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The Cardiovascular Risk of Healthy Individuals Studied by NMR Metabonomics of Plasma Samples

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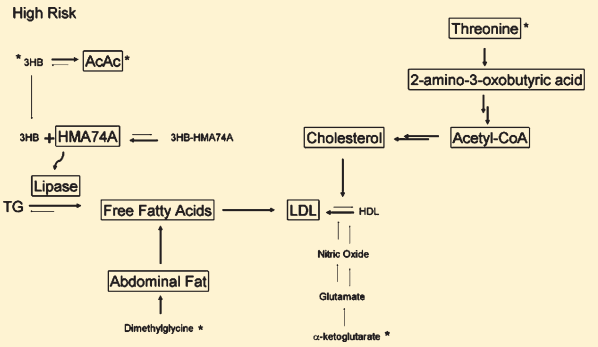
Supporting Information

ABSTRACT: The identification and the present wide acceptance of cardiovascular risk factors such as age, sex, hypertension, hyperlipidemia, smoking, obesity, diabetes, and physical inactivity have led to dramatic reductions in cardiovascular morbidity and mortality. However, novel risk predictors present opportunities to identify more patients at risk and to more accurately define the biochemical signature of that risk. In this paper, we present a comprehensive metabonomic analysis of 864 plasma samples from healthy volunteers, through Nuclear Magnetic Resonance (NMR) and multivariate statistical analysis (regression and classification). We have found that subjects that are classified as at high or at low risk using the common clinical markers can also be discriminated using NMR metabonomics. This discrimination is not only due to common markers (such as total cholesterol, triglycerides, LDL, HDL), but also to ($p < 0.05$ after Bonferroni correction) other metabolites (e.g., 3-hydroxybutyrate, α-ketoglutarate, threonine, dimethylglycine) previously not associated with cardiovascular diseases.



KEYWORDS: metabonomics, metabolomics, metabolites, cardiovascular diseases, atherosclerosis, NMR spectroscopy, oxidative stress

INTRODUCTION

Atherosclerotic cardiovascular diseases (CVD) are considered the leading cause of death for both men and women in the developed world;[1] therefore, morbidity, mortality, and socioeconomic importance of CVDs make timely and accurate diagnosis, and cost-effective management, of the utmost importance.

Atherosclerosis begins in childhood and progresses into adulthood due to multiple coronary risk factors.[2−4] The patient risk assessment, before scores such as the Framingham Risk Score[3] (FRS), was a subjective judgment that could lead to different conclusions based on the physician and patient risk perception.[5−8] Beginning from the 1990s, the CVD risk factors have been increasingly considered the target for preventive interventions.[9−12] Global risk instruments have been developed,[13] the most widely used being the FRS and its variants.[14]

The evaluation of predisposing factors, with obesity as a representative example, may reveal a number of complex interactions underlying the predisposing condition besides their role as risk factors. They are suspected to independently contribute to CVD risk in a complex network of interactions among several metabolic pathways that may start their hidden action even in early childhood. Abdominal obesity and insulin resistance (two significant predisposing factors) are a manifestation of the so-called "metabolic syndrome" which in turn is a key in the evolution of cardiovascular disease. There is debate regarding whether obesity or insulin resistance is the cause of the metabolic syndrome or if they are consequences of a more far-reaching metabolic derangement.

A healthy and relatively young population like that of Italian volunteer blood donors may be representative of a significant proportion of the young part of the general population that could have a very early and latent tendency to CVDs. They may constitute a model to prove the existence of a relationship between the CVD risk factors and a certain metabolic profile considered as the whole ensemble of metabolites, such as the metabonomic NMR spectrum of serum or plasma.

Metabonomics[15−17] has established itself in the past decade as a useful complement to the characterization of several physiological and pathological conditions. The result of the extensive analysis of the metabolites in a specific biological specimen, that is, its metabonome, can be considered the downstream end product of the complex interaction of genome, transcriptome, and proteome. Usually, metabonomic analysis does not rely on the measurement of a single metabolite-associated peak but it considers the

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spectrum as a whole.[18] The NMR profile contains qualitative and quantitative information on the hundreds of different small molecules present in the sample.[19−21] This approach offers evident advantages with respect to knowledge-guided search of metabolites, since it does not need to make any assumptions on the identity of the metabolites that are relevant for the selected pathology. The metabonome, in fact, is affected by perturbations due to physiological, pathological, and also iatrogenic factors.[22−24] Finally, the statistical analysis of the NMR profiles may provide information on the metabolite pattern alterations and their association with pathologies.[25−28]

Only few metabonomic investigations of subjects with a high cardiovascular risk profile has been yet reported; however, the usefulness of metabonomics in cardiovascular research is strongly advocated by some authors.[29,30] Nicholson et al.[31] have examined the relationship between hypertension, diet, and the metabonome alterations. Recently, Shah[32] demonstrated that the analysis of metabolic profiles can predict future cardiovascular events independently of standard clinical predictors in subjects undergoing endovascular procedures. The same author[33] has found an association of some blood metabolites with coronary artery disease, using a model built on healthy controls and patients. The application of metabonomics to the discovery of new biomarkers of acute myocardial ischemia is also reported.[34] Despite these achievements, the usefulness of metabonomics for CVD risk assessment is still debated: the first results from Brindle[35] were subsequently questioned in another paper,[36] where the authors conclude that the metabolic profile of cardiovascular disease is dominated by lipidic profile, with no evident advantage with respect to the classical analysis. However, there is certainly interest for metabonomics in the risk assessment of silent disease or for large population screening.

In line with these conclusions, we hypothesized that metabonomics would be an effective tool for a deeper investigation of the molecular mechanisms and biochemical pathways involved in the composition of cardiovascular risk at very early stages.

In this study, we have examined the metabolic profile of 864 healthy volunteers (the largest cohort among the cited studies), with neither overt cardiovascular disease nor extremely high Framingham scores. The aim of the paper is to investigate (i) whether the NMR of plasma contains a fingerprint of the cardiovascular risk status of a healthy individual and (ii) whether this fingerprint is constituted also by molecules (metabolites) not previously associated with cardiovascular risk. To do this, we have analyzed healthy individuals with increased cardiovascular risk parameters (low HDL, high LDL, high total cholesterol, high triglycerides, and in general high Framingham score) and we have compared them with the subjects with a low risk pattern, using multivariate and univariate statistics. We have found that subjects that are classified by the common markers as at high or at low risk can be discriminated also by NMR metabonomics. We show that this discrimination is due to a complex NMR fingerprint that is composed of not only the common markers (total cholesterol, triglycerides, LDL, HDL), but also other metabolites previously not associated with cardiovascular risk, reinforcing the first intuition of Brindle.[35]

These metabolites are discussed in the light of related observations in the published literature and can contribute (if validated in further studies) (i) a novel approach to CVD risk estimation and (ii) novel insights into the complex biochemical nature of CVD risk.

Table 1. Average Clinical Data for Recruited Volunteers

female = 186	mean	st. deviation	max	min
Age (years)	41.81	11.92	65	19
Glycaemia (mg/dL)	87.85	13.94	175	45
Creatinine (mg/dL)	0.773	0.108	1.14	0.54
Cholesterol (mg/dL)	211.8	39.04	366	139
Triglycerides (mg/dL)	85.15	54	556	39
HDL (mg/dL)	65.08	13.46	98	43
LDL (mg/dL)	134	40.63	219	69
Blood pressure Max (mmHg)	119.1	10.48	160	110
Blood pressure Min (mmHg)	77.77	7.05	100	65
Cardiac Frequency (bpm)	71.74	5.47	88	52
Cardiovascular risk score (%)	2.5	2.3	9.5	0
male = 678	**mean**	**st. deviation**	**max**	**min**
Age (years)	40.62	10.8	71	19
Glycaemia (mg/dL)	90.14	12.54	226	65
Creatinine (mg/dL)	0.97	0.124	1.54	0.32
Cholesterol (mg/dL)	201.8	38.05	339	75
Triglycerides (mg/dL)	106.87	64.53	635	30
HDL (mg/dL)	51.6	11.35	99	29
LDL (mg/dL)	129.86	36.39	231	42
Blood pressure Max (mmHg)	124.4	10.71	160	100
Blood pressure Min (mmHg)	81.44	7	100	60
Cardiac Frequency (bpm)	69.66	6.14	90	48
Cardiovascular risk score (%)	5	4	28	0

MATERIALS AND METHODS

Recruitment of Volunteers

In the study, a total of 864 adult healthy volunteers (678 males, 186 females, mean age 40.87 ± 11.0 yrs) were enrolled. The overall median age of the subjects is 41 years, with a minimum of 19 years and a 25th percentile of 32 years. Eighty-five percent of the patients are less than 49 years old. Only 33 patients are more than 60 years old. So, the cohort is made up of a relatively young population (Supplemental Figure S1). The subjects participating in this study were recruited in collaboration with the Tuscanian section of the Italian Association of Blood Donors (AVIS) in the Transfusion Service of Pistoia Hospital (Ospedale del Ceppo, AUSL 3 - Pistoia, Italy). The demographic features of the patients are depicted in Table 1, and match the gender and age statistics for blood donors in Italy, that is, a much larger share of male versus female donors.

The problem of unbalanced cohort in metabonomics studies was already addressed by Nicholson et al.[35] This, in fact, is a strength, not a weakness, if the aim is to avoid overemphasizing the power of the methods artificially by removing major source of variation, as it commonly occurs when using highly age- and sex-matched group. Our results did not depend on the sex basis of the patients group: performing the statistical analyses only on the men or the women group, the results are not affected. Moreover, randomly changing the proportion of men and women did not change the outcome.

Each one of the study subjects underwent, before blood withdrawal, a thorough clinical and physical examination that included the clinical history of the subject.

Plasma samples were obtained according to the Standard Operating Procedures (SOPs) defined for blood donation in Italy.

The samples were obtained after overnight fasting and Ethylenediaminetetraacetic acid (EDTA) was used as anticoagulant. The presence of EDTA does not affect the quality of the samples: the NMR spectral effects of any interactions between the added EDTA and endogenous components were found to be negligible, thus for plasma samples collected using EDTA as anticoagulant, useful biochemical information can be recovered effectively by metabolomics[37]

The plasma samples for the NMR analysis were obtained from the backup aliquots of samples that, according to the SOPs, are stored in the Transfusion Service for analytical purposes for at least 24 h after blood withdrawal. The plasma samples were stored at −80 °C immediately after collection for further metabonomic analysis.

Lipidic Fractions Determination

TC, HDL, and triglycerides were measured using a direct enzymatic essay.[38,39] LDL was derived using the Friedewald equation.[40] Further details are given in Supporting Information.

Ethical Issues

Written informed consent was obtained from all volunteers according to the Helsinki declaration.

NMR Sample Preparation

Frozen plasma samples were thawed at room temperature and shaken before use. A total of 300 μL of a sodium phosphate buffer was added to 300 μL of each plasma sample, and 450 μL of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin) for analysis. Full details are reported in Supporting Information.

NMR Analysis and Spectral Processing

One-dimensional ^{1}H-NMR spectra of plasma samples were measured on a Bruker spectrometer operating at 600 MHz proton Larmor frequency using standardized protocols. CPMG, diffusion edited and 1D-NOESY spectra were acquired (see Supporting Information). Each spectrum in the region 10.00−0.02 ppm was segmented into 0.02-ppm chemical shifts bins (buckets) prior to any statistical analysis. Bucketing is a means to reduce the number of total variables and to compensate for small shifts in the spectra. EDTA peaks were not removed from the analysis.

Statistical Analysis

The statistical procedure used is a combination of Partial Least Squares, followed by Canonical Correlation Analysis (CA), followed by Support Vector Machines (SVM) on the CA scores. For homogeneity, exactly the same analysis was performed both for classification and for regression, and both were validated in the same way, as explained hereafter: all the employed multivariate techniques can be easily used both with categorical and with continuous variables.

The accuracy for classification and regression was assessed by means of a double cross-validation scheme.[41,42] The original data set was split into a training set (90%) and a test set (10%) prior to any step of statistical analysis. No samples in the test set were used for parameter selection. The number of PLS components (from 1 to 50 components), the kernel kind (linear or radial), and the SVM parameter "C" for the cost of constrains violation (7 values from an exponential growing sequence between 10^{-3} and 10^3) were optimized using a 7-fold cross-validation scheme in training set. To avoid overtraining, all other SVM parameters were kept at the default settings. The whole procedure was repeated 100 times inside a Monte Carlo cross-validation scheme. Data classification using SVM was performed by applying the classifier on PLS scores. From this cross-validation procedure, we obtain unbiased error estimations for both classification and regression. For regression analysis, we reported also the R^2 between the true clinical values and the mean cross-validated predicted values for each sample (Figures 2 and 3).

For classification purposes, the groups are created by dividing the samples in two classes represented by the highest and the lowest quintiles, respectively, relative to the target variable: HDL <44 mg/dL and >63 mg/dL (42 vs 44 individuals, respectively), LDL <100 mg/dL and >160 mg/dL (42 vs 44 samples), TC <174 mg/dL and >232 mg/dL (153 vs153), TC/HDL ratio <3 and >4.90 (40 vs 40), triglycerides <60 mg/dL and >131 mg/dL (150 vs 151), glycemia <78 mg/dL and >105 mg/dL (148 vs 153), Framingham score <1.25 and >7.00 (40 vs 40). For regression analyses, all the available samples were used: 206 for HDL, 199 for LDL, 715 for TC, 205 for TC/HDL ratio, 709 for triglycerides, 693 for glycemia, and 201 for Framingham score. The different numbers of individuals in each class is due to the fact that all clinical parameters were not available for all individuals: although the total cholesterol was measured for all subjects, in general (for cost and speed purposes), the lipid fractions (LDL and HDL) are measured only for new blood donors or for the blood donors that expressly request them.

To assess which buckets were significantly different between different groups a univariate Wilcoxon test was used. A P-value $\leq$.0001 was considered statistically significant. This value corresponds to a nominal significance level of 0.05, after a Bonferroni[43] correction for the number of multiple comparisons, that is, 0.05/416, were 416 is the total number of buckets. The use of a conservative criterion such as the Bonferroni correction is motivated by the goal of selecting a small subset of putative but reliable biomarkers, so it is better to lower type I errors (false discoveries) at the expense of an increase of false negatives.[44]

All calculations were made using homemade scripts written in our lab using the R language.[45] Further references and information on the statistical methods are reported in the Supporting Information.

RESULTS

The study population appears to be representative of a young adult healthy proportion of Italian people. The male/female ratio of the study population is high (78.5%), comparable with that of other studies performed on Italian blood donors as well as with that of AVIS registry of donors.[46] The clinical data and the NMR spectra are publicly available via the Internet.[62]

As a first step, we have performed several thorough multivariate statistical analyses in order to ascertain whether metabolic fingerprints of the plasma samples contain predictive information about the global status of the individuals. First, a classification analysis was performed, using as predictor the full NMR profile, with respect to the total cholesterol (TC), LDL cholesterol, HDL cholesterol, triglycerides, and glycaemia. Although continuous variables were available for these clinical data, we have turned the problem into a classification using only the extreme values, in order to understand better what characterizes (from a metabolic point of view) the subjects that can be considered "most healthy" with respect to those "at higher risk". The results are shown in Tables 2 and 3 for CPMG and NOESY spectra, and in Supplemental Tables S1−S6 for diffusion edited spectra. Values of sensitivity, specificity, and accuracy, obtained from a double cross-validation procedure,[41] are reported.

Table 2. Sensitivity, Specificity, and Accuracy Results for the Various Classifications Using CPMG Spectra

CPMG classification	samples	values	sensitivity	specificity	accuracy
TC	306 (153 vs 153)	<174 and >232	96.13%	96.78%	96.50%
LDL	85 (42 vs 43)	<100 and >160	99.01%	96.73%	98.30%
HDL	82 (42 vs 40)	<44 and >63	92.87%	94.86%	92.80%
Triglycerids	351 (150 vs 151)	<60 and >131	97.77%	96.91%	97.48%
Glycaemia	301 (148 vs 153)	<81 and >96	85.89%	85.32%	85.65%
TC/HDL	80 (40 vs 40)	<3 and >4.90	98.15%	98.94%	98.19%
Framingham score	80 (40 vs 40)	<1.25 and >7.00	88.40%	88.07%	88.21%

Table 3. Sensitivity, Specificity, and Accuracy Results for the Various Classifications Using NOESY Spectra

NOESY classification	samples	values	sensitivity	specificity	accuracy
TC	306 (153 vs 153)	<174 and >232	97.60%	98.06%	98.07%
LDL	85 (42 vs 43)	<100 and >160	100.00%	97.96%	98.94%
HDL	82 (42 vs 40)	<44 and >63	98.87%	98.85%	98.81%
Triglycerids	351 (150 vs 151)	<60 and >131	98.18%	97.05%	97.37%
Glycaemia	301 (148 vs 153)	<81 and >96	76.80%	79.20%	78.02%
TC/HDL	80 (40 vs 40)	<3 and >4.90	100.00%	97.75%	98.87%
Framingham score	80 (40 vs 40)	<1.25 and >7.00	89.17%	91.17%	89.68%

All the accuracy values turned out to be greater than 95% for all classifications and types of 1-D spectra: CPMG spectra (that contain signals arising mostly from low molecular mass metabolites), diffusion edited spectra (that contain signals arising mostly from macromolecules), and NOESY spectra (that contain both kinds of signals). Glycaemia represents the only exception, since it showed an accuracy of 85%. The separation obtained by plotting the CA scores is also very clear (Figure 1).

On the basis of these encouraging results, a more sophisticated regression analysis was also attempted, aiming at establishing a quantitative relationship between clinical data and the NMR spectra. Figures 2 and 3 show the agreement between the cross-validated statistical prediction obtained for HDL and LDL cholesterol, respectively, with the values obtained from the clinical analysis. The mean relative error is less than 10% in both cases.

These results are not unexpected, since the NMR spectra (especially NOESY and diffusion edited) contain several strong peaks directly related to the lipidic components of the plasma.[47] Nevertheless, they are an excellent starting point for our subsequent analysis: to confirm that metabonomics is able to extract also subtler metabolic information, we tested whether individuals could be divided into high and low values of TC or glycaemia even after removing from the spectra all the regions with signals arising from lipids (0.45−1.00, 1.13−1.45, 1.51−1.69, 1.87−2.24, 2.55−2.87, 3.07−3.29, 3.6−5.49 ppm) or glucose (3.31−3.95 and 5.22 ppm), respectively. The accuracy obtained (75% in both cases, see CA score plot in Supplemental Figures S2, S3) is a convincing indicator that the changes in blood composition may be considered as global phenomena that involve several metabolic alterations, and thus, metabonomics could be able to outline all these effects simultaneously. This unexpected result demonstrates that plasma profiles contain other cardiovascular risk indicators (metabolites).

As it is well-known that low levels of HDL cholesterol and high levels of TC correlate with high risk of developing CVDs, we performed the classification and regression analysis also for the TC/HDL ratio, using the full spectra (i.e., without removing lipids). Again the results, both for classification and regression, are extremely good, with accuracy for classification even higher than for the two risk factors taken separately (Tables 2 and 3), and R^2 of 0.88 for regression (Figure 4). Analogously, very good values are obtained for the HDL/LDL ratio.

On the basis of the clinical data of the subjects, the FRS was calculated for each individual, using the tables and equations of Wilson et al,[12] that give an accurate score (c-statistic of 0.75−0.77) in predicting CVD using risk categories. A classification was again performed by splitting the data into the quintiles with the highest and the lowest score. Also in this case, no peaks (except the water peak) are removed. The accuracy is high (over 89% with the NOESY spectra) but lower than in the previous analysis (Tables 2 and 3). This may due to the fact that FSR takes into account also nonanalytical variables, but it can be also due to the fact that the analysis here performed was able to extract a richer and more complex metabolic pattern.

As a further step of our analysis, in order to identify the metabolites that are most characteristic of the metabolic fingerprint associated to classical cardiovascular risk markers, we applied a univariate Wilcoxon test on the different groups, using a significance level of $P < .0001$, after applying the Bonferroni correction (see Materials and Methods section). This was because univariate models look at one metabolite-at-a-time and are easy to interpret but lack an overall view on the data, while multivariate models provide a full view but often lack interpretability, especially in high-dimensional data cases.[48]

Individuals with high plasma levels of HDL were found to be characterized by low levels of creatinine and threonine and high levels of 3-hydroxybutyrate (3-HB). The subjects with high plasma levels of LDL showed low levels of α-ketoglutarate and dimethylglycine, while the ones with high levels of TC were characterized by low levels of α-ketoglutarate, dimethylglycine, and serine.

From the analysis performed, it also resulted that the subjects with high levels of triglycerides (TRI) have a more complex metabolic pattern, with low levels of several metabolites: acetate, creatine, creatinine dimethylglycine, 3-HB, isoleucine, methionine,

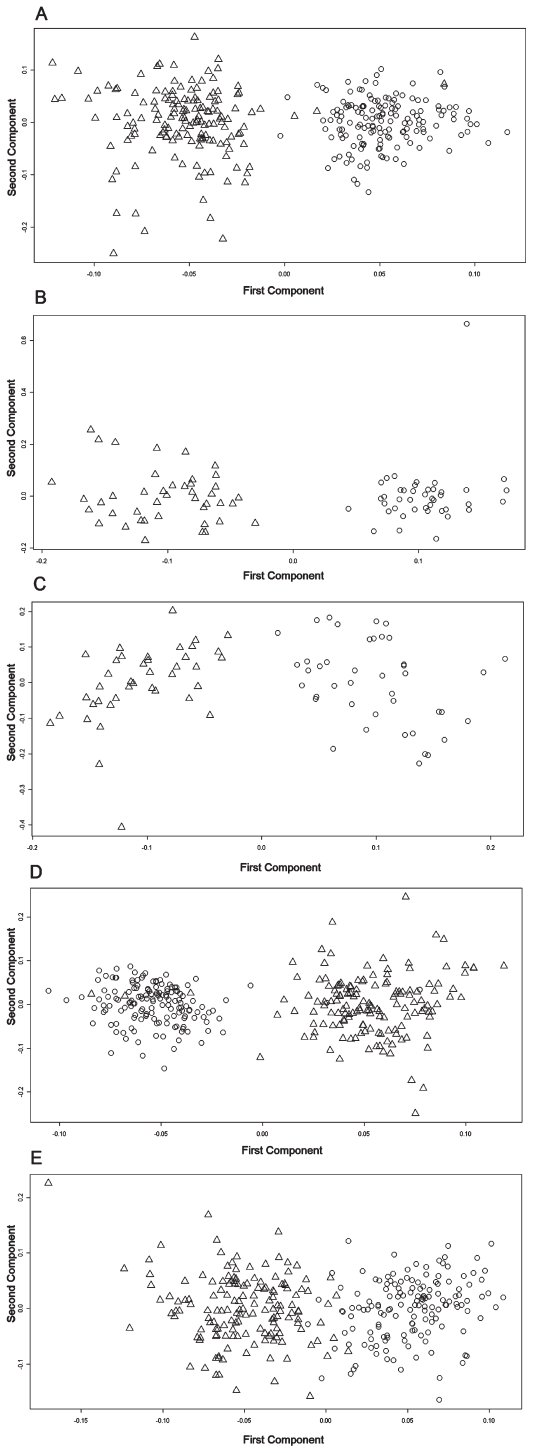

Figure 1. Clusterization of high (triangles) and low (circles) quintiles of TC (A), LDL (B), HDL (C), triglycerides (D), and glycaemia (E) values, using CPMG spectra. The cross-validation results provide accuracies (Table 2) between 85 and 98%, confirming the striking predictive power of CPMG spectra.

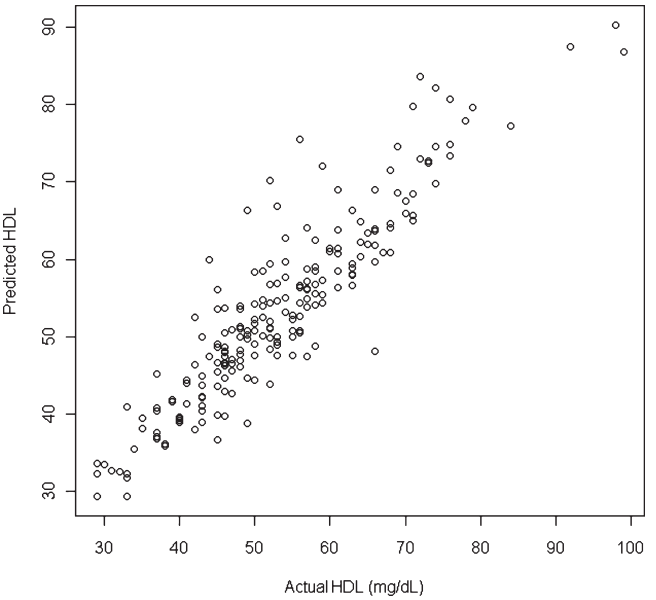

Figure 2. Correlation between actual (x axis) and predicted (y axis) HDL levels on the basis of NOESY spectra (R^2 = 0.83).

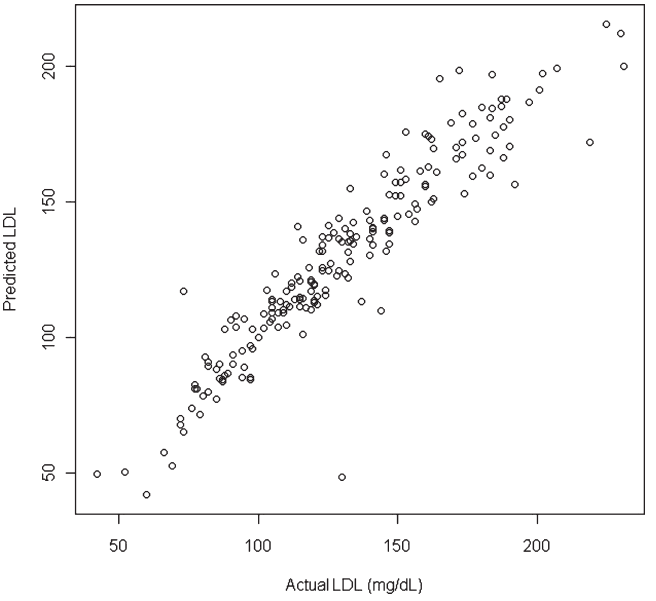

Figure 3. Correlation between actual (x axis) and predicted (y axis) LDL levels on the basis of NOESY spectra (R^2=0.88).

phenylalanine, and serine, and high levels of threonine. Intriguingly, with the exception of threonine, the concentrations of all amino acids are lower in these subjects.

Since the signal deriving from 3-HB in the 1-D spectra is covered in part by other signals, the significance value of 3-HB has been also confirmed using a J-RES projection where this signal is well resolved.

The complete list of the most significant metabolites found to vary with clinical data is reported in Table 4.

Because our aim is to find a metabolic pattern that correlates with an increase of classical cardiovascular risk markers, we performed the same analysis by only considering subjects with high level of HDL and low levels of LDL cholesterol (i.e., the "lowest risk" subjects of the study cohort), ordered by the HDL/LDL ratio. Their metabolic pattern was again robustly characterized by low levels of creatinine and threonine, and by high levels of 3-HB.

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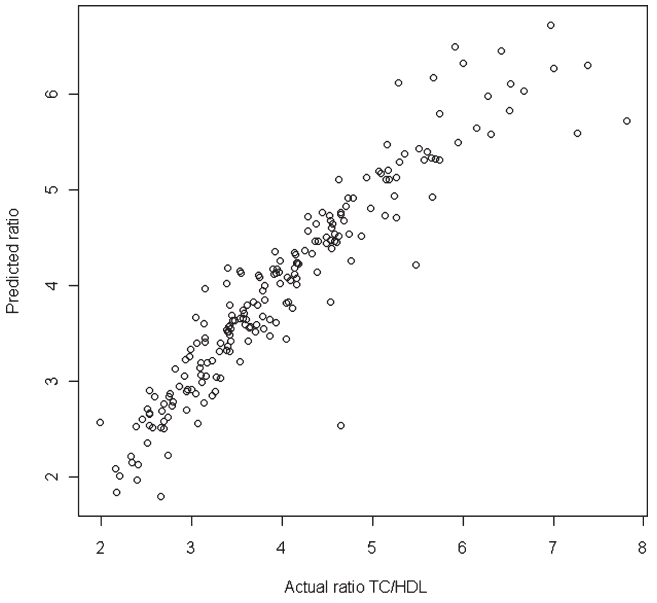

Figure 4. Correlation between actual (x axis) and predicted (y axis) TC/HDL levels on the basis of NOESY spectra ($R^2 = 0.88$).

DISCUSSION

The statistical results obtained by classification and regression analysis are extremely encouraging, suggesting that the whole plasma spectrum profile contains meaningful information about the metabolic status of individuals. Also the CPMG spectra (where peaks associated with lipoproteins are attenuated) are able to predict with high accuracy the clinical data. This reinforces the view that we are effectively following global metabolic changes and not only obvious patterns of lipids and lipoproteins peaks. To prove this statement, we also tried to completely remove spectral regions with peaks associated with lipid fractions (for TC analysis) and with glucose (for glycaemia analysis). Although the accuracy decreases to about 75% (both for TC and glycaemia), it is still a very meaningful result, especially if we consider that, in the case of TC, we have removed as much as 20% of the total buckets in order to be on the safe side. This is a main finding of this work because, although the relationships between HDL, LDL, and NMR spectra were already investigated and are well-known, we are showing here that there are other metabolites involved in the discrimination.

These promising results prompted us to explore the metabolite composition of plasma profiles to characterize what (apart from the trivial lipoproteins levels) are the typical features of an undoubtedly "low risk" status (high HDL levels, low LDL levels, low triglycerides, and low glycaemia) and what are the typical ones of a high risk status (the reverse).

From the point of view of this comprehensive metabolic analysis, it seems that the "low risk" pattern is mostly constituted by high levels of 3-HB and low levels of threonine and creatinine. The "high risk" pattern is more complex, and is characterized by low levels of several metabolites, in particular α-ketoglutarate, dimethylglycine, and serine. The present findings highlight a role for 3-HB, α-ketoglutarate, threonine, and dimethylglycine in the fingerprint associated to high or low cardiovascular risk markers in our population. As discussed below, all of these metabolites are related to mitochondrial metabolism.

3-HB is generated by fatty acid β-oxidation, and is known to have a protective role in a broad spectrum of cerebral injuries through a process involving a mitochondrial oxido-reductase superfamily, with fatty acid-CoA thioesters as substrates.[49] Our data indicate that high levels of circulating 3-HB are significantly related to a high HDL/LDL ratio that, in turn, is related to a low risk profile for cardiovascular events. A role of mitochondria can therefore be invoked. The acetoacetate to 3-hydroxybutyrate ratio in the blood is a good indicator of the NAD^+/NADH redox ratio in the mitochondria of hepatic cells.[49,50] Usual values of the ratio in healthy subjects are highly variable and range between 0.96 and 2.55.[51] The mean value of this ratio in our cohort was 2.02 with a standard deviation of 0.78.

In our findings, since the level of acetoacetate seems not to vary as a function of the HDL/LDL ratio, but 3-HB shows a positive correlation, the final ratio between acetoacetate and 3-HB decreases in the case of the low risk profile.

Although the use of acetoacetate/3-HB ratio is not new in the literature, to the best of our knowledge this is the first time that this figure is related with LDL and HDL levels in the blood and, in turn, with metabolic alterations associated with an enhanced risk for cardiovascular disease. We may hypothesize that the oxidative stress may enhance the oxidation of 3-HB to acetoacetate, reducing its plasma levels. The mechanism underlying the correlation between the plasma parameters of individuals at low risk and high levels of 3-HB may be related to the ability of 3-HB to bind the HM74A receptor expressed in adipocytes, resulting in the inhibition of the lipolysis and thereby reducing free fatty acids plasma levels. This receptor binds nicotinic acid, that is known to cause a significant fall of the LDL levels and, concomitantly, a raise of the HDL levels.[52] The receptor responds also to 3-HB, and the latter is thought to be the probable endogenous ligand for HM74A. Hence, the occupancy of HM74A receptor by circulating 3-HB could represent a physiological feedback cycle for the control of lipolysis rate.[53]

In our findings, low levels of α-ketoglutarate are associated with increased cardiovascular risk. α-Ketoglutarate is a key intermediate in the Krebs cycle, coming after isocitrate and before succinyl CoA. The cycle can be filled at this junction by the biosynthesis of α-ketoglutarate through the action of glutamate dehydrogenase on glutamate. Early observations[54] demonstrated a role of oral administration of α-ketoglutarate in lowering blood serum cholesterol and body weight in an animal model. We may speculate that an imbalance of the concentrations of the redox substrates of glutamate dehydrogenase due to some alteration of the mitochondrial redox potential may play a role. Our findings are also related to the studies by He et al.[55] that link citric cycle intermediate with atherosclerosis, diabetes, and renal failure.

In addition to these considerations, it is noteworthy that the concentrations of all amino acids but threonine are lower in subjects with high levels of triglycerides. Threonine is actually higher in these subjects. Intriguingly, the catabolism of threonine is regulated by threonine dehydrogenase (THD), which is a mitochondrial enzyme that catalyzes NAD^+-dependent oxidation of threonine to 2-amino-3-oxobutyric acid, which in turn is very rapidly metabolized by 2-amino-3-oxobutyrate-CoA ligase to glycine and acetyl-CoA.[56] The formation of acetyl-CoA from ligase is so fast that acetyl-CoA can be considered a direct product of TDH.[56] Acetyl-CoA is not only derived from TDH, but also from fatty acids by β-oxidation. Excessive formation of acetyl-CoA by TDH would inhibit both the β-ketothiolase reaction and β-oxidation by the law of mass action, playing a biological role in the regulation of fatty acid breakdown, biosynthesis,

Table 4. Means, in Arbitrary Units, of Statistically Significant ($p < 0.05$) Metabolites for the Groups Where the Levels of the Clinical Variables Are Low or High (First and Second Values, Respectively)

	LDL	TC	TRI	HDL	HDL/LDL
3-HB				4.8×10^{-2} vs 5.4×10^{-2}	1.5×10^{-2} vs 2.0×10^{-2}
Acetate			2.5×10^{-1} vs 1.7×10^{-1}		
Apha Ketoglutarate	4.9×10^{-1} vs 3.9×10^{-1}	4.6×10^{-1} vs 3.8×10^{-1}			
Citrate			1.3×10^{-1} vs 8.6×10^{-2}		
Creatine			1.9×10^{-1} vs 1.4×10^{-1}		
Creatinine			1.1×10^{-1} vs 1.5×10^{-1}	1.7×10^{-1} vs 1.3×10^{-1}	1.6×10^{-1} vs 1.3×10^{-1}
Dimethylglycine	2.1×10^{-1} vs 1.7×10^{-1}	2.0×10^{-1} vs 1.62×10^{-1}	2.1×10^{-1} vs 1.4×10^{-1}		
Glucose			1.3 vs 0.9		
Isoleucine			7.2×10^{-1} vs 5.9×10^{-1}		
Methionine			4.8×10^{-1} vs 3.4×10^{-1}		
Phenylalanine			9.8×10^{-2} vs 6.7×10^{-2}		
Serine		3.6×10^{-1} vs 2.9×10^{-1}	3.7×10^{-1} vs 2.5×10^{-1}		
Threonine			1.9×10^{-1} vs 2.4×10^{-1}	1.7×10^{-1} vs 1.3×10^{-1}	1.6×10^{-1} vs 1.3×10^{-1}
Tyrosine			1.1×10^{-1} vs 8.0×10^{-2}		

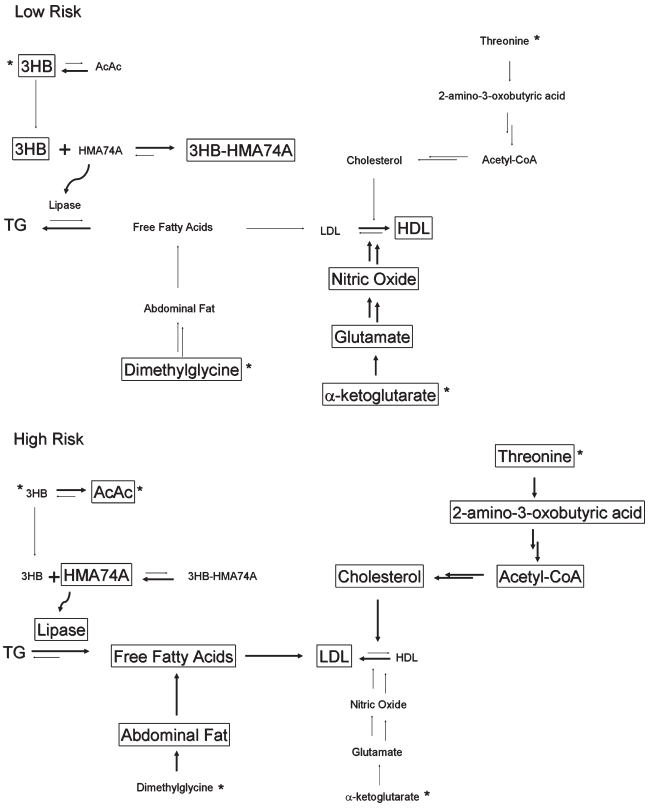

Figure 5. Metabolic fingerprint in low and high risk subjects (metabolites whose amount differs significantly between the low- and high-risk subjects are marked with an asterisk).

and in ketone bodies formation. A fine regulation balances threonine concentration under different conditions, and TDH is the target of selective feedback inhibition by all compounds derived from its major product, acetyl-CoA and, remarkably, by 3-HB.[56]

From the present data, it appears that subjects with high levels of triglycerides show low levels of plasma dimethylglycine. Dimethylglycine is a tertiary amino acid, which is an intermediary metabolite in the choline pathway, as it is formed in liver mitochondria by removal of one methyl group from betaine.[57] It can be metabolized in liver mitochondria, loosing both its methyl groups through transmethylation of tetrahydrofolate, and yielding free glycine.[58,59] A dietary supplement of dimethylglycine in chickens' diet was shown to reduce the deposition of dietary fat into abdominal depot tissue and the fasted nonesterified fatty acid plasma level significantly decreased with increasing dimethylglycine level.[60]

Although all the discussed metabolites can have a dietary origin, they are subjected to mitochondrial cycles that cannot be controlled only with diet. Moreover, the statistically significant differences observed between the investigated groups can be hardly explained with differences in the dietary habits.

Taken together, the results of the present study point to mitochondrial metabolic dysfunctions as a mechanism that may at least in part explain some of the metabolic features of subjects at high risk for CVD. A mitochondrial dysfunction is considered to be one of the major determinants of insulin resistance in adolescents with familial history of type-1 diabetes.[61] Moreover, an association between mitochondrial activity and CVDs is hypothesized by Shah et al.[33] These observation could be linked to our findings, thus moving back the starting point of obesity, insulin resistance, and of the risk of developing cardiovascular injury, to an alteration of the mitochondrial activity.

Although a full clarification of a so complex machinery is still far away, we may speculate that an imbalance in the mitochondrial redox potential (mainly related to oxidative stress), may be linked to an alteration of the fatty acids β-oxidation pathway with

an increased secretion of LDL lipoproteins in the blood. In addition, we can also hypothesize that this mechanism amplifies itself by the complementary action of the above-mentioned metabolites, as shown in Figure 5. Although the suggested mechanism is admittedly speculative, nevertheless it is based on experimentally observed metabolic alterations, all of which can be coherently linked to literature data.

CONCLUSION

In the present paper, we have presented a metabonomic approach to the analysis of the cardiovascular risk in a cohort of relatively healthy and young subjects.

We have found (by regression and classification analysis), that all the common blood analytes used for cardiovascular risk estimate correlate with NMR metabonomics. Moreover, subjects that are classified as at high or at low risk using these clinical markers can also be discriminated using NMR metabolomics. This discrimination is due to a complex NMR fingerprint that is composed of not only the common markers (total cholesterol, triglycerides, LDL, HDL), but also other metabolites previously not associated with CVD and that may be related to the biochemistry of cardiovascular risk.

Hopefully, in the future, novel cardiovascular scores could be formulated that are also based on the metabolic fingerprinting, or that at least consider the alteration of the metabonomic profile as an early predisposing condition. Therefore, the untargeted approach, that is, the unraveling of the whole disease-linked metabonome of a subject, can suggest targeted analyses of a small subset of metabolites that could be included in the standard clinical practice. At this stage, due to the lack of follow-up samples, we are not able to demonstrate a real improvement in risk assessment by the addition to the CVD risk panel of all or some of the new metabolites described in this work.

ASSOCIATED CONTENT

Supporting Information

Supplemental methods, tables, and figures as detailed in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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