2	3.5-22.0	4 (12)
Lac	100.0%	21.8	86.1	2.1-926.6	0.0-192.2	20.0-101.0	2 (101)
Metmal	90.2%	1.4	0.8	1.0-6.0	0.0-3.0	0.0-1.0	5 (43)
3-MetGlut	88.5%	2.1	1.4	0.8-7.3	0.0-4.8		

[a]Counts based on estimated 95% CI (on known 95% CI). OA, organic acid; SD, standard deviation; CI, confidence interval; 5-HIAA, 5-hydroxy-indole acetic acid; 3-OH Pr, 3-hydroxy propionic acid; Pim, pimelic acid; EMA, ethylmalonic acid; 3-OH Glut, 3-hydroxyglutaric acid; HVA, homovanillic acid; Glyc, glyceric acid; Pyrog, pyroglutamic acid; 3-OH Isob, 3-hydroxyisobutyric acid; 2-OH Glut, 2-hydroxyglutaric acid; ICit, isocitric; Cit, citric acid; Mal, malic acid; Suc, succinic acid; 2-ketog, 2 ketoglutaric acid; Pyr, pyruvic acid; Lac, lactic acid; MetMal, methylmalonic acid; 3-MetGlut, 3-methylglutaric acid.
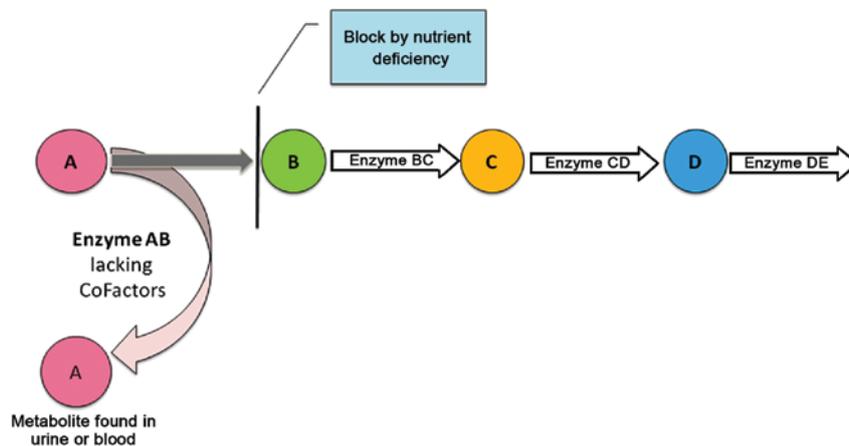

Figure 1. Micronutrient deficiency.

mean. The Kolmogorov-Smirov test was applied for examining the data normality. It has been previously demonstrated that 'normalization to partial sums' (or similar methods) has better performance as compared to normalization to creatinine concentration (17). Differences in OA levels between two or more subgroups were examined using parametric (t-test, ANOVA) and non-parametric tests (Mann-Whitney, Kruskall-Wallis). IBM SPSS Statistics 22.0 (IBM SPSS, Armonk, NY, USA) was used for analysis of the data.

Results

The chemical analysis characteristics (precision, linearity and recovery) of 22 selected urine OAs are documented in Table I. To determine the RVs for each metabolite, the 5% percentile lower limit and 95% percentile upper limit was defined. In some cases, to achieve a more effective clinical sensitivity, changes in determining the lower and upper RVs were applied (18). The detection limit of the urine OA concentration is 1 μmol/l.

Table III. Percentiles and quartiles of urine OAs.

	1st quartile			2nd quartile (median)	3rd quartile		
	5th percentile	10th percentile	25th percentile	50th percentile	75th percentile	90th percentile	95th percentile
5-HIAA	1.0	1.0	1.5	2.0	4.3	8.1	16.6
3-OH Pr	1.6	1.9	3.3	5.0	10.2	18.6	25.5
Pim	1.0	1.0	1.1	1.3	2.6	3.0	3.0
EMA	1.0	1.0	1.1	1.5	2.3	4.8	6.2
3-OH Glut	1.0	1.0	1.0	1.0	1.0	1.2	1.8
HVA	1.0	1.0	1.3	1.7	2.7	3.8	5.1
Glyc	1.0	1.0	1.6	2.0	2.0	2.0	2.0
Pyrog	5.9	12.0	18.3	26.0	33.6	43.1	55.5
3-OH Isob	2.9	4.6	5.2	7.8	13.0	18.0	22.7
2-OH Glut	1.0	1.3	1.9	2.8	4.2	5.6	7.5
ICit	1.7	2.3	3.0	4.3	5.5	8.5	10.0
Cit	19.8	26.8	43.8	65.3	115.6	171.0	204.7
Mal	1.0	1.0	1.0	1.0	1.0	1.2	2.0
Suc	1.2	1.4	1.9	2.8	5.1	8.9	11.6
2-ketog	2.4	2.9	6.5	13.2	25.8	39.4	52.2
Pyr	2.9	4.0	5.4	8.4	11.5	16.6	21.1
Lac	3.3	3.6	5.0	8.4	13.8	26.3	54.6
Metmal	1.0	1.0	1.0	1.0	1.5	2.6	3.0
3-MetGlut	1.0	1.0	1.1	1.8	2.4	4.2	5.0

OA, organic acids; 5-HIAA, 5-hydroxyindole acetic acid; 3-OH Pr, 3-hydroxypropionic acid; Pim, pimelic acid; EMA, ethylmalonic acid; 3-OH Glut, 3-hydroxyglutaric acid; HVA, homovanillic acid; Glyc, glyceric acid; Pyrog, pyroglutamic acid; 3-OH Isob, 3-hydroxyisobutyric acid; 2-OH Glut, 2-hydroxyglutaric acid; ICit, isocitric; Cit, citric acid; Mal, malic acid; Suc, succinic acid; 2-ketog, 2 ketoglutaric acid; Pyr, pyruvic acid; Lac, lactic acid; MetMal, methylmalonic acid; 3-MetGlut, 3 methyl glutaric acid.

Our study population consisted of 122 volunteers of which 71 were females (58.2%). The mean age of all the participants was 42.0±9.6 years with a range of 21-81 years. There was no significant difference in age between the sexes. For analytical purposes and with respect to age, the participants were divided into 4 groups (≤30, 30-40, 40-50 and 50+) consisting of 18, 28, 56 and 20 subjects, respectively. Table II shows the basic descriptive parameters of the analyzed OA urine levels and the estimated physiological 95% confidence intervals. A zero concentration was set for 95% lower limit of urine OA concentration when a negative concentration was estimated. Only 3 of the urine OAs [pimelic acid (Pim), 3-hydroxyglutaric acid (3-OH Glut) and malic acid (Mal)], showed a positive (non-zero) 95% percentile lower limit. In Table III the 5th, 10th, 20th, 25th, 50th (median), 75th, as well as the 90th percentile of specific metabolites are presented. The mean levels of OAs over the age groups exhibited no significant differences (P>0.05) among the females and males (Table IV).

Moreover, Pearson's and Spearman's rho coefficients were applied to estimate bivariate associations between urine OAs (Table V). Of note, there was a positive correlation (P<0.05), between the levels of discrete OA metabolites (Table V). Thus, homovanillic acid (HVA) correlated with all of OAs except Pim, 3-OH Glut, glyceric acid (Glyc) and Mal. Similar correlations were detected for ethylmalonic acid (EMA). By contrast, Mal only correlated significantly, but with a moderate intensity with methylmalonic acid (MetMal) (rs=0.22), 3-hydroxyisobutyric acid (3-OH Isob) (rs=0.22) and 3-hydroxypropionic acid (3-OH Pr) (rs=0.20).

Discussion

The rising significance and potential utilization of metabolomics details illustrated by the fact that since the year 2000, 12.272 articles have been published containing the word 'Metabolomics' (Fig. 2). Importantly, it has been established that alterations in the levels of the metabolites in the quantified metabolic pathways correlate with the patient clinical status. In particular, the citric acid cycle (CDC)-metabolites in healthy individuals reflect the performance of the enzymes, which catalyze specific reactions (Fig. 3). Thus, according to studies where CDC metabolites were quantified and correlated to mitochondrial dysfunction in chronic diseases, it was shown that the presence of CDC antagonists or the lack of specific nutrients can alter CDC enzyme efficiency, and may thus provide vital information on the causative nature of a disease in a healthy North Indian pediatric population (14). Indeed, mitochondrial dysfunction has been associated with a number of chronic diseases. Heavy metals, such as mercury, can disrupt acotinase for example, in an antagonistic manner. The lack of fumarase

Table IV. Descriptive statistics of urine OAs in the different age groups and according to sex.

	Age group (females)								P-value[a]	Age group (males)								P-value[a]
	≥30		31-40		41-50		51+			≤30		31-40		41-50		51+		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
5-HIAA	4.4	5.9	3.7	1.7	3.4	3.3	10.0	19.1	0.228	7.6	13.9	2.5	1.6	5.0	12.6	7.2	11.1	0.735
3-OH Pr	5.6	3.5	7.5	7.4	12.0	12.3	9.2	7.4	0.432	6.0	2.5	4.6	1.9	6.9	5.6	5.8	2.9	0.529
Pim	1.8	0.8	1.9	0.8	1.8	0.8	2.1	1.0	0.957	1.5	0.8	1.8	1.2	1.1	0.1	1.7	0.9	0.585
EMA	2.4	2.2	2.7	2.0	2.2	1.3	2.7	2.3	0.699	1.3	0.4	2.3	1.8	1.4	0.5	2.7	2.8	0.082
3-OH Glut	1.1	0.2	1.0	0.0	1.1	0.4	1.0	0.0	0.698	1.3	0.5	1.0	0.0	1.0	0.0	1.0	0.1	0.071
HVA	2.2	1.1	2.4	1.1	2.5	1.8	2.8	1.1	0.507	2.1	0.6	1.6	0.6	2.0	1.7	1.7	0.8	0.761
Glyc	1.9	0.2	1.9	0.2	1.8	0.4	9.0	17.5	0.649	8.9	17.2	1.8	0.3	1.6	0.5	1.7	0.4	0.096
Pyrog	24.8	16.6	33.8	18.3	27.2	15.3	36.0	25.4	0.331	30.3	11.1	23.7	7.8	22.4	10.6	30.9	11.5	0.103
3-OH Isob	10.1	6.1	12.6	8.8	12.4	13.2	11.6	8.0	0.882	9.7	5.2	9.0	4.1	7.0	3.4	7.5	3.7	0.303
2-OH Glut	3.1	1.9	4.2	2.5	4.2	3.0	3.4	1.3	0.437	3.5	1.5	2.0	1.1	2.6	1.9	2.5	2.0	0.384
ICit	4.4	2.5	5.6	3.1	5.1	3.4	5.8	3.8	0.493	4.6	1.3	3.6	1.2	6.4	12.3	5.2	1.7	0.824
Cit	79.0	52.3	92.3	37.6	118.1	92.1	131.6	63.3	0.117	64.1	36.4	37.8	30.9	71.6	99.7	64.9	18.2	0.616
Mal	1.0	0.0	1.1	0.4	1.1	0.2	1.0	0.0	0.717	1.0	0.0	1.0	0.1	1.2	0.4	1.1	0.3	0.613
Suc	3.4	2.1	4.6	2.6	7.1	12.6	5.8	3.7	0.375	2.6	1.5	3.8	2.4	3.5	2.5	2.7	1.5	0.536
2-ketog	22.5	18.7	28.8	20.4	25.3	25.2	23.8	13.1	0.739	11.7	7.8	7.6	5.2	9.3	13.7	12.0	7.4	0.764
Pyr	9.2	5.3	13.1	12.2	11.5	7.5	9.6	4.7	0.769	8.2	3.5	8.5	3.0	6.1	3.2	8.8	3.8	0.084
Lac	10.6	5.7	72.1	228.0	29.2	42.5	13.2	6.4	0.267	5.4	3.4	5.9	3.0	5.4	2.4	5.5	2.2	0.969
MetMal	1.4	0.6	1.4	0.8	1.5	0.9	1.4	0.4	0.858	1.4	0.8	1.3	0.4	1.3	1.1	1.2	0.3	0.982
3-MetGlut	3.4	2.0	2.3	1.2	v2.2	1.5	2.5	1.0	0.247	1.3	0.4	1.7	1.1	2.0	1.3	1.3	0.4	0.278

[a]Mann-Whitney P-value. SD, standard deviation; 5-HIAA, 5-hydroxyindole acetic acid; 3-OH Pr, 3-hydroxypropionic acid; Pim, pimelic acid; EMA, ethylmalonic acid; 3-OH Glut, 3-hydroxyglutaric acid; HVA, homovanillic acid; Glyc, glyceric acid; Pyrog, pyroglutamic acid; 3-OH Isob, 3-hydroxyisobutyric acid; 2-OH Glut, 2-hydroxyglutaric acid; ICit, isocitric; Cit, citric acid; Mal, malic acid; Suc, succinic acid; 2-ketog, 2 ketoglutaric acid; Pyr, pyruvic acid; Lac, lactic acid; MetMal, methylmalonic acid; 3-MetGlut, 3 methyl glutaric acid.

Table V. Bivariate associations of measured OAs.

Correlation coefficients[a]	5-HIAA	3-OH Pr	Pim	EMA	3-OH Glut	HVA	Glyc	Pyrog	3-OH Isob	2-OH Glut	ICit	Cit	Mal	Suc	2-ketog	Pyr	Lac	MetMal	3-MetGlut
5-HIAA		-0.04	0.13	0.16	0.09	0.43	-0.03	0.14	-0.08	0.20	0.57	0.47	-0.02	-0.02	0.27	0.00	-0.01	0.36	-0.004
3-OH Pr	0.02		0.11	0.12	0.46	0.34	0.01	0.35	0.53	0.39	0.13	0.41	0.13	0.13	0.42	0.45	0.23	0.36	0.04
Pim	0.27	0.02		0.07	0.28	0.32	0.01	0.31	0.05	0.12	0.43	0.27	0.07	-0.10	0.24	0.21	0.06	0.28	0.32
EMA	0.35	0.25	0.25		0.24	0.20	0.23	0.25	0.26	0.13	0.12	0.23	-0.01	0.02	0.31	0.24	0.04	0.27	0.01
3-OH Glut	0.28	0.04	0.19	0.18		0.33	-0.02	0.33	0.05	0.51	0.22	0.44	0.07	0.35	0.46	0.21	-0.04	0.40	0.33
HVA	0.28	0.26	0.27	0.27	0.33		0.00	0.42	0.12	0.46	0.64	0.73	-0.03	0.03	0.54	0.22	0.03	0.49	0.32
Glyc	0.12	0.11	0.01	0.28	0.19	0.01		0.11	0.11	0.02	-0.03	-0.03	-0.05	0.00	0.00	0.06	-0.01	0.15	-0.01
Pyrog	0.40	0.30	0.32	0.36	0.23	0.48	0.18		0.29	0.50	0.37	0.40	0.05	-0.06	0.47	0.55	0.36	0.45	0.26
3-OH Isob	0.14	0.65	0.14	0.45	0.04	0.32	0.16	0.32		0.27	0.03	0.16	0.13	0.01	0.31	0.68	0.44	0.52	0.04
2-OH Glut	0.18	0.13	-0.05	0.21	0.21	0.38	0.08	0.35	0.34		0.49	0.61	0.09	0.15	0.75	0.42	0.26	0.48	0.24
ICit	0.40	0.29	0.41	0.34	0.35	0.55	-0.05	0.67	0.32	0.48		0.73	0.04	0.03	0.47	0.15	0.00	0.67	0.18
Cit	0.20	0.32	0.22	0.34	0.31	0.56	0.12	0.33	0.33	0.47	0.57		-0.04	0.13	0.68	0.24	0.03	0.55	0.23
Mal	0.16	0.20	0.12	0.19	0.06	0.07	0.03	0.12	0.22	0.17	0.15	0.07		0.00	0.12	0.08	0.02	0.05	-0.12
Suc	0.00	0.20	0.04	0.07	0.09	0.22	0.11	0.03	0.14	0.24	0.05	0.21	0.16		0.04	0.02	0.09	0.01	0.02
2-ketog	0.24	0.10	0.15	0.43	0.12	0.47	0.14	0.45	0.30	0.62	0.46	0.61	0.13	0.06		0.32	0.09	0.38	0.16
Pyr	0.15	0.44	0.28	0.46	0.23	0.30	0.15	0.41	0.70	0.26	0.40	0.32	0.12	-0.06	0.36		0.71	0.60	0.11
Lac	0.20	0.37	0.21	0.52	0.06	0.42	0.08	0.27	0.62	0.39	0.34	0.51	0.09	0.34	0.46	0.65		0.35	0.04
MetMal	0.21	0.38	0.20	0.39	0.50	0.30	0.02	0.43	0.58	0.32	0.34	0.24	0.22	0.02	0.24	0.58	0.42		0.16
3-MetGlut	0.09	0.03	0.22	0.03	0.18	0.28	0.16	0.22	0.09	0.24	0.24	0.22	-0.02	0.15	0.19	0.12	0.27	0.00	

[a]Up diagonal coefficients: Pearson's rho. Down diagonal coefficients: Spearman's rho. Numbers in bold and italics refer to significant correlations (p<0.05). 5-HIAA, 5-hydroxyindole acetic acid; 3-OH Pr, 3-hydroxypropionic acid; Pim, pimelic acid; EMA, ethylmalonic acid; HVA, homovanillic acid; Glyc, glyceric acid; Pyrog, pyroglutamic acid; 3-OH Isob, 3-hydroxyisobutyric acid; 2-OH Glut, 2-hydroxyglutaric acid; ICit, isocitric; Cit, citric acid; Mal, malic acid; Suc, succinic acid; 2-ketog, 2 ketoglutaric acid; Pyr, pyruvic acid; Lac, lactic acid; MetMal, methylmalonic acid; 3-MetGlut, 3 methyl glutaric acid.
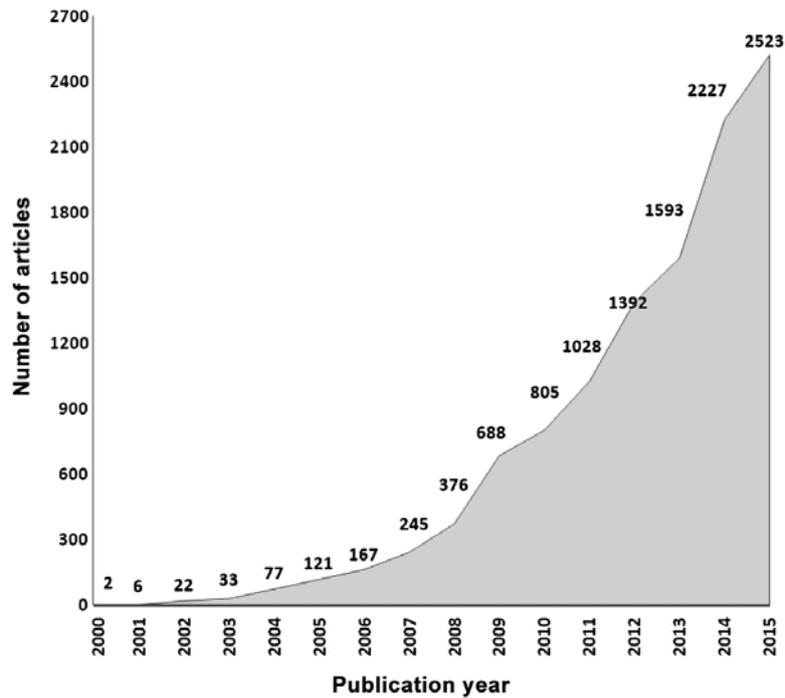
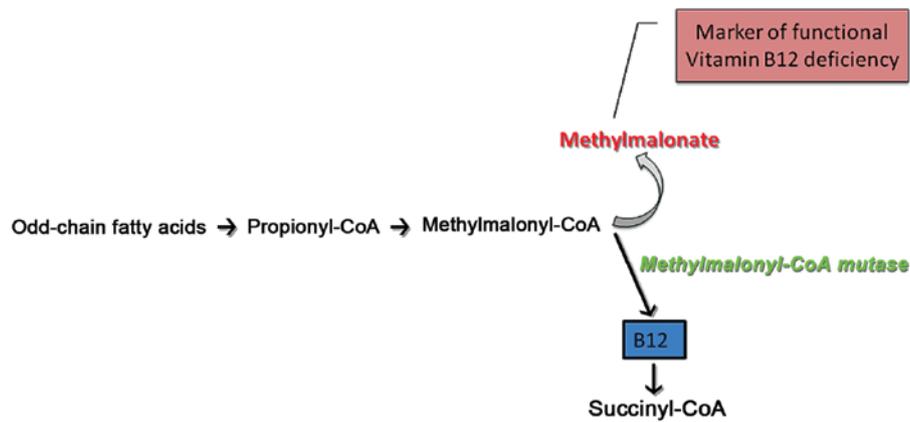Figure 2. PubMed metabolomics articles published in the period 2000-2015.

Figure 3. Methylation Marker-Vitamin B12 deficiency.

co-factors, such as ubiquinol may disrupt fumarate conversion to malate with concurrent fumarate elevation (19). Another important application of the measurement of OAs is the case of 3-hydroxybutyric acid, the levels of which increase in urine due to the failure of glucose utilization in both fasting and diabetic subjects, and can thus be used as a marker for subclinical keto-acidosis, insulin sensitivity and complications associated with diabetes (13,20). In a recent clinical study, children presenting with non-alcoholic fatty liver disease (NAFLD) were treated with probiotic VSL#3 and subsequently their urinary OAs, including amino-acid metabolites and nucleic acid degradation metabolites were estimated. The data obtained in that study suggested that the urinary OAs levels of children with NAFLD may be utilized as efficient biomarkers to evaluate the response to therapy (21). It has recently been discussed that both genetic and biochemical data indicate a contribution of the mitochondrial disease during the pathological evolvement of autism spectrum disorder (ASD) in a specific subgroup of children (22). Indeed, significant differences in urinary 4-hydroxyphenylacetic, 2-oxoglutaric, isocitric, citric, 4-hydroxybenzoic, hippuric, adipic and suberic levels have been detected in autistic children, as compared to age-matched healthy controls (23,24). Furthermore, urine homovanillic acid and vanilmandelic acid levels have shown to be related to neurotransmitter metabolism in subjects suffering from occupational stress (25). Moreover, a utilization of metabolic profiling for the detection of early chronic kidney disease has recently been highlighted. Such an approach could significantly decrease the existing time lapse between the onset of the pathology and the time point when symptoms are of adequate intensity as to initiate therapy (25). Moreover, metabolic profiling, including the evaluation of 2-hydroxyisobutyric acid, has been suggested as a feasible strategy in the evaluation of renal disease progression (26). A separate study demonstrated a correlation between urinary

amino acid metabolites' levels and chronic kidney disease progression (27). Moreover, changes in arginine metabolism have likewise been indicated as a biomarker for the 2, 3 and 4 stages of chronic kidney disease (28). As these patients constitute a significant subgroup of total human pathologies, improved early detection and accurate disease monitoring could increase the efficiency of treatment and significantly improve the socioeconomic aspects of disease. Another feasible application of metabolic profiling is the evaluation of intestinal disease. Thus, 4-hydroxyphenylacetic acid, an intestinal bacterial flora metabolite, was found to be a marker of small-bowel disease due to bacterial overgrowth as and in the case of Giardia infestation (29). The utilization of OAs as disease markers is still limited due to the lack of stringent, reproducible RVs of healthy controls, as well as to difficulties when comparing data from different platforms (30). At present, RVs are mainly based on studies and measurements performed with the aim to detect inborn errors of metabolism (31). In patients presenting with congenital metabolic diseases, the lack or substantial dysfunction of an enzyme participating in a specific metabolic pathways markedly alters the levels of the metabolites involved, allowing the use of a wider interval for RVs. However, in patients suffering from a metabolic pathway dysfunction due to epigenetic factors, there are smaller variations of the metabolite levels between the healthy controls and subjects with pathologies. This, demands strictly defined RVs in order to detect and correctly interpret minor to moderate deviations. The human metabolome database has contributed significantly to the increased application of metabolic profiling in clinical settings. Moreover, the accumulation of high resolution metabolomics data from a variety of platforms is approaching the critical threshold in assembling a central, reference human metabolome (11,30). This study, therefore aimed to qualitatively contribute to existing data bases of urine metabolites with the utmost goal being to increase sensitivity in detecting metabolic pathway deviations in chronic diseases.

Over the past 6 years, we have performed more than 15,000 OA profiles, targeting metabolites that relate to critical metabolic pathways, such as the CDC, neurotransmitter turnover, bacterial flora markers, protein metabolism, ketones metabolism, fatty acid β-oxidation methylation markers, vitamin B sufficiency and carbohydrate metabolism. Therefore, according to our experience, more sensitive RVs are required to identify smaller variations in individuals presenting with various pathologies but not suffering from inborn errors of metabolism.

This study was designed to determine the RV for a set of key OA metabolites in a population of healthy Greek adult males and females, who were on an unrestricted diet. The establishment of a successful analytical profiling technique involves the simultaneous analysis of numerous metabolites with various chemical characteristics. Furthermore, the separation, identification and quantification of these metabolites need to be performed with the highest possible accuracy. It is imperative to be able to detect very low concentrations of metabolites in order to detect any deviation with respect to normal levels. According to the above-mentioned characteristics, the technical method of choice needs to exhibit high sensitivity and repeatability. In previous studies, the analytical techniques that have been used are GC-MS, HPLC, TANDEM-MS and

others. However, the gold standard for the evaluation of OAs in urine is GC-MS with the chemical modification of metabolites, separated by chromatography and identified by mass spectrometry (32-34). Prior to application in each respective laboratory, the method needs to be validated with specific conclusions about the value ranges, the repeatability and the linearity of measurements. This methodology needs to be carefully applied for the obtaining of accurate and representative RV. To the best of our knowledge, this study is the first of its kind conducted in Greece. The concentrations of OAs may vary among populations from different geographical regions due to genotype, different diet and various other environmental and epigenetic factors. Moreover, there is extensive individual variation in metabolite secretion following specific dietary intake (28). Along these lines, our results are in agreement with those of similar studies performed on different ethnic groups with minor differences attributed to the details of laboratory methods (35). A recent study performed on a healthy north Indian pediatric population utilizing mass GS-MS indicated that the concentrations of OAs were higher (14) as compared to matched age-groups in Turkish and Swiss (36,37) pediatric populations. Thus, Kumari *et al*, putatively attribute the higher RVs of some metabolites to dietary deficiency, as protein intake was inadequate in the North Indian pediatric population (14). Moreover, studies on pediatric populations have shown a strong age dependent effects on RVs of OA metabolites (14,37).

Our study contributes to the available data with the aim to narrow the RV range, and the increase sensitivity and the clinical application of urine metabolites in detecting deviation from normal metabolism. However, further studies with well-defined groups of patients presenting specific symptoms or diseases, are warranted in order to test the ability of this method to discriminate between normal and pathological values.

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