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Metabolomic correlates of coronary atherosclerosis, cardiovascular risk, both or neither. Results of the 2 × 2 phenotypic CAPIRE study

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ABSTRACT

Background: Traditional cardiovascular risk factors (RFs) and coronary artery disease (CAD) do not always run parallel. We investigated functional-metabolic correlations of CAD, RFs, or neither in the CAPIRE (Coronary Atherosclerosis in Outlier Subjects: Protective and Novel Individual Risk Factors Evaluation) 2 × 2 phenotypic observational study.

Methods: Two hundred and fortyone subjects were included based on RF burden, presence/absence of CAD (assessed by computed tomography angiography), age and sex. Participants displayed one of four phenotypes: CAD with ≥3 RFs, no-CAD with ≥3 RFs, CAD with ≤1 RF and no-CAD with ≤1 RF. Metabolites were identified by gas chromatography–mass spectrometry and pathways by metabolite set enrichment analysis.

Results: Characteristic patterns and specific pathways emerged for each phenotypic group: *amino sugars* for CAD/high-RF; *urea cycle* for no-CAD/high-RF; *glutathione* for CAD/low-RF; glycine and serine for no-CAD/low-RF. Presence of CAD correlated with *ammonia recycling*; absence of CAD with the *transfer of acetyl groups into mitochondria*; high-risk profile with *alanine metabolism* (all $p < 0.05$).

The comparative case-control analyses showed a statistically significant difference for the two pathways of phenylalanine, *tyrosine and tryptophan biosynthesis* and *phenylalanine metabolism* in the CAD/Low-RF vs NoCAD/Low-RF comparison.

Conclusions: The present 2 × 2 observational study identified specific metabolic pathways for each of the four phenotypes, providing novel functional insights, particularly on CAD with low RF profiles and on the absence of CAD despite high-risk factor profiles.

1. Introduction

Atherosclerosis is currently the leading cause of death and disability worldwide. Its primary expression is coronary artery disease (CAD), accounting for over 8 million deaths per year [1]. Symptoms and consequences of CAD depend on location, extent, stability of lesions, as well as collateral circulation and myocardial responsivity. The presence of CAD is a significant marker and harbinger of adverse cardiovascular (CV) events. Although traditional CV risk factors (RFs) for atherosclerosis (hyperlipidemia, diabetes mellitus, smoking and hypertension) have been identified [2–7], considerable evidence shows that the local microenvironment, determined by arterial mechanics, matrix remodelling, lipid deposition and vasoactive metabolites, plays a crucial role in the individual susceptibility to plaque development and progression [8,9]. Accordingly, people with an unfavourable risk profile do not constantly develop CAD, while others, classified at low or intermediate risk based on the aforementioned traditional RFs, may develop CAD. These

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findings suggest mechanisms acting independently of CVRFs or that modulate the local vascular response to systemic CVRFs. Although recent basic and translational research has analysed the molecular mechanisms associated with the onset of atherosclerosis, the exact modalities governing the transition from CVRFs to plaque lesion remain unclear.

To gain insight into the complex relationship between traditional CVRFs and CAD development, the CAPIRE (Coronary Atherosclerosis in Outlier Subjects: Protective and Novel Individual Risk Factors Evaluation) 2 × 2 phenotypic project compares imaging and biochemical variables across four mutually exclusive phenotypes, defined by presence/absence of RFs and CAD [10]. Metabolomics is a recent tool that identifies and quantifies large numbers of metabolites in biological samples, providing direct functional information and potentially leading to the translation of metabolic profiles into personalised therapeutic strategies [11–14]. The present substudy of CAPIRE investigated whether plasma metabolic pathways might provide insights on unexpected relations between traditional RFs and CAD.

2. Materials and methods

Consecutive subjects referred for coronary computed tomography angiography (CCTA) were screened. Coronary atherosclerotic plaque was defined as any discernible structure assigned to the coronary artery wall and discriminated from surrounding pericardial tissue and epicardial fat. Coronary arteries were defined as normal if no atherosclerotic plaque (including focal and eccentric calcified plaques) was detected in any segment within the vessel wall or lumen. A five-coronary-segment involvement cut-off defined diffuse CAD [10].

The authors assessed CV risk using different scores (Framingham Risk Score, SCORE, Pooled Cohort Equations). Definitions were: family history as a history of CAD in first-degree relatives, with onset <55 years for men and < 65 years for women; arterial hypertension as a history of hypertension, ongoing antihypertensive treatment, or recent blood pressure > 140/90 mmHg; hypercholesterolemia as total cholesterol >200 mg/dL or < 200 mg/dL with lipid-lowering medications; diabetes mellitus as fasting plasma glucose >126 mg/dL, two-hour oral glucose tolerance test of ≥200 mg/dL, isolated glycated haemoglobin ≥6.5%, or current use of insulin or oral hypoglycemic agents; smoking as current cigarette smoking or abstention <1 year.

The choice of risk cut-offs for the different populations was based on the number of RFs according to data reported in the literature; patients without any RFs or one single RF belong to a risk group <10% of events at ten years according to Framingham Study, whereas patients with three or more RFs belong to a risk group >20% of events at ten years. Subsequently, we used these cut-offs to assign the patients to the Low- and High-RF Groups.

A peripheral venous blood sample was collected from the whole study population; over 80% of patients were fasting, and, in this regard, there were no differences between the four groups. The samples were treated and stored in a freezer at −70 °C. Samples of each subject enrolled in the study were collected in a single dedicated biological bank (SATURNE-1; Mario Negri Institute of Pharmacological Research, Milan, Italy) [10].

2.1. Study population

The original CAPIRE study included 544 patients. The present analysis comprised 241 subjects, based on rigorous criteria of age (± 5 years), sex (1:1 for case: control Groups), presence/absence of CVRFs and CAD, falling into four predefined groups (Table 1): CAD/High-RF, i.e. with CAD in >5/16 segments according to the AHA classification and ≥3 CVRFs (case); No-CAD/High-RF, i.e. without CAD but ≥3 CVRFs (control); CAD/Low-RF, i.e. with CAD in >5/16 segments according to the AHA classification and ≤1 CVRF, excluding type 1 or type 2 diabetes mellitus as single RF (case); No-CAD/Low-RF, i.e. without CAD and ≤1 CVRF, excluding type 1 or type 2 diabetes mellitus as single RF (control). Table 2 reported the drugs taken by the enrolled patients; the only significant differences concern, as by protocol, the number of subjects without therapy and those taking ASA in the Case-Control High-Risk Groups (CAD/High-RF vs No-CAD/High-RF).

Given the high risk associated with diabetes mellitus, the authors discarded the latter as a single risk factor for the low RF groups. The Ethics Committee of each participating centre approved the study, which was performed according to the Helsinki Declaration. Written informed consent was obtained before inclusion.

2.2. Untargeted gas chromatography–mass spectrometry (GC–MS) metabolomic analysis

Sample preparation. The procedure followed a modified version of the one by Dunn et al., [15]. Plasma was collected in EDTA and stored at −80 °C. Thawed plasma (400 μL) was treated with cold methanol (1200 μL), mixed and centrifuged for 15 min at 14000 rpm (16.9 G). Supernatants (400 μL) were evaporated to dryness overnight in Eppendorf vacuum centrifuges. A solution of methoxylamine hydrochloride (0.24 M, 20 mg/mL) in pyridine (50 μL) was added, and samples mixed and left to react for 17 h at room temperature. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (50 μL) was then added and left to react for one h at room temperature. The derivatised samples were diluted with hexane (100 μL) and tetracosane (0.01 mg/mL) as the internal standard, just before GC–MS analysis.

GC–MS analysis. Samples were analysed using an Agilent 5975C system, interfaced to the GC 7820 and equipped with a DB-5ms J&W column (Agilent Technologies, Palo Alto, CA, USA). Injector temperature was 230 °C, detector temperature 280 °C, and helium carrier gas flow 1 mL/min. The GC program was set at 90 °C initial temperature with 1 min hold time, ramping at 10 °C/min to a final temperature of 270 °C with 7 min hold time. The derivatised sample (1 μL) was injected in split (1:5) mode. After a solvent delay of 3 min, mass spectra were acquired in full scan mode using 2.28 scans/s with a mass range of 50–700 Amu (atomic mass units). Each acquired chromatogram was analysed using the free software AMDIS (Automated Mass Spectral Deconvolution and Identification System, http://chemdata.nist.gov/mass-spc/amdis), which identified each peak by comparison of the relative mass spectra and retention times with those stored in an in-house library comprising 255 metabolites. Other metabolites were identified using NIST08 (National Institute of Standards and Technology's mass spectral database) and the Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/). Through this approach, 113 compounds were accurately identified, while 28 other metabolites were tentatively assigned, relying on the GMD and NIST libraries. AMDIS analysis produced an Excel datasheet that underwent chemometric analysis.

Statistical analysis. Principal Component Analysis (PCA) and orthogonal partial least square-discriminant analysis (OPLS-DA) were conducted on SIMCA P+ 13 software (Umetrics, Umea, Sweden) and used to observe data variance in unsupervised and supervised modes. Data analysis was preceded by Pareto scaling, which reduced the relative importance of large values, maintaining the overall data structure [16]. The quality of the model was described by cumulative modelled variation in the X matrix R^2_X, cumulative modelled variation in the Y matrix R^2_Y, and cross-validated predictive ability Q^2 values. A cross-validation analysis of variance (ANOVA) was applied to assess the significance of the model. Discriminant metabolites, identified through variable importance in projection scores from the OPLS-DA, were considered specific for differences among phenotypic groups. Metabolic pathways were determined by metabolite set enrichment analysis (MSEA) using MetaboAnalyst 3.0 and graphically ranked by decreasing *p*-values. The

Table 1
Demographic, Anthropometric and clinical data of the study population described as per 2 × 2 case-control protocol.

	CAD/High-RF (Case, $N = 56$)	No-CAD/High-RF (Control, $N = 56$)	CAD/Low-RF (Case, $N = 64$)	No-CAD/Low-RF (Control, $N = 65$)
Age	61.8 ± 6.8	60.9 ± 7.5	62.5 ± 7.4	61.5 ± 8.4
M/F	34/22	34/22	56/8*	56/9*
Height (cm)	167.95 ± 8.75	167.61 ± 8.53	172.65 ± 8.23*	172.55 ± 7.58*
Weight (kg)	79.40 ± 15.08	76.98 ± 13.68	81.51 ± 17.5	75.86 ± 10.72#
BMI (Kg/m^2)	22.59 ± 5.51	21.95 ± 5.66	24.70 ± 7.45#	22.82 ± 4.69
Abdominal circumference (cm)	100.04 ± 12.90	97.17 ± 11.60	99.41 ± 11.71	95.56 ± 9.10§,†
CAD family history	34/56	34/56	7/64*	7/65*
Hypertension	52/56	53/56	26/64*	17/65*
Hypercholesterolemia	54/56	52/56	14/64*	18/65*
Diabetes Mellitus	20/56	17/56	0/64*	0/65*
Tobacco	33/56	24/56	7/64*	4/65*
No CV-RFs	0/56	0/56	10/64**	19/65*

Data are reported as mean ± SD for continuous variables and as numbers of affected/Group sample.
* $p < 0.001$ vs CAD/High-RF and No-CAD/High/RF;
** $p < 0.002$ vs CAD/High-RF and No-CAD/High/RF;
# $p < 0.05$ vs CAD/Low-RF;
§ $p < 0.05$ vs CAD/Low-RF;
† $p < 0.05$ vs CAD/High-RF.

Table 2
Drugs use in study population described as per 2 × 2 case-control protocol. Data are reported as numbers of affected/Group sample.

	CAD/High-RF (Case, $N = 56$)	No-CAD/High-RF (Control, $N = 56$)	CAD/Low-RF (Case, $N = 64$)	No-CAD/Low-RF (Control, $N = 65$)
No Therapy	11/56	24/56*	19/64	15/65
β-Blockers	10/56	16/56	16/64	19/65
ACE-Inhibitors	15/56	10/56	15/64	14/65
ARBs	8/56	13/56	10/64	14/65
CCB - Dihydropyridines	5/51	3/53	5/64	5/65
CCB – No Dihydropyridines	2/54	2/54	6/64	7/65
Diuretics	7/56	7/56	7/64	7/65
Potassium-sparing diuretics	0/56	0/56	0/64	0/65
Other antihypertensive drugs	0/56	0/56	1/64	2/65
Antiarrhythmic drugs	0/56	1/56	0/64	0/65
ASA	22/56	11/56**	17/64	17/65
Clopidogrel	1/56	0/56	1/64	0/65
Statins	21/56	13/56	24/64	20/65
Others hypolipidemics drugs	1/56	5/56	1/64	1/65
Insulin	0/56	0/56	4/64	0/65
Other hypoglycemic drugs	5/56	7/56	7/64	7/65
Allopurinol	0/56	0/56	2/64	1/65

* $p < 0.01$ vs CAD/High-RF;
** $p < 0.05$ vs CAD/High-RF.

False Discovery Rate (FDR) correction was implemented, and an adjusted p-value <0.05 was considered statistically significant.

3. Results

A cross-validation ANOVA test was performed to validate the OPLS-DA models, with resulting p values indicating statistical validity (Table 3). PCA and OPLS-DA score plots of all samples, taken together, did not show outlier values (data not shown), nor did they yield any spontaneous separation among groups. In contrast, case-control pairwise analyses led to identifying different patterns of metabolites, as shown in Fig. 1 (panels A–B). The R^2 and Q^2 values of the corresponding analyses are reported in Table 3. Clear clustering was seen comparing the two case-control sets, suggesting different metabolic profiles and pathways involved.

Variable importance in projection scores allowed further identification of the metabolites responsible for separation among the phenotypic groups. Metabolic patterns, quantified in terms of abundance, were further investigated by metabolite set enrichment analysis, thus providing information on metabolic pathways produced by metabolite interdependence. Over ten pathways were identified as distinct by metabolite set enrichment analysis (Table 4). The five most abundant pathways in each phenotypic group, ranked by p values, are shown in Table 4. Pathways shared by three or more phenotypes were attributed to general metabolism rather than to a specific clinical condition; we, therefore, focused on pathways specifically identifying each phenotypic group, i.e. the presence/absence of CAD or presence/absence of CVRFs.

Unique to the CAD/High-RF group was an amino-sugar pathway that included fructose and cytidine. At the other extreme, unique to the No-CAD/Low-RF group was the amino acid metabolism pathway involving glycine and serine. Glutathione metabolism was unique to CAD/Low-RFs, whereas the urea cycle was specific to No-CAD/High RF. Transfer of acetyl groups into mitochondria characterised the absence of CAD, whereas ammonia recycling was typical of the presence of CAD (Table 3). The alanine pathway was found in the high RF groups. Glycerolipid metabolism, glucose-alanine cycle and methionine metabolism were identified in three phenotypic groups (Table 4).

Moreover, we performed MSEA relative to the two comparative case-control analyses (Figs. 2 and 3). Although both the OPLS-DA results were statistically significant, MSEA showed corrected p-values <0.05 only for the two pathways of phenylalanine, tyrosine and tryptophan biosynthesis and phenylalanine metabolism in the CAD/Low-RF vs NoCAD/Low-RF case-control comparison.

Table 3
Parameters of the OPLS-DA models derived from the GC–MS spectra of plasma samples and relative p-values evaluated by ANOVA.

Multivariate statistical models	CAD/Low-RF vs No-CAD/Low-RF	No-CAD/High-RF vs CAD/High-RF
R^2X	0.454	0.417
R^2Y	0.449	0.744
Q^2	0.313	0.271
p-value (CV-ANOVA)	0.0008	0.013

CAD = coronary artery disease; CV-ANOVA = cross-validation analysis of variance; GC–MS = gas chromatography–mass spectrometry; OPLS-DA = orthogonal partial least squares discriminant analysis; RF = risk factor profile.

4. Discussion

It is commonly accepted that individuals with medium-high CV risk develop clinical ischemic syndrome when coronary atherosclerosis reaches a critical threshold. Not all subjects, however, follow this course. We focused on two extreme subpopulations: patients with diffuse CAD despite a low CV risk profile and subjects without CCTA-based CAD despite the presence of multiple CVRFs. Two conventional groups – one positive for both CAD and CVRFs and the other negative for both CAD and RFs – were included for comparison. Unique metabolic profiles emerged for each of the four clinical phenotypes, suggesting specific metabolic milieus associated with CAD (particularly with low RF burden) or, conversely, with the absence of CAD (particularly despite high-risk profiles). To identify metabolic profiles, we used the most advanced MS chromatography technology in terms of stability and reproducibility and multiple databases containing information on over 200.000 compounds [17]. Metabolite set enrichment analysis was applied to cluster the metabolomic profiles around known metabolic pathways. Of note, there was a high degree of commonality across the identified pathways. A glutamate signal, for instance, was found in seven of ten pathways (Table 3). This is not surprising when one considers the interconnections of metabolism.

The most abundant pathway in the CAD/High-RF group was amino sugar metabolism, involving glutamate, pyruvate, fructose and phosphate. Altered glutamate homeostasis has been found to precede CAD [18] and associated with an increased risk of CV events [19]. High plasma levels of other carbohydrate metabolites, such as pyruvate and fructose, recall the metabolic signature of insulin resistance, metabolic syndrome and obesity [20]. Increased phosphate has been associated with CV disease [21], and a provisional interpretation is based on its effects on vascular calcification [22].

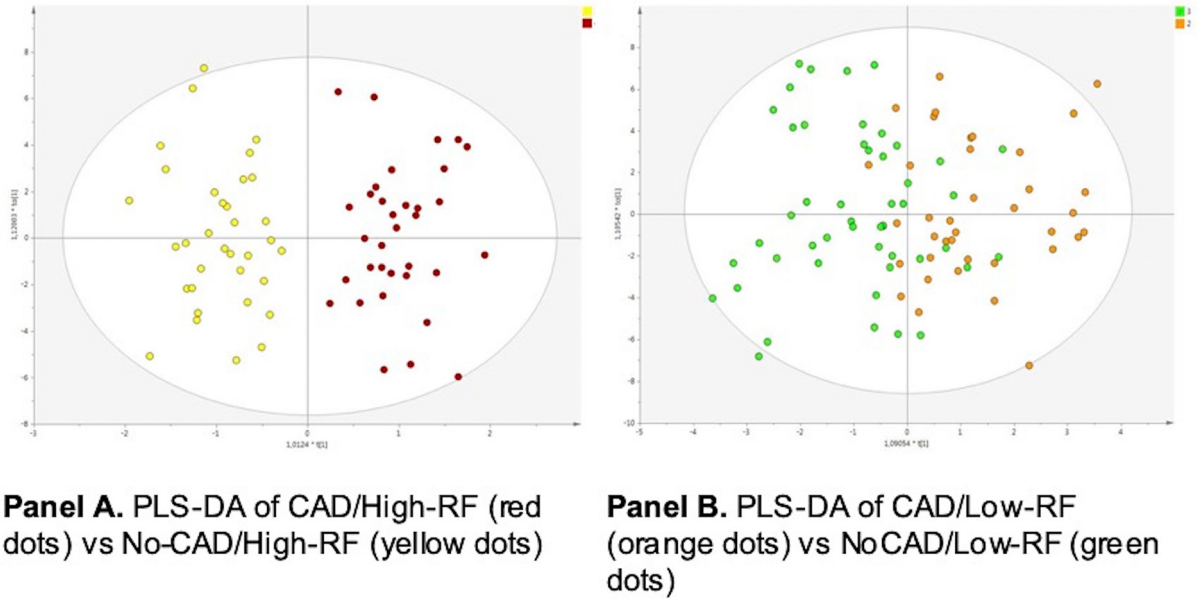


Fig. 1. Score plots of the metabolomic case-control comparisons of phenotypes using OPLS-DA. Each dot represents a patient. CAD = coronary artery disease; OPLS-DA = orthogonal partial least squares discriminant analysis; RF = risk factor profile.

The urea cycle was specific for the No-CAD/High-RF group. The urea cycle is closely related to the synthesis of nitric oxide (NO) insofar as citrulline, a by-product of the NO synthase reaction, can be recycled to arginine by the urea cycle enzymes [23–25]. Reduced urea cycle metabolites have been associated with hypertension [26] through endothelial dysfunction [27]. Alterations of the urea cycle have been found in animal and human models of pulmonary arterial hypertension [28]. Reduced arginine bioavailability, secondary to perturbation of the urea cycle, can be induced by hyperlipidemia [29], smoking [30], and diabetes [31,32]. The absence of CAD despite a high RF profile suggests that this pathway may constitute a protective milieu towards the CV system, able to prevent the development of atherosclerosis and to mitigate the effects of traditional RFs.

On the other hand, the CAD/Low-RF group was specifically associated with enhanced turnover of glutathione (the ubiquitous tripeptide glycine-glutamate-cysteine), which contributes to preserving intracellular redox homeostasis. In the absence of traditional RFs, activation of glutathione is consistent with an endogenous vascular oxidative stress microenvironment associated with CAD. Previous studies have correlated oxidative stress markers, such as isoprostane [33] and thiobarbituric acid [34], with endothelial dysfunction and CAD, potentially not related to traditional RFs. Glutathione pathway dysregulation has been detected in human plaque-containing aortic tissue compared to plaque-free samples [35]. The CAPIRE 2 × 2 phenotypic analysis is the first to indicate a specific correlation of this oxidative stress pathway with CAD and low RF profile (Table 3).

The No-CAD/Low-RF group was specifically characterised by the non-essential amino acids glycine and serine. Glycine is a component of glutathione and originates from both diet and endogenous synthesis [36]. Glycine has been found to exert anti-inflammatory and antioxidant effects [37] and inversely associated with traditional CVRFs [36,38–41]. Importantly, plasma levels of glycine and serine have been found to correlate inversely with the risk of acute myocardial infarction [42,43].

The two CAD groups were characterised by ammonia recycling, suggesting an association with coronary lesions rather than multiple RFs. Ammonia recycling has been related to CAD in a previous well-conducted GC–MS metabolomic study, where it was correlated with a high risk of subsequent CV events [44]. The CAPIRE 2 × 2 phenotypic analysis is the first to indicate a specific correlation of this pathway with CAD, independently of RF profile (Table 3).

On the other hand, the two Control (No-CAD) groups were characterised by the transfer of acetyl groups into mitochondria, involving pyruvate and citrate. The latter metabolites have been inversely correlated with CAD [45–47] and are higher in healthy subjects than in patients with peripheral artery disease [48]. This metabolic pathway, shared by the two disease-free groups, may play a protective role against the development of atherosclerosis, mitigating the effects of CVRFs when present.

The enhanced alanine metabolism in patients with high RF profile is supported by previous findings[23–24,8] showing greater myocardial release of alanine in CAD patients than in healthy controls, or alanine blood levels correlating with severity of coronary artery stenosis [49], or alanine independently associated with major adverse CV events [50]. We previously showed significantly higher coronary plasma levels of alanine in patients with CAD versus no-CAD controls or subjects with microvascular disease [8]. The CAPIRE 2 × 2 phenotypic analysis is the first to indicate a specific correlation of this amino acid with a high RF burden, independently of CAD (Table 3).

The direct case-control comparison suggests a relevant role of phenylalanine. This essential amino acid has already been associated with the presence of coronary artery calcium evaluated by computed tomography as a marker of subclinical atherosclerosis in a recent work of Tzoulaki et al., [51]. In addition, phenylalanine has been proposed as a biomarker of both myocardial infarction [52] and stable angina [53] and, more recently, it has been associated with the early onset of coronary atherosclerosis [53]. On the other hand, phenylalanine and tyrosine were decreased in both arterial and venous serum in a myocardial ischemia and reperfusion model to assess the early systemic metabolic response to balloon-induced transient coronary ischaemia [54].

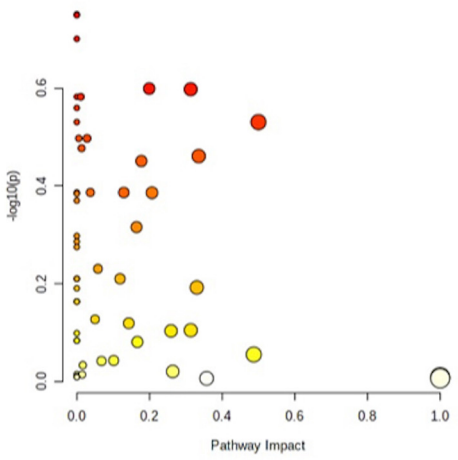
Even the metabolic pathway of tyrosine and tryptophan in the OPLS-DA was able to discriminate Low-RF patients, with and without CAD. These pathways have been already found to be involved in myocardial energetics and inflammation; moreover, they are associated with mortality in patients with CAD [55].

Tryptophan and tyrosine metabolism is regulated by interferon-gamma (IFN-γ), a pro-inflammatory cytokine that plays a significant role in the development and progression of CAD [20,56–58]. IFN-γ can

Table 4
Summary of the 5 most abundant metabolic pathways in the four phenotypic groups (all $p < 0.05$).

CAD/High-RF	CAD/Low-RF	No-CAD/High-RF	No-CAD/Low-RF
Amino sugar metabolism (Glutamate, Pyruvate, Fructose, Tetraphosphate and Cytidine)	**Glutathione metabolism** (Glutamate, Alanine, Pyroglutamate, Cysteine and Glycine)	**Glucose-alanine cycle** (Glucose, Glutamate, Alanine and Pyruvate)	**Glycine/serine metabolism** (Glycine, Glycerate, Glutamate, Alanine, Threonine, Serine, Pyruvate, Cysteine, Methionine and Phosphate)
Methionine metabolism (Serine, Cysteine, Methionine, Phosphate and Glycine)	**Methionine metabolism** (Serine, Cysteine, Methionine, Phosphate and Glycine)	**Transfer of acetyl groups into mitochondria** (Citrate, Glucose, Pyruvate and Phosphate)	**Methionine metabolism** (Serine, Cysteine, Methionine, Phosphate and Glycine)
Glucose-alanine cycle (Glucose, Glutamate, Alanine and Pyruvate)	**Glucose-alanine cycle** (Glucose, Glutamate, Alanine and Pyruvate)	**Alanine metabolism** (Glycine, Glutamate, Alanine, Pyruvate and Phosphate)	**Glycerolipid metabolism** (Glycerol, Glycerate, Palmitate and Phosphate)
Ammonia recycling (Glutamate, Asparagine, Pyruvate, Serine, Glycine, Phosphate and Ammonia)	**Ammonia recycling** (Glutamate, Asparagine, Pyruvate, Serine, Glycine, Phosphate, and Ammonia)	**Urea cycle** (Glutamate, Alanine, Pyruvate, Phosphate, Ornithine, Arginine and Citrulline)	**Glucose-alanine cycle** (Glucose, Glutamate, Alanine and Pyruvate)
Alanine metabolism (Glycine, Glutamate, Alanine, Pyruvate and Phosphate)	**Glycerolipid metabolism** (Glycerol, Glycerate, Palmitate and Phosphate)	**Glycerolipid metabolism** (Glycerol, Glycerate, Palmitate and Phosphate)	**Transfer of acetyl groups into mitochondria** (Citrate, Glucose, Pyruvate and Phosphate)

CAD = coronary artery disease; RF = risk factor profile.



Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact	Details
Histidine metabolism	1/16	0.17725	0.75142	1.0	0.98578	0.0	KEGG SMP
Butanoate metabolism	3/15	0.17806	0.74943	1.0	0.98578	0.0	KEGG SMP
Propanoate metabolism	2/23	0.199	0.70115	1.0	0.98578	0.0	KEGG SMP
Citrate cycle (TCA cycle)	4/20	0.25157	0.59934	1.0	0.98578	0.19926	KEGG SMP
Alanine, aspartate and glutamate metabolism	7/28	0.2524	0.59792	1.0	0.98578	0.3133	KEGG SMP SMP SMP
Valine, leucine and isoleucine biosynthesis	5/8	0.26143	0.58264	1.0	0.98578	0.0	KEGG SMP
Valine, leucine and isoleucine degradation	4/40	0.26151	0.5825	1.0	0.98578	0.01084	KEGG SMP
Selenocompound metabolism	1/20	0.27555	0.5598	1.0	0.98578	0.0	KEGG SMP

Fig. 2. Analysis of metabolic pathways determined by metabolite set enrichment graphically ranked by decreasing *p*-values in the CAD/High-RF vs No-CAD/High-RF comparison.

induce the indoleamine-2,3-dioxygenase enzyme, which catabolises tryptophan to kynurenine [59], and it has been shown that the kynurenine:tryptophan ratio correlates with the presence of CAD [60].

On the other hand, IFN-γ-stimulated macrophages cause a reduced production of tyrosine from phenylalanine [61], and higher phenylalanine:tyrosine ratio in subjects with CAD appeared to correlate with C-reactive protein levels, a biomarker of adverse CV outcomes [61].

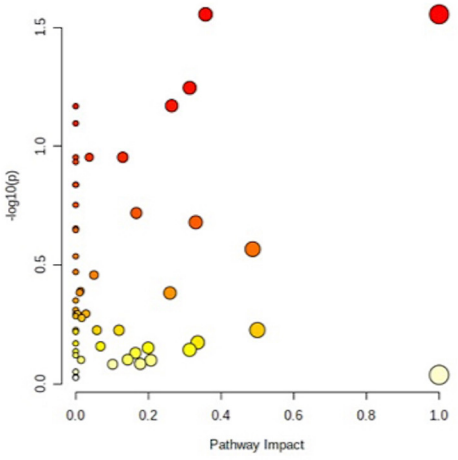
4.1. Limitations

An apparent limitation of the present metabolomic study is the relatively small number of subjects. Our sample size, however, is comparable or superior to that of other studies in the field. Moreover, CAPIRE is the first to compare mutually exclusive clinical phenotypes in a 2×2 design. Additionally, the presence/absence of CAD was systematically investigated by noninvasive angiography. Finally, the four groups were well balanced in terms of number, age and sex. CCTA was prompted by a clinical indication that may have differed from case to case. However, none of the CAPIRE subjects had a history of acute CV event, allowing our analyses to focus specifically on the presence/absence of coronary atherosclerosis. Given the observational nature of CAPIRE, cause-effect relations cannot be inferred from the identified associations.

The lack of a validation group is a limitation of this study; however, beyond the high statistical significance, we performed cross-validation to better evaluate the goodness of the developed model. Moreover, this study was not aimed to be exhaustive or conclusive but rather hypothesis-generating to stimulate further research on the individual susceptibility to the development of CAD.

4.2. Implications and conclusions

To our knowledge, this is the first systematic investigation of subjects with CAD, CVRFs, both or neither of them that identified metabolic pathways able to interpret the favouring or protective mechanisms for CAD, beyond traditional RF. The metabolic pathways that characterise each group suggest a complex interaction between traditional CVRFs and the biological substrates on which they act. Specific metabolites



Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact	Details
Phenylalanine, tyrosine and tryptophan biosynthesis	2/4	0.027892	1.5545	1.0	0.6177	1.0	KEGG SMP
Phenylalanine metabolism	2/10	0.027892	1.5545	1.0	0.6177	0.35714	KEGG SMP
Arachidonic acid metabolism	1/36	0.056834	1.2454	1.0	0.6177	0.3135	KEGG SMP
Cysteine and methionine metabolism	5/33	0.067636	1.1698	1.0	0.6177	0.26401	KEGG SMP SMP
Sphingolipid metabolism	1/21	0.067938	1.1679	1.0	0.6177	0.0	KEGG SMP

Fig. 3. Analysis of metabolic pathways determined by metabolite set enrichment graphically ranked by decreasing *p*-values in the CAD/Low-RF vs No-CAD/Low-RF comparison.

were associated with the mitigation or even prevention of CAD (eg, glycine/serine) or, conversely, exacerbation or progression of the disease (eg, glutathione or amino sugars). Furthermore, case-control comparisons have highlighted the role of phenylalanine, tyrosine and tryptophan metabolisms in the development and progression of CAD in patients with a low risk profile. This observation suggests a predisposing metabolic profile that, even in the absence of traditional CVRFs, makes the subject prone to the atherosclerotic process.

Overall, our results provide functional correlations for unconventional "outlier subjects", possibly underlying the mechanisms of CAD expression or prevention. In the future, these metabolic profiles could be used to identify subjects at higher risk of CAD, despite low clinical risk profiles, to optimise individual patient management and characterise new therapeutic targets.

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Declaration of Competing Interest

Roberto Latini, Attilio Maseri, Marco Magnoni, Daniele Andreini, Martino Deidda, Antonio Noto, Christian Cadeddu Dessalvi and Giuseppe Mercuro have no conflict of interest to report.

Felicita Andreotti reports receiving consultancy/speaker fees, outside the present work, from Amgen, Bayer, — BMS/Pfizer and Daiichi Sankyo;
Aldo P. Maggioni reports receiving fees, outside the present work, from Bayer, Fresenius, Novartis for participation in study committees;
Eleuterio Ferrannini reports receiving consultancy/speaker fees, outside the present work, from Boehringer Ingelheim, Lilly&Co., AstraZeneca, and Sanofi.

Appendix A. Appendix

Steering Committee: A. Maseri (Chairman; Firenze), D. Andreini (Milano), S. Berti (Massa), M. Canestrari (Fano), G. Casolo (Lido di Camaiore), D. Gabrielli (Roma), R. Latini (Milano), M. Magnoni (Milano), P. Marraccini (Pisa), T. Moccetti (Lugano), M.G. Modena (Modena).

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