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ORIGINAL ARTICLE

Metabonomics and population studies: age-related amino acids excretion and inferring networks through the study of urine samples in two Italian isolated populations

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Abstract The study of two different Italian isolated populations was combined with a metabonomic approach to better understand tubular handling of amino acids. Levels of amino acids and metabolites have been analyzed by Nucleic Magnetic Resonance and expressed as ratio vs urinary creatinine concentration (mmol/mol). For most of the amino acids there is an age-related U shape pattern of excretion, with the peaks during childhood and old age, and a significant reduction in the adult age. Hierarchical cluster analysis has clearly identified three groups clustering the same amino acids: His, Thr and Ala (group one); Gly and Phe (group two) and a third larger one. Results have been further confirmed by factor and regression analysis, and used to confirm and, in some cases, infer new amino acids networks. As a matter of facts, the identification of strong evidences for clustering of urine excretion of several neutral amino acids suggests the predominant impact of relevant and common transporters

Keywords Amino acids · Tubular handling · Metabonomics · Population studies

Introduction

Complex and quantitative traits are due to the combined effects of multiple interacting genes and environmental factors. Genetic analyses of complex diseases have not had widespread success due to genetic heterogeneity, phenotype

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complexity, inadequate statistical and genotyping strategies and small sample size (Risch 2000). The identification of genetic and environmental risk factors for complex and quantitative traits can be simplified studying isolated populations since it is expected that an association can be detected with a smaller sample of patients in an inbred population than in a panmictic one, in the presence of a more homogeneous environment and genetic background. Usefulness of investigations of these populations has already been proven in identifying associations with complex diseases and quantitative traits (Varilo and Peltonen 2004). We have identified, on the basis of their location (geographically isolated), history (few founders and high rate of endogamy) and genealogic data (few surnames and high level of inbreeding), two isolated villages, Carlantino, located in the South, and Stoccareddo, in the North of Italy. Haplotype analysis of chromosome X and Y, and of mithochondrial DNA as well as increased homozigosity of several multiallelic DNA markers has confirmed their given genetic structure. Metabonomic approaches are believed to have the capability of revolutionizing diagnosis of diseases. NMR spectroscopy has become one of the main tools for measuring changes in bio-fluids since an NMR spectrum can accurately identify metabolites and their concentrations. Proton magnetic resonance spectrum of a urine sample contains all the information useful to analyze a great variety of metabolites such as: aminoacids, ketoacids, keton bodies, creatinine and N-containing compounds. Here we have coupled two very powerful approaches such as metabonomics and the use of isolated populations to better understand the tubular handling of amino acids (aa) and their age dependent excretion, to infer possible new networks, common transporters and pathways.

Materials and methods

Sample selection

A total of 571 first-morning urine samples after overnight fasting have been randomly collected in two different isolated populations, 387 from Carlantino (South Italy) and 184 from Stoccareddo (North Italy). A total of 44 different parameters including 34 aa and metabolites plus 10 general ones have been analyzed. Analysis of the latter was carried out using the Bayer Diagnostics CLINITEK STATUS instrument coupled with dipsticks MULTISTIX 10SG. Samples were then divided in aliquots and frozen at −20°C.

Urinary samples

Prior to NMR spectral acquisition, 450 μL of urine (defrosted at room temperature) were mixed with 150 μL of 1 mM standard solution of sodium-3-trimethylsilyl-[2,2,3,3-$^{2}H_4$]-propionate (TSP; Sigma-Aldrich) in D_2O (99.9%; Avanti Polar Liquids). The pH value was adjusted to <1.5 by adding HCl 3 M solution. The urine specimens, about 600 μL, were then transferred into a 5 mm NMR tube for spectral acquisition.

NMR spectral acquisition

Spectra were acquired on a Bruker Avance 300 spectrometer, operating at 300 MHz resonance for proton. One-dimensional spectra were acquired at 295 K by means of a pre-saturation pulse sequence, used for peak water suppression purpose. In this commonly used technique, one irradiates the H_2O resonance frequency long enough to achieve equally populated spin states and starts the sampling pulse and data acquisition, immediately after the water irradiation is turned off. The duration and the intensity of the continuous wave irradiation for pre-saturation, were optimized in order to have the best suppression of the water signal intensity, with the minimum B1 field strength (40 dB). The optimized 90° pulse was 9.5 μs. The spectral width was 12 ppm for all data collection and typically 32 FIDs were accumulated for routine analysis into 32 k digital data points. The acquisition time per scan was 4.56 s. All measurements were recorded with spinning at 10 Hz and the lock signal was provided by the heavy water (D_2O) used in urine sample preparation. All collected FIDs were apodized prior to Fourier Transform, with an exponential decay corresponding to a line broadening of 0.4 Hz. Spectra were zero filled to 64 K. TSP was taken as a qualitative standard for chemical shift scale as well as a quantitative internal standard for peak area calculation. The chemical shift assignment of each signal in a spectrum was made by comparison with standard amino acid solutions and also by addition of the standard aa into the urine sample (co-peaking). Quantification of metabolites was done measuring the height (h) of the peak and taking into account the multiplicity and the number of protons of the signal. Peak heights were normalized as respect to creatinine to point to tubular handling of aa and avoid timed collection of urinary samples, a very difficult task in a population study. The following formulas were used to calculate the amount of a metabolite (M):

$$M\left(\frac{\text{mmol}}{\text{mol}_{\text{creatinine}}}\right) = \frac{h_M}{h_{\text{creatinine}}} 10^{-3} \quad (1)$$

$$[M]_{(\text{mmol/L})} = \frac{h_M}{h_{\text{TSP}}} [\text{TSP}] \quad (2)$$

which respectively provide the mmol of the metabolite M per mol of creatinine and the absolute value of the concentration of M expressed in mmol/L. Measures obtained

using Eq. 1 are more accurate, because no (lowering accuracy) addition of any external standard of known concentration (as TSP in Eq. 2) is required, being the creatinine always present in urine. The concentration values of creatinine, were calculated by means of Eq. 2. The mean value and standard deviation are 9.41 ± 7.46 mmol/L. By means of this method, the urine components of Table 1 and 2 were estimated. Among these, 17 protein amino acids are present. Unfortunately, Asp, Arg, Pro and their derivatives were hardly detectable with satisfactory accuracy at normal levels. Few data were available, thus not allowing a statistical evaluation. Nevertheless, if high amounts of such amino acids should appear in urines (i.e. therapy in cardiovascular diseases), these should be easily detected.

Statistical analyses

To assess influence of age and sex of aa levels a quantile normalization, which has minimal impact on results for traits that are approximately normally distributed, was performed using R software (version 2.6). For other traits, normalization will not bring to misleading results (i.e. correlations not already present in the original data) and thus should not lead to erroneous inferences (Pilia et al. 2006). Observations were ranked and the percentile of each observation matched to the corresponding percentile in a standard normal distribution. Using the resulting percentiles, we replaced each observation with the corresponding *z*-score from the standard normal distribution. Normalized phenotypes where then tested by covariance (ANCOVA) with age and sex as covariates. Hierarchical cluster analysis on normalized levels was performed using "complete linkage" agglomeration method as implemented in the hclust library of R. Cluster Analysis is a multivariate analysis technique that seeks to organize information about variables so that relatively homogeneous groups, or "clusters," can be formed. The main outcome of a cluster analysis is a dendrogram (tree) with the Euclidean distance used to understand the similarities between aa. We grouped aa in clusters in a hierarchical way with two different methods: the agglomerative and the divisive ones. The agglomerative method builds the hierarchy from the individual elements by progressively merging clusters. Agglomerative algorithms begin at the top of the tree. The first step is to determine which elements to merge in a cluster. On the contrary, with the divisive method clusters are repeatedly subdivided rather than joined. This cluster analysis is an attractive option when is essential to group into a large cluster. The analysis starts from one group done by all aa and goes ahead with consecutive divisions to obtain groups formed by one amino acid. Factor analysis was performed on normalized levels using *R* with a "varimax" rotation of factors. A regression analysis was also carried out on the most relevant data arising from hierarchical cluster and factor analyses. In regression

Table 1 Descriptive statistical analysis for each urinary parameter investigated by NMR

Metabolite	Mean	Median	Min	Max	N
1MeHis	22.8	18.0	0.0	138.8	179
3Hiv	6.5	5.9	0.4	24.5	450
3MeHis	21.9	19.1	0.0	69.1	174
Acet	70.2	46.4	9.4	424.3	184
Ala	34.9	29.1	0.0	193.2	571
Asn	8.9	7.1	0.6	41.8	173
Btai	2.1	1.3	0.0	47.6	452
Carn	11.6	8.7	0.4	84.9	446
Citr	274.9	241.7	18.5	977.7	496
Coli	6.8	5.2	0.0	46.1	448
Crtn	41.8	33.7	2.4	262.4	184
Cys	12.4	8.8	1.2	63.0	151
Form	70.7	62.2	11.0	348.2	458
Glcl	39.0	32.8	1.2	192.2	449
Gln	25.8	22.1	0.0	139.0	567
Glu	17.9	14.2	0.4	91.4	343
Gly	82.6	67.9	0.0	444.5	438
Hipp	153.9	107.3	3.1	1254.8	459
His	68.7	58.1	0.0	297.7	569
Il_Le	7.0	5.3	0.0	40.9	522
Lact	19.6	14.9	0.0	218.3	449
Lys	41.5	32.6	0.0	241.7	566
Met	4.1	3.2	0.0	24.7	548
Oxbu	11.3	10.1	1.2	34.9	184
Phe	25.9	20.7	0.0	171.9	569
Prop	11.5	9.1	0.0	54.4	453
Pyrv	6.4	5.0	0.1	35.5	448
Ser	87.5	67.7	1.4	473.1	450
Thr	16.2	13.3	0.0	92.7	567
TMA	3.1	2.2	0.7	14.0	179
TMAO	51.9	36.4	0.7	394.1	436
Trp	25.5	20.4	0.0	165.1	441
Tyr	20.0	14.9	0.0	212.1	564
Val	7.9	6.7	0.0	44.1	568

Values are reported as mmolmetabolite/molcreatinine ratio

Ala alanine, *Met* methionine, *Phe* phenylalanine, *Trp* tryptophan, *Gly* glycine, *Tyr* tyrosine, *Val* valine, *Ser* serine, *Il_Le* isoleucine + Leucine, *His* histidine, *Thr* threonine, *Lys* lysine, *Glu* glutamic acid, *Gln* glutamine, *Hypp* hyppuric acid, *Lact* lactic acid, *Btai* trimethylglycine, *Carn* carnitine, *Prop* propionic acid, *Pyrv* pyruvic acid, *Form* formic acid, *Glcl* glycolic acid (or hydroxyacetic acid), *3Hiv* 3-hydroxyisovalerate, *Coli* coline, *TMAO* Trimethylamine n-oxide, *TMA* trimethylamine, *Citr* citric acid, *Crtn* creatine, *Cys* cysteine, *Acet* acetic acid, *1MeHis* 1-methylhistidine, *3 MeHis* 3-methylhistidine, *Oxbu* 3-Hydroxybutyric acid, *Asn* asparagine

Table 2 Urinary excretion of single aa and metabolites according to the age. Data (median) are reported as mmol metabolite/mol creatinine ratio

	<12	12–19	19–35	35–50	50–65	≥65
1MeHis	16.6	16.7	14.3	16.4	20.1	24.5
3Hiv	6.2	7.1	4.7	5.6	5.9	6.5
3MeHis	18.3	19.3	17.3	19.8	23.4	20.6
Acet	46.4	35.2	39.1	37.8	63.5	88.0
Ala	31.8	29.2	20.6	26.0	31.3	37.9
Asn	7.2	7.9	5.8	6.2	7.5	8.3
Btai	1.3	1.1	0.8	1.0	1.4	2.0
Carn	3.5	7.9	7.6	8.0	11.1	9.4
Citr	203.2	173.4	181.3	217.6	270.9	273.9
Coli	2.7	4.6	3.7	4.9	4.8	7.2
Crtn	53.6	35.0	32.8	34.3	33.6	28.6
Cys	13.4	12.3	7.3	6.7	10.6	10.9
Form	56.2	50.4	45.7	57.6	67.0	78.6
Glcl	58.4	48.0	25.3	34.4	29.4	33.2
Gln	24.6	22.3	16.1	17.3	23.4	27.7
Glu	13.4	18.0	9.6	12.0	16.4	19.2
Gly	89.9	51.1	57.2	68.3	78.0	81.9
Hipp	47.3	55.9	77.4	123.2	145.1	122.3
His	62.8	81.4	43.7	58.1	59.2	57.8
Il_Le	5.4	4.3	4.2	4.3	6.1	8.1
Lact	11.4	13.7	10.4	13.1	16.1	21.0
Lys	34.0	28.5	24.3	28.7	33.4	42.2
Met	2.8	3.1	2.2	2.3	3.1	4.5
Oxbu	11.4	9.2	9.7	12.9	9.7	12.8
Phe	20.4	16.7	17.9	19.4	19.3	27.0
Prop	9.0	9.3	7.1	7.7	9.1	12.8
Pyrv	4.2	4.4	3.3	3.4	5.6	7.3
Ser	66.7	47.1	46.0	60.6	73.1	96.8
Thr	14.8	14.9	9.8	10.3	13.6	17.3
TMA	1.9	2.3	1.7	2.2	2.4	2.8
TMAO	30.6	19.8	27.0	34.6	40.0	48.2
Trp	18.3	17.3	13.4	15.3	20.6	31.1
Tyr	13.1	9.0	11.8	12.4	16.0	20.7
Val	6.4	5.8	4.9	5.8	7.3	8.2

Ala alanine, *Met* methionine, *Phe* phenylalanine, *Trp* tryptophan, *Gly* glycine, *Tyr* tyrosine, *Val* valine, *Ser* serine, *Il_Le* isoleucine + Leucine, *His* histidine, *Thr* threonine, *Lys* lysine, *Glu* glutamic acid, *Gln* glutamine, *Hypp* hyppuric acid, *Lact* lactic acid, *Btai* trimethylglycine, *Carn* carnitine, *Prop* propionic acid, *Pyrv* pyruvic acid, *Form* formic acid, *Glcl* glycolic acid (or hydroxyacetic acid), *3Hiv* 3-hydroxyisovalerate, *Coli* coline, *TMAO* Trimethylamine n-oxide, *TMA* trimethylamine, *Citr* citric acid, *Crtn* creatine, *Cys* cysteine, *Acet* acetic acid, *1MeHis* 1-methylhistidine, *3 MeHis* 3-methylhistidine, *Oxbu* 3-Hydroxybutyric acid, *Asn* asparagine

analysis, the R^2 coefficient of determination is a statistical measure of how well the regression line approximates the real data points, in which 1.0 indicates that the regression line perfectly fits the data.

Results

The evaluation of the ten standard urine parameters clearly demonstrates that all of them are usually within the normal range and in agreement with those previously reported for large European population cohorts (UK, Sweden, etc) (Mant and Fowler 1990). Kidney function assessed by measuring serum creatinine revealed that the selected population had a normal renal function; indeed serum creatinine was 0.813 ± 0.18 mg/dl, ranging from a minimum of 0.38 to a maximum of 1,33 mg/dl. As regards to the 34 parameters investigated by NMR, after a descriptive statistical analysis for each parameter (Table 1), specific analysis to estimate the effect of age and gender on the levels of aa were carried out. Regression analysis for age were performed. The asymmetric distribution of values due to the decreased number of individuals subdivided in the several age-related classes was overcome by the use of median which is more robust as compared to mean (Table 2). The levels of almost all aa seem to be influenced by age. There is a U shape pattern of excretion, with the peaks during childhood and old age, and a significant reduction in the adult age (Table 2). To detect possible similarities between urine aa Hierarchical Cluster Analysis has been applied. Using the agglomerative method it is possible to distinguish three major groups that cluster together. The first one is characterized only by Gly and Phe; the second one by His, Thr and Ala; and the last one by Il_Le, Val, Glu, Ser, Trp, Met on one branch and Gln, Lys and Tyr on the other, as shown in Fig. 1a. As regards to statistical significance, in a cluster graph, the strongest associations are in the lower part, while going up they became less strong. With the divisive method again the identification of three clusters was possible: a first one characterized by His, Thr and Ala, a second one only by Gly and Phe, and a third one by Il_Le, Val, Glu, Trp, Tyr and Lys on one branch, Gln and Ser on another branch, and Met alone (Fig. 1b). By comparing the data obtained using the two above mentioned methods we could easily identify two groups clustering the same aa: His, Thr and Ala characterize the first one; Gly and Phe, the second one. As regards to the third group there are strong similarities but not a complete overlap among the analysis (i.e. Met). Moreover, in order to confirm these results, we have also carried out a factor analysis, which is used to uncover the latent structure (dimensions) of a set of variables. This analysis reduces attribute space from a larger number of variables to a smaller number of factors. Results obtained are reported in Fig. 2. Factor analysis clearly demonstrates the presence of two different groups of aa (the ones with the highest loadings in each factor). One group comprises Il_Le, Val, Lys, Gln, Ser, Glu, Tyr, Trp, Met while the other contain only Thr, Ala and His. We have also tested

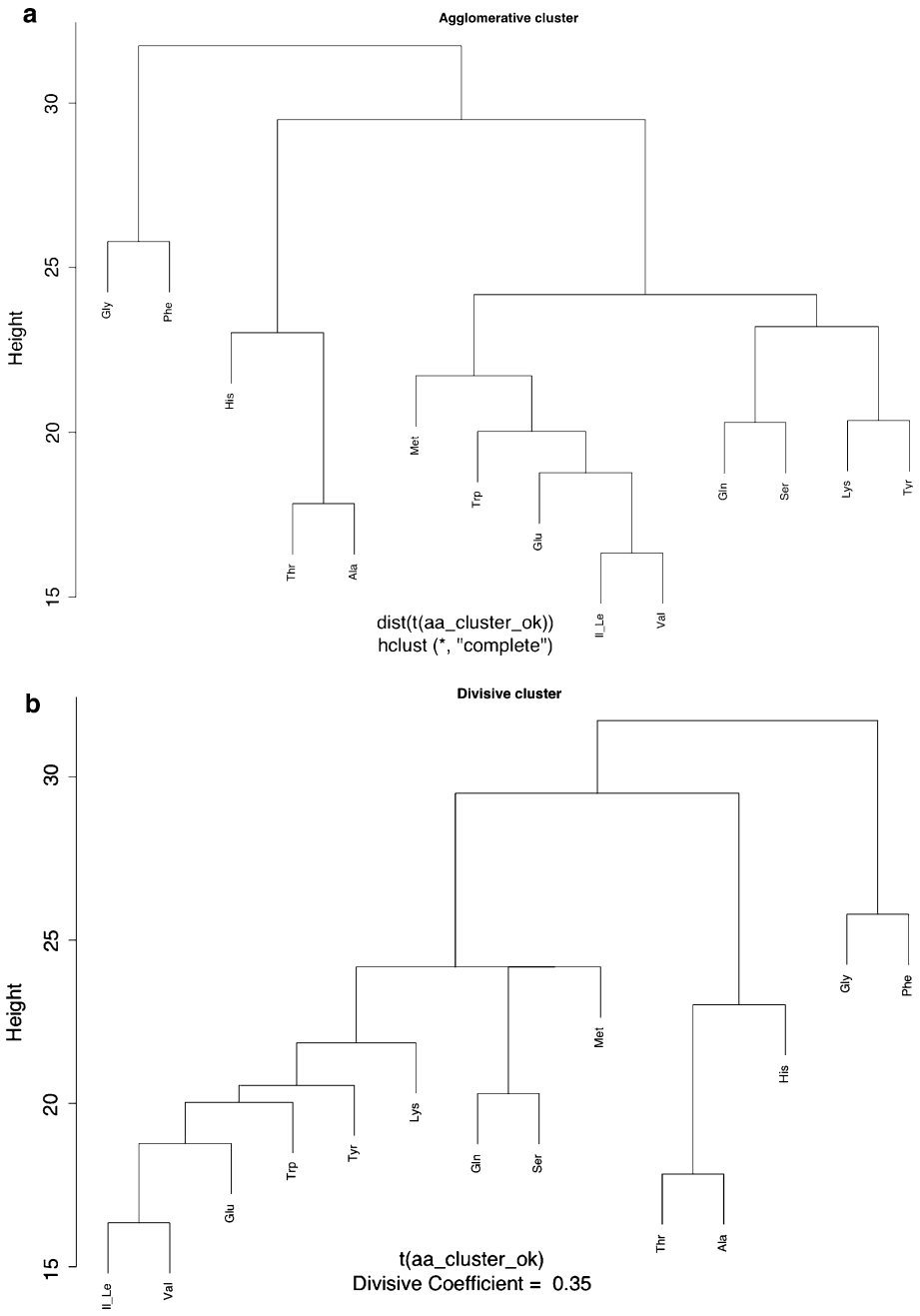

Fig. 1 Hierarchical cluster analysis. Dendrogram of an agglomerative cluster (**a**) and of a divisive cluster analysis (**b**)

the hypothesis that 2 factors are sufficient. The chi square statistical is 145.34 on 64 degrees of freedom and a *P* value of 2.89e-08. The analysis with 3 factors gave not significant results (*P* value of 0.118) due to the increased complexity of the model. These results are largely comparable with cluster analysis, only the group with Gly and Phe remain undetected. Finally, to further confirm the correlation between the closest aa in the clusters according to the Euclidean distance, a linear regression model with R was applied to evaluate the possible association between Val and Il_Le and between Thr and Ala (Fig. 3 a, b). Strong significant correlation values (*P* value $<$ 2e-16) in both cases have been obtained. The adjusted R^2 values are: 0.5174 for the association between Thr and Ala, and 0.5772 for the association between Val and Il_Le. This means that $\sim$58% of the variability of Il_Le can be predicted using Val (quite high). Less significant correlations were obtained for Gln and Ser (0.3738) (Fig. 3c) and for Gly and Phe (Fig. 3d). In this last case there is a significant correlation (*P* value $<$ 2.2e-16), but with a R^2 of only 0.1406, in agreement with the results of the cluster. A possible explanation for this finding is the presence of a correlation

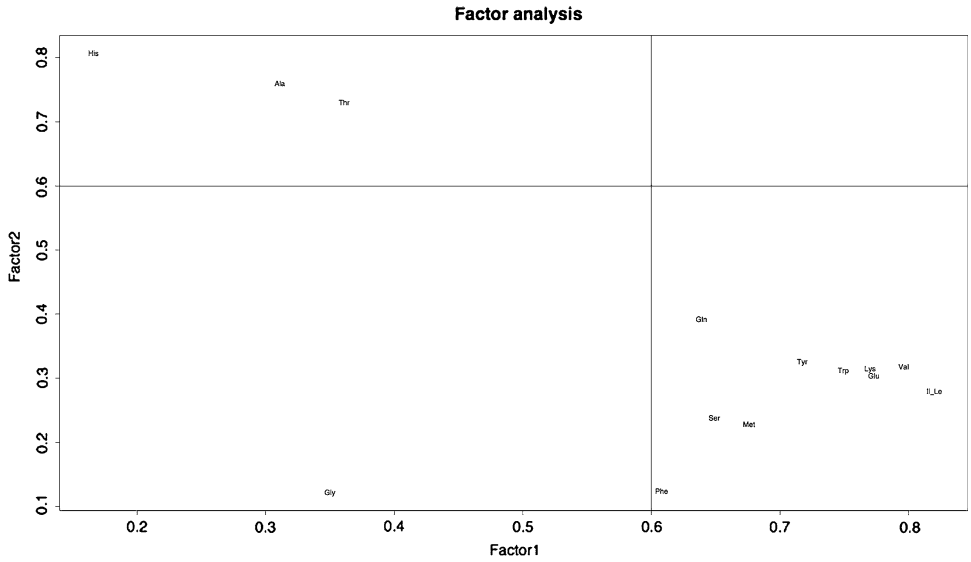

Fig. 2 Factor analysis

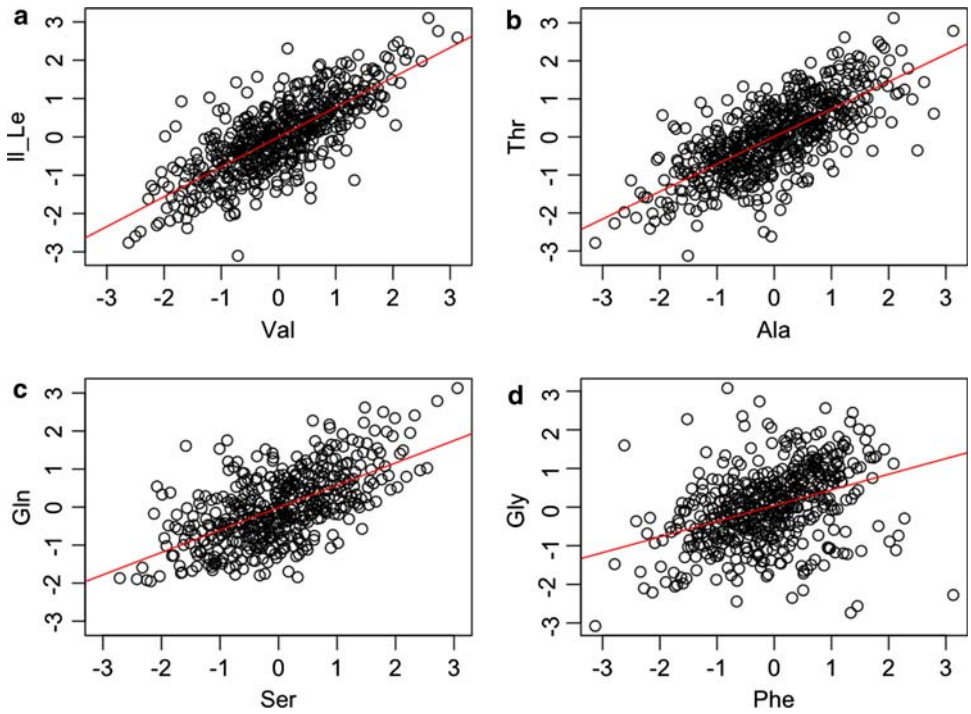

Fig. 3 Regression analysis. Graphs show regression analyses results (conducted on normalized and centered values) for: **a** Il_Le and Val, **b** Thr and Ala, **c** Gln and Ser and **d** Gly and Phe. Formulas were: (Il_Le $\sim$ Val): $y = 0.778245x - 0.005845$; (Thr $\sim$ Ala): $y = 0.721970x + 0.006113$; (Gln $\sim$ Ser): $y = 0.58829$ $x - 0.01594$; (Gly $\sim$ Phe): $y = 0.40595\ x + 0.03908$

with "confounding factors" such as, for example, one transporter in common among the two aa and another specific for one of them.

Discussion

To better understand the tubular handling of aa, NMR spectroscopy has been coupled with the use of genetic homogenous healthy populations. Multivariate analyses have been carried out using urinary amino acid/creatinine ratio that is the most practical approach to standardize the urinary excretion of various substances in epidemiological studies. In this light, NMR can be proposed as the best available technique to quantify metabolites as respect to creatinine, since they are all measured by means of homogeneous parameters coming from the same spectrum. Our data demonstrate that the level of aa excretion shows an age-related U shape pattern that may be related to the process of maturation/senescence of the various amino acid transport systems. Dependence from plasma amino acid level and/or tubular amino acid load looks less likely, first because the data are normalized for creatinine excretion, and second since the glomerular filtration rate (GFR)

generally increases during childhood and decreases in the senescence (Baylis and Schmidt 1996). This age dependent aa excretion in a large population cohort extends and confirms previous data obtained mostly in young infants (Parvy et al. 1995). This study, for the first time, delineates a reliable amino acid transport network in the kidney of a model healthy Caucasian population. Since urine standard parameters in the two studied populations behave as the general one, it is very likely that the study has wide potential use and implications for all other Caucasian populations. The hypothesis that amino acid transporters of the reabsorptive cells might be at the basis of the clustering of urine excretion of aa found is supported by two facts: (1) strong associations occur among aa with similar structure (Il_Le/Val, neutral branched aa; Thr–Ala, small neutral aa) and (2) the degree of linear correlation increases with essential aa (one essential and one not essential amino acid (Gly–Phe, Thr–Ala) < two essential aa (Il_Le–Val). Identification of the transporters lying at the basis of the clustering of amino acid urine reabsorption is not trivial since amino acid transport is very promiscuous (i.e., a particular transporter handles several aa and a particular amino acid is transported by several carriers) (Palacin et al. 1998). Figure 4 depicts the known amino acid reabsorption machinery of the renal proximal tubule. The role of these transporters in renal reabsorption has been established by the study of human aminoacidurias and animal models. Thus, mutations in system $b^{0,+}$ ($b^{0,+}$AT/rBAT) cause human, murine and canine cystinuria (hyperexcretion of cystine and dibasic aa) (Calonge et al. 1994; Feliubadalo et al. 1999, 2003; Peters et al. 2003; Harnevik et al. 2006) mutations in B^0AT1 (system Neutral Brush Border) cause Hartnup disorder (general hyperexcretion of neutral aa) (Kleta et al. 2004; Seow et al. 2004), mutations in system y^+L1 (y^+LAT1/4F2hc) cause Lysinuric protein intolerance (hyperexcretion of dibasic aa) (Borsani et al. 1999; Torrents et al. 1999). Moreover, the mouse knockout of the glutamate transporter EAAC1 presented dicarboxylic aminiaciduria (Peghini et al. 1997), the mouse knockout of XT2 resulted in glycinuria together with hyperexcretion of several neutral aa (Quan et al. 2004), and finally the knockdown of LAT2 (LAT2/4F2hc) resulted in the accumulation of cystine and depletion of serine, threonine and alanine in the proximal tubule epithelial cell model OK (Fernandez et al. 2003). Finally other amino acid transporters expressed in the apical [SIT1 (System IMINO), PAT1 (and PAT2; imino acid carriers), and ASCT2)] and basolateral (TAT1) plasma membrane of the proximal tubule are expected to have a role in renal reabsorption. Thus, SIT1, PAT1and/or PAT2 are candidate transporters to have a role in familiar iminoglycinuria (hyperexcretion of glycine and imino acids) (Broer et al. 2006) and TAT1 has been proposed to drive the exchange of neutral aa via LAT2/4F2hc by the facilitated efflux of aromatic aa

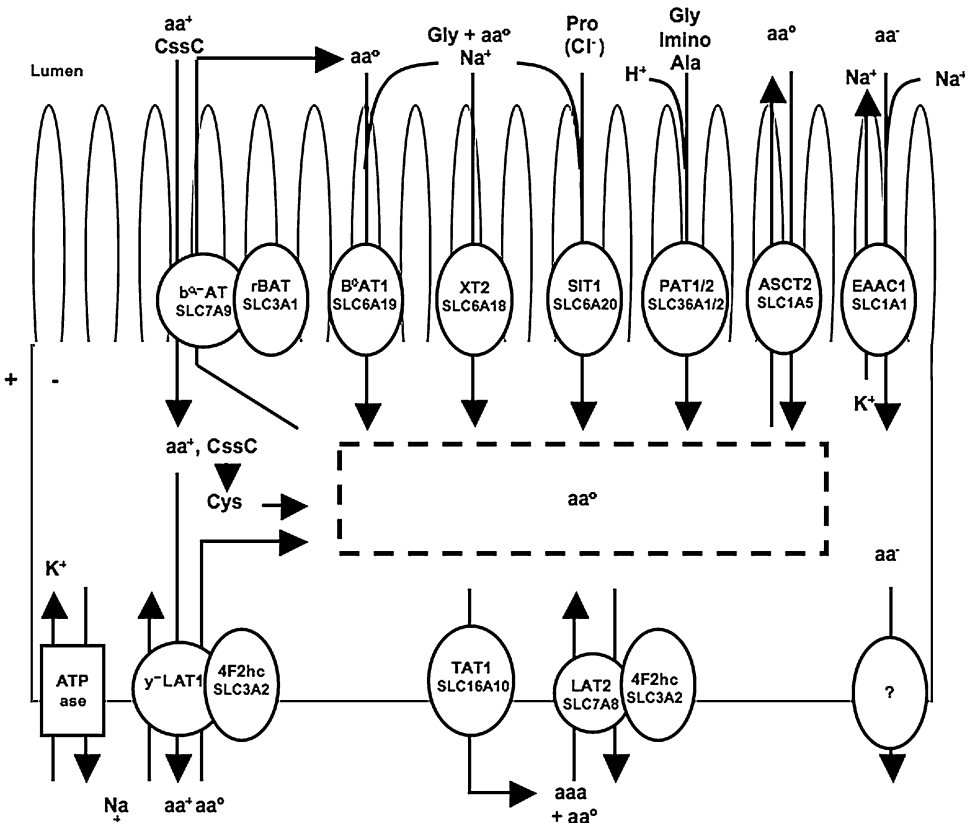

Fig. 4 Transport machinery for amino acid renal reabsorption. Schematic representation of an epithelial cell from the renal proximal tubule. All the indicated amino acid transporters (trivial name with HUGO nomenclature below) are mainly expressed in the proximal convoluted tubule (S1-S2 segments of the nephron) with the exception of XT2, expressed in the proximal straight tubule (S3 segment of the nephron) and ASCT2, which distribution in kidney has not been studied. The main proximal tubule imino acid carrier (PAT1, SLC36A1 or PAT2, SLC36A2) is at present unknown. The ancillary protein collectrin of B^0AT1 (Danilczyk et al. 2006) is not depicted for simplicity. *aaa* aromaric amino acids, *aa°* neutral amino acids, *aa+* dibasic amino acids, *aa−* dicarboxylic amino acids, *CssC* cystine, Imino, imino acids proline and hydroxiproline. Specific amino acids are abbreviated with the 3

(Ramadan et al. 2007). With the present knowledge, four of the above mentioned transporters for neutral aa might be at the bases of the most significant correlations identified in this study because their expression in proximal tubule and transport activities: (1) The most conspicuous transporter for neutral aa in the apical membrane of the reabsorptive cells of the proximal convoluted tubule corresponds to B^0AT1. This transporter handles all neutral aa with a range of affinities (Bohmer et al. 2005). (2) The apical transporter ASCT2, which expression has been reported in rat kidney cortex (Pinho et al. 2007), exchange neutral aa with a range of affinities (Ala, Ser, Cys, Thr, Gln, Asn > Met, Gly, Leu and Val) (Broer et al. 1999). (3) The apical transporter XT2, expressed in the proximal straight tubule, has not been expressed functionally in foreign expression systems, but its knockout in mouse resulted in hyperglycinuria but also moderate hyperexcretion of tyrosine, methionine, phenylalanine, leucine/isoleucine and even lower of alanine and valine (Quan et al. 2004). (4) Basolateral LAT2/4F2hc from the proximal convoluted tubule exchange neutral aa of any size with apparent affinity in the low-medium micromolar range, including histidine (Pineda et al. 1999; Segawa et al. 1999). Thus, polymorphic variations in the activity of any of these four transporters might result in the correlation of the urine excretion of Ile–Leu–Val, being B0AT1 the best candidate for this correlation since these three aa show the highest affinity for this transporter. Similarly, B^0AT1, XT2 and LAT2 might be responsible for the Gly–Phe correlation and B^0AT1, ASCT2 and LAT2 might be responsible for Ala–Thr correlation because these transporters share these substrates, respectively. However, one of the identified clusters contains neutral, dibasic (Lys) and acidic (Glu) aa. None of the mammalian identified transport systems carry this variety of amino acid substrates (Palacin et al. 1998). Then, either this study is revealing an unknown functional interaction between the transporters or, in addition, the clustering of amino acid urine excretion has in part a metabolic basis. Thus, Ile, Leu and Val metabolism share, at least in part, common pathways. In the catabolism for example, the first three metabolic steps are common: (1) Transamination that leads to the corresponding α-ketoacid (with the concomitant transformation of α-ketoglutarate into Glu). (2) Oxidative decarboxylation of the α-ketoacid into the corresponding acyl-CoA. (3) Dehydrogenation into the relative α,β-unsaturated acyl-CoA. In the subsequent steps, Leu is transformed into acetoacetate and acetyl-CoA, Ile into acetyl-CoA and propionyl-CoA and Val into propionyl-CoA (and finally in succynil-CoA), that are intermediates of the citric acid cycle or glycolysis. Such preliminary observations pose the question of how to obtain an overall view of the activities (transport of aa and metabolism) of the epithelial cells of the kidney. In this perspective, it should be of great value extending the hierarchical cluster analysis over other urinary components. Moreover, association studies between amino acid renal tubular handling and transporter polymorphisms might result in the identification of the transporters with a higher impact on the renal reabsorption of aa. In conclusion, the present study provides a first set of reference data on amino acid levels detected by NMR in a healthy population, generates accurate metabolite concentration data, and provides, for the first time, data that can be used to help understanding metabolic differences in a healthy population. Moreover, our approach shows that clustering analysis might suggest models for tubular handling of aa and also new metabolic networks and pathways. All these findings open new perspectives in the research of tubular aa handling and in defining related quantitative and qualitative traits.

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