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Metabolomics signature improves the prediction of cardiovascular events in elderly subjects

S. Rizza [a], M. Copetti [b], C. Rossi [c,d], M.A. Cianfarani [a], M. Zucchelli [c,d], A. Luzi [a], C. Pecchioli [a], O. Porzio [e], G. Di Cola [a], A. Urbani [d,e], F. Pellegrini [b], M. Federici [a,*]

[a] Department of Systems Medicine, University of Rome "Tor Vergata", 00133 Rome, Italy
[b] Unit of Biostatistics, IRCCS, "Casa Sollievo della Sofferenza" San Giovanni Rotondo, FG, Italy
[c] Department of Clinical and Experimental Sciences, G. D'Annunzio University, Chieti, Pescara, Italy
[d] Center of Excellence on Aging (Ce.S.I.), University Foundation, Chieti, Italy
[e] Department of Experimental Medicine and Surgery, University of Rome "Tor Vergata", Rome, Italy

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ABSTRACT

Aims: Age is one of the most important determinants of cardiovascular health, therefore the management of cardiovascular diseases (CVD) in elderly people entails great challenge. A possible explanation of vascular senescence process is the mitochondrial damage and dysfunction. We hypothesized that metabolomic profiling would identify biomarkers predicting major cardiovascular events (MACEs) in elderly people, improving the clinical standard cardiovascular risk factors.

Methods and results: Targeted-mass-spectrometry-based profiling of 49 metabolites was performed in a group of very old participants ($n = 67$, mean age $= 85 \pm 3$ years) with a high rate of previous CVD (68%). Principal Component Analysis, Random Survival Forest analysis and Cox proportional hazards regression modeling were used to evaluate the relation between the metabolite factors and recurring MACEs. We tested discrimination ability and reclassification of clinical and metabolomic models.

At follow-up (median = 3.5 years), 17 MACEs occurred (5 cardiovascular deaths, 1 nonfatal myocardial infarction, 7 nonfatal strokes and 4 peripheral artery surgeries) (incidence = 7.3% person-years). Metabolite factor 1, composed by medium- and long-chain acylcarnitines, and factor 7 (alanine) were independently associated with MACEs, after adjustment for clinical CV covariates [HR = 1.77 (95% CI = 1.11–2.81, $p = 0.016$) and HR = 2.18 (95%CI = 1.17–4.07, $p = 0.014$), respectively]. However, only factor 1 significantly increases the prediction accuracy of the Framingham *Recurring-Coronary-Heart-Disease-Score*, with a significant improvement in discrimination (integrated discrimination improvement = 7%, $p = 0.01$) and correctly reclassifying 41% of events and 37% of non-events resulting in a cNRI = 0.79 ($p = 0.005$).

Conclusions: Aging mitochondrial dysfunction evaluated by metabolomic profiling is associated with MACEs, independently of standard predictors.

1. Introduction

Aging is a very complex process because many transformations happen to the human organisms, leading to a wide variety of altered functions. Very often this process is accompanied by several disabilities and chronic diseases such as Parkinson's and Alzheimer disease as well as cardiometabolic disturbances including diabetes and atherosclerosis [1]. Even though most of these clinical manifestations are well known, the complex biomolecular networks contributing to aging are only beginning to be uncovered. Genetic and environmental influences seem to be involved, but additional experimental approaches are needed to understand aging mechanisms [2].

Cardiovascular disease (CVD) is expected to remain the leading cause of death and disability in the USA and worldwide [2]. One of the most important determinants of cardiovascular health is aging. By 2030, approximately 20% of the world population will be aged 65 or older [3]. In this age group, CVD results in 40% of all deaths and rank as the leading cause [3]. Hence, it remains vital to understand why age is such a critical component of CVD etiology. Moreover, directing advanced therapies to elderly patients who are most likely to benefit from them will require advances in risk stratification [3].

* Corresponding author. Tel.: +39 0672596889; fax: +39 0672596890.
E-mail address: federicm@uniroma2.it (M. Federici).

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Metabolomics is a key technology of modern systems biology that focuses on obtaining an integral depiction of the current metabolic status of an organism, associated with physiological and pathophysiological processes [4,5]. Metabolomics is therefore a valuable tool for investigating in a single approach all the various ways by which metabolism is influenced, thus a very promising tool for capturing the complexity of the aging process. Therefore, the development of this technology could help in the search of novel biomarkers for the relationship between aging and CVD.

The aim of the current study is to characterize the metabolic profile of a small population of elderly patients followed for about 42 months and consequently creates an integrated clinical-metabolomic model which could improve the prediction of incident cardiovascular events in aged people.

2. Methods

2.1. Baseline study population

The baseline study population has been previously described [6]. Briefly, it is composed by one-hundred-eight subjects (mean age 77.3 ± 3.5), enrolled from the outpatient geriatric ambulatory clinic of the Department of Medicine at Policlinico "Tor Vergata". All patients were Italian and living in central Italy in or around Rome and were referred to the center for metabolic diseases or CVD. Fifty patients (46.3%) had a well-documented history of coronary heart disease whereas nine (8.3%) suffered from a precedent transient ischemic attack or ischemic stroke. None had a diagnosis of chronic renal or heart failure. A local ethics committee approved the study, and the reported investigations were conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000. All patients gave written consent to participate in the study.

All the patients had a routine follow-up at six months and one year after inclusion, then yearly thereafter. Follow-up included the development of one major cardiovascular event (MACE) such as nonfatal stroke, nonfatal myocardial infarction, peripheral vascular surgical procedures and cardiovascular death.

Since the study patients had a high rate of previous history of CHD (68%), we used the *Recurring Coronary Heart Disease Score* [7] to calculate the cardiovascular risk of a recurring cardio-vascular event (RCHD score). To this model we add the BMI (RCDH score + BMI) because of "obesity paradox" typical of elderly people [8].

2.2. Laboratory methods

As previously described [9,10], using a targeted mass spectrometry-based study we determined the serum levels of 18 amino acids, free carnitine and 30 acylcarnitines. Serum samples were stored at −80 °C until analysis. Serum samples (3.2 μL) were transferred into 1.5 mL tubes (Eppendorf, Hamburg, Germany) and then extracted with a solution of methanol/water (75:25, v:v) and 0.01% oxalic acid (100 μL) containing the stable isotope labeled internal standards. The stable isotope labeled internal standards as well as the extraction solution were obtained from the NeoBase Non-derivatized MSMS Kit (Perkin Elmer Life and Analytical Sciences, Turku, Finland). The tubes were then capped and vortex mixed. The samples were centrifuged (15,600 rpm at 4 °C for 15 min), and the supernatant was analyzed by direct infusion mass spectrometry (DIMS). A quality control (QC) serum pool was prepared from the serum patient samples, then extracted and analyzed as described for the serum samples in study. A total of 10 QC serum pool were analyzed during the run. Method accuracy was assessed for each analyte, precision being evaluated as repeatability in terms of coefficient of variation (CV) for the QC samples. The calculated mean CV for the amino acids and acylcarnitines was 9.5% and 9.0%, respectively. The DIMS analysis for the evaluation of metabolite profile, in serum samples was performed using a Liquid Chromatography-tandem Mass Spectrometry (LC/MS/MS) system consisting of an Alliance HT 2795 HPLC Separation Module coupled to a Quattro Ultima Pt ESI tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The instrument operated in positive electrospray ionization mode using MassLynx V4.0 Software (Waters) with auto data processing by NeoLynx (Waters Corporation, Milford, MA, USA) [9].

2.3. Statistical methods

Principal Component Analysis (PCA) was used to reduce the large number of correlated metabolites to a smaller number of uncorrelated factors. Varimax rotation [11] was used to identify significant factors: only factors with an eigenvalue of ≥1.5 were considered. For each factor, we computed the metabolomic factor scores as the sum of standardized metabolites, weighted on their factor loadings, for each patient. We reported metabolites with a factor load ≥0.60 as component of that given factor. We chose, conservatively, a large cut-off to avoid false discoveries. Univariable and multivariables time to event analysis was performed using the Cox proportional hazards model. Risks were reported as hazard ratios (HR) along their 95% confidence interval (95%CI).

To validate the PCA and time to event analyses results we performed a Random Survival Forest (RSF) analysis [12], a powerful machine learning statistical algorithm which is the appropriate framework to validate results from small sized samples. Then, we used PCA factors as covariates and time to MACE as the outcome. Therefore, we computed the ranking of PCA factors based on RSF variable importance to validate our results.

Furthermore, since the RCHD score is a well established risk score, we assessed if the addition of metabolomic factors with the highest importance could improve its discriminative power.

Models' calibration, i.e. the agreement between observed outcomes and predictions, was assessed using the survival-based Hosmer–Lemeshow (HL) goodness-of-fit test [13]. Models' discrimination, i.e. the ability to distinguish subjects who will develop an event from those who will not, was assessed by computing the modified C-statistic for censored survival data [14] and the Integrated Discrimination Improvement (IDI) [14]. Relative IDI (RIDI) was also reported.

Reclassification improvement offered by metabolomic factors was quantified using the survival-based continuous net reclassification index (cNRI) following the KM approach [15]. The time horizon of risk prediction was set to 4 years.

3. Results

3.1. Characteristics of the metabolomic-study population

Patients were followed until 4 years from enrollment. Seven (6%) patients dropped out of the study within the second and two (2%) within the third year. Ninth-nine out of one-hundred-eight enrolled patients completed the follow-up. For sixty-seven of them a metabolomic profiling composed by 18 amino acids, free carnitine and 30 acylcarnitines was available. The selection of this subpopulation was random. As compared to the original population, this subpopulation was significantly older (85 ± 3 vs 77.3 ± 3 y, $p = 0.01$) and with a lower rate of diabetes (39% vs 53%, $p < 0.01$) but no other differences were observed. The baseline clinical characteristics of study metabolomic patients are listed in Table 1.

During follow-up (mean 42 ± 13 months, range 5–63 months), 17 major adverse cardiovascular events (MACEs) occurred: 5

Table 1
Baseline clinical characteristics of study metabolomic patients.

Characteristic	Overall (n = 67)	No event subjects (n = 50)	Event subjects (n = 17)	p-Value
Age (y)	85.3 ± 3.2	86.6 ± 4.0	84.2 ± 3.6	0.78
Gender (% female)	29 (43)	23 (46)	6 (35)	0.44
Current/ex smokers (%)	63 (94)	50 (100)	13 (76)	0.01
BMI (kg/m^2)	27.4 ± 4.1	27.8 ± 4.4	26.0 ± 2.9	0.09
Diabetes (%)	26 (39)	18 (36)	8 (47)	0.42
Hypertension (%)	65 (97)	48 (96)	17 (100)	0.40
Hypertension duration (y)	22 ± 15	21 ± 14	24 ± 18	0.47
Hypertension stages (1/2/3)[a]	40/27/0	31/19/0	10/7/0	0.33
Previous CVD (%)	48 (68)	37 (74)	11 (64)	0.32
Systolic press (mmHg)	138.7 ± 16.3	138.8 ± 14.6	138.5 ± 20.9	0.87
Diastolic press (mmHg)	79.9 ± 9.5	80.3 ± 9.2	78.8 ± 10.5	0.52
A1c (%)	5.8 ± 0.7	5.8 ± 0.6	5.9 ± 0.8	0.68
Tot cholesterol (mg/dl)	190.3 ± 39.7	194.6 ± 36.2	183.4 ± 45.2	0.46
HDL cholesterol (mg/dl)	51.7 ± 14.1	51.4 ± 12.9	52.4 ± 17.5	0.90
LDL cholesterol (mg/dl)	123.2 ± 34.9	124.6 ± 36.1	119.0 ± 31.9	0.50
Triglycerides (mg/dl)	124.7 ± 57.4	125.8 ± 57.1	121.6 ± 59.6	0.75
C-RP (mg/l)	4.2 ± 4.8	3.8 ± 4.5	5.3 ± 5.4	0.45

Data presented as mean ± standard deviation or numbers, with percentage in parentheses. BMI, body mass index; C-RP, C-reactive protein; CVD, cardiovascular disease.
[a] Hypertension stages: 1 = prehypertension, 2 = hypertension stage 1, 3 = hypertension stage 2.

cardiovascular deaths, 1 nonfatal MI, 7 nonfatal strokes and 4 peripheral artery surgeries were observed (incidence of MACEs was 7.3% person-years). Table 2 appendix online shows pharmacological therapies of metabolomic population according to the presence of MACEs.

3.2. Association of individual metabolites with cardiovascular events

Baseline characteristics of the followed-up study population according to event or no event are displayed in Table 1. Patients who experienced an event during the follow-up were less frequently smokers or ex-smokers as compared to no event individuals ($p = 0.01$) whereas no other differences were observed between the two groups. In adjusted analysis, controlled for smoke, diabetes, total and HDL cholesterol, SBP, age, sex and BMI, the levels of some medium- and long-chain acylcarnitines including decadienoylcarnitine C10:2 ($p = 0.02$), myristoylcarnitine C14 ($p = 0.02$), palmitoylcarnitine C16 ($p = 0.02$) and oleylcarnitine C18:1 ($p = 0.05$) were different between event and no event subjects whereas no significant differences were observed among the amino acids between the two groups (Table 1 appendix online).

3.3. Unbiased PCA

Principal Component Analysis identified 7 meaningful metabolomic factors (Table 2).

In multivariable analyses (Table 3), adjusting for clinical covariates such as age, systolic blood pressure, smoking, total and HDL cholesterol, diabetes diagnosis and BMI, factor 1 (medium- and long-chain acylcarnitines) and factor 7 (alanine) remained as independent predictors of MACEs with an HR = 1.77 (95%CI = 1.11–2.81, $p = 0.016$) and HR = 2.18 (95%CI = 1.17–4.07, $p = 0.014$), respectively.

Survival Random Forest analysis validated these findings since factor 7 and factor 1 resulted (Fig. 1) as the most important predictors of time to MACE after building 100,000 trees with bootstrap sub-samplings.

Table 2
Principal Component Analysis.

Factor	Description	Components	Eigenvalue	Variance
1	Medium–long chain acylcarnitines	C2, C6, C8, C10, C10:1, C12, C12:1, C14, C14:1, C14:2, C16, C16:1, C18:1, C18:2	12.78	0.26
2	Amino acids/short chain acylcarnitine	Pro, C5, Val, Leu/Ile/Pro-OH	6.6	0.13
3	Amino acids	Asn, Orn	4.22	0.09
4	Amino acids/short chain acylcarnitine	C2, Glu	2.57	0.05
5	Amino acids	Tyr	2.34	0.05
6	Amino acids/long chain acylcarnitines	Asn, C18:OH	1.85	0.04
7	Amino acids	Ala	1.72	0.04

The table lists the 7 metabolomic factors identified by Principal Component Analysis and the associated individual components, description, eigenvalue and variance. Pro, proline; Val, valine; Leu/Ile/Pro-OH, leucine/isoleucine/hydroxyproline; Asn, asparagine; Orn, ornithine; Glu, glutamic acid; Tyr, tyrosine; Ala, alanine.

Then, we tested if the addition of metabolomic factors 1 and 7 to the model including the "RCHD score + BMI" could improve the 4-year prediction of MACEs. We found that only factor 1 significantly increases the prediction accuracy. The survival C-index of "RCHD score + BMI" was 0.70 (95%CI = 0.59–0.81) and such a model resulted calibrated (HL, $p = 0.94$). Adding the metabolomic factor 1 into this model, survival C-index increased up to 0.75 (95% CI = 0.64–0.86) and we found a statistically significant improvement in discrimination evaluated by IDI = 7% (95%CI = 1%–6%, $p = 0.01$) and RIDI = 67% (95%CI = 6%–156%, $p = 0.01$). Moreover, the introduction of factor 1 into the model correctly reclassified 41% of events and 37% of non-events resulting in a cNRI = 0.79 (95% CI = 0.17–1.36, $p = 0.005$).

4. Discussion

In this study we found that a particular baseline metabolomic profile independently predicts cardiovascular events in very old individuals after adjustment for established clinical cardiovascular risk factors included in the *Recurring Coronary Heart Disease Score* (RCHD score) [7]. RCHD algorithm stems from the Framingham Heart Study and it is a useful tool for the CVD risk appraisal in individuals who have had a prior CHD event or stroke. In our study, we added BMI to the well established variables included in RCHD; in fact, our population is composed by elderly people in which higher BMI is associated with lower mortality when compared to normal weight subjects, a fact commonly known as the "obesity paradox" [8]. To gain insight into the nature of the CV risk in elderly people we used a metabolomic assay to map amino acids and acylcarnitines. Both these metabolites could give information on future development of some chronic metabolic diseases or frailty in

Table 3
Univariable and multivariable association of metabolomic factors with cardiovascular event.

Factor	Univariable		Multivariable[a]	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
1	1.49 (0.95–2.34)	0.08	1.77 (1.11–2.81)	0.02
2	1.01 (0.65–1.57)	0.97	1.10 (0.63–1.92)	0.73
3	1.42 (0.88–2.30)	0.15	1.54 (0.85–2.77)	0.15
4	0.97 (0.56–1.66)	0.90	0.75 (0.40–1.40)	0.37
5	1.45 (0.93–2.27)	0.10	1.67 (0.97–2.87)	0.06
6	0.90 (0.57–1.43)	0.67	0.94 (0.53–1.64)	0.82
7	2.03 (1.19–3.46)	0.01	2.18 (1.17–4.07)	0.01

[a] Multivariable association adjusted for age, gender, smoke, systolic blood pressure, total- and HDL-cholesterol, diabetes diagnosis.

Variable	Importance	Rank
Factor 7	0.162	1
Factor 1	0.057	2
Factor 5	0.021	3
Factor 4	0.00	4
Factor 8	-0.006	5
Factor 9	-0.006	6
Factor 10	-0.006	7
Factor 3	-0.007	8
Factor 6	-0.010	9
Factor 2	-0.022	10

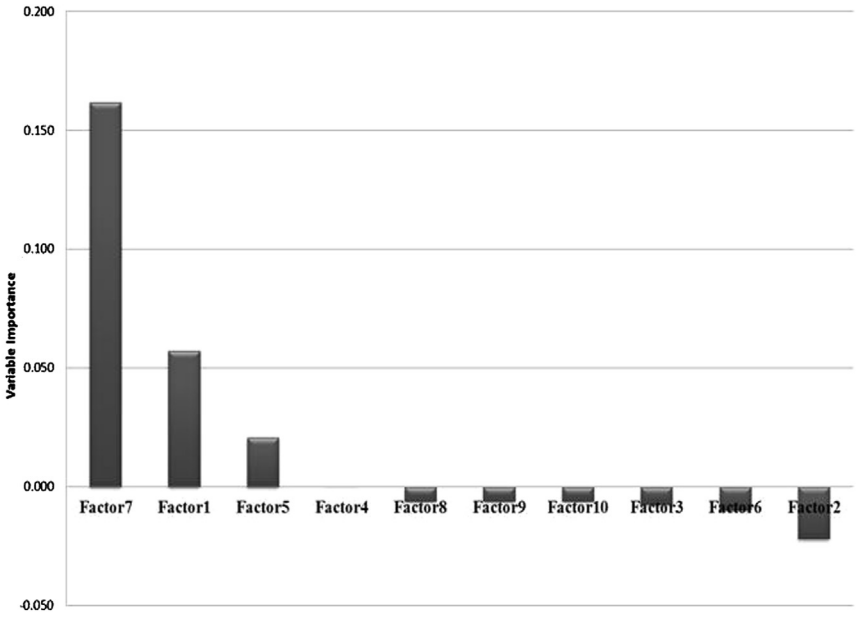

Fig. 1. Principal Component Analysis factors importance estimated using Random Survival Forest Analysis. Variables with the highest variable importance values are the best predictors of MACEs.

aged people, in particular branched chain and aromatic amino acids emerged as predictors of future development of diabetes [16] as well as a cluster of medium and long-chain acylcarnitines were able to identify a group of elderly men at high risk due to a lower measure of physical performance [17].

Vascular senescence results in increased arterial thickening and stiffness both contributing to a variety of age-related pathologies, such as hypertension, ischemia and age-related macular degeneration. However, the extent to which metabolites resulting from a mitochondrial dysfunction might directly contribute to CVD in aged humans is still unclear [18,19].

Our findings revealed that a metabolomics signature, mainly constituted by medium- and long-chain acylcarnitines, which are intermediates of fatty acid oxidation, significantly adds an incremental discriminative capability for recurring CVD events. Given the uncertainty about risk management in very old subjects our findings could help to design new ad hoc intervention studies to test the correction of specific metabolic pathways in older people [20,21].

Actually, accumulation of acylcarnitines may be indicative of inefficient β-oxidation and mitochondrial dysfunction and recently, elevation of acylcarnitines and branched chain amino acids has been shown to act as a biomarker of insulin resistance and of CVD [22,23].

In a recent report including a large cohort of subjects aged from 54 to 70 y old enrolled from the CATHGEN biorepository the authors identified some metabolic profiles composed of long chain dicarboxylacylcarnitines, medium-chain acylcarnitines, and fatty acids that independently predict future CV events [4]. Similarly, Houtkooper RH and colleagues identified a factor composed by medium and long chain acylcarnitines as discriminating predictor for aging in mice [24]. Our Principal Component Analysis revealed that medium and long chain acylcarnitines are associated to CV events in old subjects suggesting that these specific metabolites could have a biomarker role to be tested in larger cohorts. We think that among the wide variety of molecular and metabolic mechanisms which may happen during the aging process, the reduced entry in and flux through the mitochondrial fatty acid oxidation pathway should reflect a mitochondrial dysfunction typical of aged tissues including vasculature. Notably, mitochondrial dysfunction can result in the altered ability of the cell to generate energy, and can also alter redox signaling and a variety of important functions regulated by mitochondrial oxidant generation and response [25,26]. Therefore, during advanced age the mitochondrial production of ROS may significantly enhance both in the heart and in the vasculature increasing the vascular inflammation and contributing to alterations in the composition of the plaque and to its restenosis or rupture. Thus, age-dependent mitochondrial dysfunction is closely correlated with abnormal mitochondrial ROS production which in turn increases CVD risk. The impaired flux of medium- and long-chain acylcarnitines might be the metabolomic footprint of mitochondrial dysfunction in elderly people who more likely will have a cardiovascular event. It is well established that caloric restriction (CR) is the most robust and efficient intervention for both increasing lifespan and improving healthspan across a wide range of species including humans [27]. This potent property has been confirmed by some epidemiological studies which have supported the relationship between CR and the reduction of chronic disease risk factors including CVD [28]. Accordingly, De Guzman JM and colleagues reported that CR is able to significantly modify fatty acids metabolism in aging mice [29]. Another possible strategy to revert the mitochondrial function should be the use of some anti-ischemic drugs such as trimetazidine which mainly acts by affecting the fatty acid oxidation and restoring ATP production by shifting the energy substrate reference from fatty acid oxidation to glucose oxidation [21].

The main limitation of this study is the small number of participants and we acknowledge that the significance level of our findings is compatible with preliminary results. Therefore, our findings need further replication in larger samples before they can be considered as established. However, to increase our confidence in the results we carried out the Survival Random Forest analysis because it represents a validated powerful tool aimed at strengthening results from small sized samples [30]. Furthermore, since the main finding of our report composed by medium- and long-acylcarnitines has been already associated with cardiovascular events in other subset of patients [4], and in our population was able to still improve a well established cardiovascular risk

algorithm such as RCHD, we think that this metabolomic profile is very likely involved in aging progress of the vasculature and deserves carefully attention.

In summary, we suggest that medium- and long-chain acylcarnitines are independently associated with occurrence of subsequent cardiovascular events. This metabolomic profile, improving cardiovascular risk stratification, points toward potential novel pathophysiological mechanisms of vascular senescence in elderly subjects.

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All the authors declare to have no financial and non-financial conflict of interest regarding this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.10.029.

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