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Targeted and non-targeted metabolic time trajectory in plasma of patients after acute coronary syndrome

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ABSTRACT

Metabolite fingerprinting (metabolomics/metabonomics) is perfectly suited for assessing the biological response following acute coronary syndrome (ACS) as relevant information can be identified in both the change and the absence of change in metabolite concentrations as time progresses post syndrome. During this study the metabolic pattern of plasma from patients at time points 0, four days, two months and six months after the onset of ACS were compared to controls using a non-targeted approach with gas chromatography mass spectrometry (GC–MS). Fatty acid profiles of the sample set were also analysed in a targeted way. The methods were employed with the aim to identify specific biomarkers, which vary with time. Using the non-targeted approach 27 statistically significant metabolites of interest were found: glucose, fructose, myoinositol, pyruvate, lactate, oxalate, citrate, isocitrate, succinate, malate, valine, alanine, serine, glycine, cysteine, threonine, aspartate, tryptophan, tyrosine, 4-hydroxyproline, 2-hydroxybutyrate, 2-aminobutyrate, 2,3,4-trihydroxybutyrate, 3-hydroxybutyrate, creatinine and aminomalonate. In addition, the targeted analysis of 21 fatty acids revealed patients within the group ACS at day 0 had the highest values for all 21. After 4 days, values decreased and were maintained at a lower level during the 6 months. Whereas the overall fatty acid profile did not change, different patterns of concentration trajectories over time were identified, which can reflect the underlying metabolic alterations as a result of the initial ACS, interestingly these levels had not fully reverted six months later.

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1. Introduction

Improvements in our understanding of the pathophysiology of atherosclerosis and acute coronary syndrome (ACS) are necessary for a better prevention and treatment. There is a growing body of literature about non-targeted analysis of metabolites (metabolic fingerprinting/metabolomics/metabonomics) as a research tool to understand mechanisms of pathology and as an approach for biomarker discovery in cardiovascular disease. Recently, metabolomic analysis has been reported to discriminate in the prognosis and diagnosis of other human diseases, including diabetes, blood pressure and cancer [1–3].

As proof of principle, Sabatine et al. [4] used mass spectrometry-based methods to identify differences in plasma metabolites among 18 subjects suffering from ischemia induced by exercise stress testing compared with non-ischemic individuals who also undertook regular exercise. Results demonstrated specific changes in 6 metabolites, including citric acid, between disease and control subjects ($p < 0.0001$). In additional support to this, two further studies have reported the use of multivariate analysis combined with ^{1}H NMR spectroscopy of serum and plasma samples to predict the presence and severity of angiographically defined coronary artery disease and of hypertension [5,6]. However it has been argued that results such as these not only depend

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on the major lipid regions of the spectra, but also many variables, including gender and drug treatment, which are capable of affecting lipid composition and are potential interferants [7]. In a previous preliminary study [8] GC–MS fingerprinting was applied to plasma samples from patients with ACS and compared them to healthy subjects, looking for marker molecules capable of disease diagnosis when analysed with pattern recognition techniques. Identification of variables which account for the classification observed from the loading plot showed that citric acid, 4-hydroxyproline, aspartic acid, and fructose were decreased and lactate, urea, glucose, and valine were increased in non-ST elevation ACS (NSTEACS) patients *vs.* healthy controls. NSTEACS describes a type of ACS where ischemia affects only the inner region of the myocardium, as opposed to what happens in ST-elevation ACS, where the coronary occlusion is more severe and ischemia affects the whole thickness of the myocardium. The term ST elevation refers to a segment of the electrocardiogram which becomes elevated in the last case, but not in NSTEACS. GC–MS was selected as analytical technique because, although the drawbacks related with exhaustive sample treatment are well known, the efficiency in the separation of small molecules and the ability to identify them through libraries are important advantages that outweighed the disadvantages of the technique [8].

Previous investigations [9] also studied the plasma of patients with stable carotid atherosclerosis as compared to healthy subjects with both GC–MS and ^{1}H NMR. The results showed at least 24 metabolites that were significantly modified in the group of atherosclerotic patients by this non-targeted procedure. Most of these changes could be attributed to alterations in metabolism which are typical in cases of insulin resistance, which is a condition strongly related to the metabolic syndrome. Supporting this hypothesis, Mayr et al. [10] concluded that inefficient vascular glucose and energy metabolism coincides with increased oxidative stress in hyperlipidemic animals. This was evident as the oxidation of redox-sensitive proteins was identified via the completion of both proteomic and metabolomic analysis of aortas from apolipoprotein E deficient homozygous recessive mice.

Typically, biomarker studies of ACS patients have reported findings on the basis of a single sample collected early in the course of an ACS hospital stay [11], however there is emerging data about the importance of temporal changes in biomarker levels.

There is also previous evidence regarding the involvement of fatty acids in the ACS process. An increase of free fatty acids (FFA) has been linked to an increase in plasma norepinephrine with increases occurring within minutes of the onset of ACS and remaining high for up to 20 h [12]. This can have a negative effect because an excessive delivery of plasma FFA to the heart, may contribute to myocardial dysfunction [13]. In fact, previously high fatty acid levels were associated with all-cause and cardiovascular mortality in a cohort of patients who were referred for a coronary angiography who had a high prevalence of coronary artery disease [14].

According to Mozaffarian [15], in ACS profound changes in systemic metabolism occur, and these may result in the myocardium no longer receiving the optimum balance of energy substrates allowing it to contract and function normally. In addition, circulating free fatty acid levels provide an additional indication of higher clinical risk for ACS [15].

The present study described here addresses this issue in two ways. Firstly, by obtaining the metabolic pattern from plasma samples of patients at time 0, four days, two months and six months after the onset of ACS and compared them to matched controls in a non-targeted approach. Once the profiling of the fatty acids was undertaken this was investigated further with respect to changes versus time for their potential use as specific biomarkers.

2. Material and methods

2.1. Study design

Nineteen patients suffering from a NSTEACS, defined as anginal pain with troponin elevation and/or ST-T changes on the electrocardiogram, entered the study. Exclusion criteria for the study included those affected by inflammatory or neoplasic disease, coagulation disorders, other significant heart diseases except left ventricular hypertrophy secondary to hypertension, those undergoing chronic treatment procedures (except drugs for pre-existing clinical atherosclerosis or its risk factors), or those who have undergone surgical procedures, major traumatisms, thromboembolic events, or revascularization procedures in the previous 6 months. Patients with an ejection fraction of less than 0.45 were also excluded, given that left ventricular dysfunction may enhance inflammation, adding a potential confounder to the results [16]. The ejection fraction is defined as the percentage of end-diastolic blood volume, which is expelled by the left ventricle during systole. The normal values range between 55% and 75%. At the moment the diagnosis was made, the patients were asked to participate in the study. In case of acceptance, they signed the informed consent form, and 28 mL of blood was withdrawn for analysis and introduced immediately to EDTA-prepared collection tubes (Venoject, Terumo Europe). In cases diagnosed during the evening, blood was extracted at 9 a.m. the next morning. Management included coronary angiography in all cases. A second blood sample was taken on day 4 to investigate if there was a fast reversion of the changes observed on admission. An intermediate blood sample was withdrawn at 2 months. The last blood sample was performed at 6 months, given that and inflammation ceases at this time [17]. Moreover, we have reported previously that, on admission, circulating monocytes of NSTEACS patients show abnormal expression of 17 proteins as compared to stable subjects with coronary artery disease [18]. However, the number of abnormally expressed proteins was decreasing progressively with time and, at six months the proteome of these cells was identical to that of stable patients with this disorder. Given the relevance of monocytes in plaque inflammation, we estimate that biological phenomena leading to an ACS are over at six months. Blood was also collected from 6 healthy subjects without significant differences in age. They did not have previous or family history of CAD, and were not receiving any pharmacological treatment. Moreover, they had none of the characteristics of the patients (smoking, hypertensions, diabetes, etc.), and showed normal physiological values of cholesterol, triglycerides and lipoproteins. Well-characterized controls were difficult to obtain, but the experiment was mainly focused on the evolution of patients as compared with themselves.

The basal characteristics of the patients are described in Table 1. The final diagnosis was unstable angina in 5.6%, and non-Q wave myocardial infarction in 94.4%, defined as troponin T levels higher than 0.20 ng/mL. All patients underwent coronary angiography: the percentages of patients with 1-, 2-, and 3-vessel disease were 36.8%, 31.6%, and 31.6%, respectively. The ejection fraction was normal in all cases. Fifty-eight percent of the patients were treated with percutaneous coronary intervention, 16% with coronary artery bypass graft, and 26% received only medical treatment. At discharge, 89.5% received aspirin and/or clopidogrel and 5.3% clopidogrel plus acenocumarol, 89.5% were treated with statins, 22.2% with oral antidiabetic drugs, 52.7% with angiotensin converting enzyme inhibitors, 68.4% with beta blockers, 15.8% with calcium channel blockers and 31.6% with nitroglycerin.

The patients and the controls were recruited in the Fundación Hospital de Alcorcón and Móstoles Hospitals, whose Ethics Committees approved the study. Blood was centrifuged at $1000 \times g$

Table 1
Baseline characteristics of patients.[a]

	ACS	Controls	p
Age (years)	60.0 (54.0–67.0)	65.5 (51.8–73.3)	0.514
Sex (male/female)	16/3 (84%/16%)	3/3 (50%/50%)	0.125
Smoker: current/past/no	9/5/5 (47.4%/26.3%/26.3%)	2/1/3 (33.3%/16.7%/50.0%)	0.554
Hypertension	10 (53%)	0 (0%)	0.063
Diabetes	6 (32%)	0 (0%)	0.278
Obesity/overweight	42.1%	0 (0%)	0.129
Body mass index	28.4 (25.9–32.6)	25.9 (24.8–26.64)	0.071
Family history of early CAD	17.6%	0 (0%)	0.539
Total cholesterol (mg/dL)	197 (187–218)	198 (163–214)	0.581
LDL (mg/dL)	129 (112–142)	124 (83–147)	0.595
HDL (mg/dL)	36 (33–39)	55 (45–63)	0.001
Triglycerides (mg/dL)	159 (133–219)	92 (71–113)	0.001
Glucose	104 (86–131)	91 (84–112)	0.274
Previous CAD	21.1%	0 (0%)	0.540
Statin	21.1%	0 (0%)	0.540
Antithrombotic therapy	21.1%	0 (0%)	0.681
Peak troponin I (ng/mL)	0.68 (0.27–2.66)	–	–

[a]Quantitative data are expressed as median (interquartile range). Comparisons were performed using a Mann–Whitney for quantitative variables and a chi square test (Fisher's exact test when appropriate) for qualitative data. Statin and antithrombotic therapy refer to treatments received by the patients previously to the present event. *Abbreviations*: ACS, acute coronary syndrome; CAD, coronary artery disease.

(Heraeus Instruments, Langenselbold) for 10 min at 4 °C. Plasma was stored at −80 °C until the day of analysis.

2.2. Solvents and reagents

Reference compounds were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Steinhein, Germany). The compounds and reagents were all of analytical grade except where stated otherwise. N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) were purchased from Pierce Chemical Co. (Rockford, IL, USA). O-methylhydroxylamine hydrochloride, pyridine (silylation grade) and heptane (analytical grade) were obtained from Sigma. Methanol, acetone, and acetonitrile (HPLC grade) were from Supelco (Bellefonte, PA, USA). Ultra pure water was produced by a Milli-Q Reagent Water System (Millipore, Billerica, MA, USA).

Stock solutions of the reference compounds were prepared either in Milli-Q water or in methanol at equal concentrations of 25 mM. These solutions were kept at −80 °C. Before the analysis the solutions were thawed and diluted (1/100) with methanol for GC–MS.

2.3. Sample treatment for plasma fingerprinting with GC–MS and instrumental conditions

Sample treatment and instrumental conditions for GC–MS were as previously described [8]. Briefly, plasma was thawed, vortex-mixed and filtered through a 0.22-μm filter (Millipore). Plasma was deproteinized, dried and derivatized first with O-methylhydroxylamine hydrochloride and then with BSTFA with TMCS used as a catalyst. C18:0 methyl ester was added as internal standard (IS).

Samples were analysed by GC–MS with a Varian instrument 3900 Series GC (Varian, Palo Alto, CA, USA). Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1} through the column. The injector temperature was 250 °C and split ratio 1:10. The initial oven temperature of the column (Varian FactorFour capillary column, VF-5 ms, 30 m × 0.25 mm ID, DF = 0.25 μm,) was 50 °C; 1 min after injection, the temperature was raised at a rate of 3.3 °C min^{-1} to reach a final temperature of 340 °C. The mass spectrometer detector was a Saturn 2110T (Varian) with ion trap. The mass spectrometer was operated in full-scan mode only (scanning from m/z 50 to m/z 650) at a rate of 5 spectra s^{-1}.

Metabolites were identified in the profiles by retention time and by comparison with the mass spectra either in NIST library or with an in house library built using the pure standards.

2.4. Data alignment and treatment

The whole signal profile from total ion mode was employed to compare the fingerprints. Multialignment of the GC–MS chromatograms was performed with an in-house program developed in Matlab® 7.0 (MathWorks, Natick, MA, USA), using the correlation optimised warping (COW) method previously described [19]. The first treatment applied to each profile was baseline correction; it is part of the routine developed in Matlab and always poses an improvement in the raw signal. The second step was the multialignment of the profiles. Finally samples were normalised with the internal standard to avoid solvent evaporation effects and autoscaled and subsequently, data were analysed with SIMCA P+ 12.0 (Umetrics, Umea, Sweden) for multivariate data analysis.

2.5. Sample treatment for fatty acid analysis in plasma

All transesterification reactions were carried out in Pyrex tubes (Nirco, Barberá del Vallès, Spain) with a total capacity of 10 mL and capped with Teflon caps. The process was completed using a microwave with a method developed and validated in our own laboratory. Plasma samples and the internal standards (C15:0 acid form and C17:0 methyl ester) were added to the pyrex tube followed by the addition of 1.5 mL methanol/toluene (4:1) and 250 μL of sulphuric acid. Each individual tube was then vortexed. Microwave irradiation was carried out at 600 W for 80 s (Anton Paar Microwave digestor Multiwave, Graz, Austria). Following that, 1 mL of heptane was added as the extraction solvent along with as 500 μL NaCl 5% (w/v) to aid separation of the layers due to its higher ionic concentration. Each tube was vortexed for 1 min and then centrifuged for 10 min at 1000 × *g* at 4 °C. Finally, 700 μL of the organic layer was removed and dried under vacuum before resuspension in 100 μL of toluene/BHT (50 mg/L) to be analysed by GC–MS.

The fatty acid methyl ester (FAME) analysis was performed on the same equipment previously described. Helium was used as carrier gas at a constant flow rate of 0.8 mL min^{-1} through the column. The injector temperature was 200 °C and split ratio 1:30. The initial oven temperature of the column (Varian FactorFour capillary column, VF-23 ms 30 m × 0.25 mm ID DF = 0.25 μm) was 150 °C, after 1 μL of injection the temperature was raised at the rate of

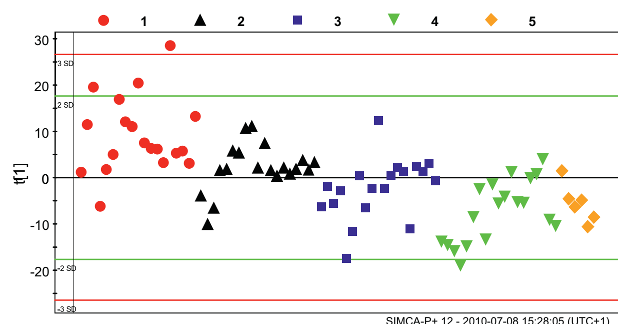

Fig. 1. Score plot of PLS-DA for the 1st component. R^2Y(cum) = 0.123, Q^2(cum) = 0.0296. (1) Time zero, (2) 4 days, (3) 2 months, (4) 6 months after ACS, and (5) control group.

2 °C min^{-1} to reach 210 °C. It was then raised at a rate of 3 °C min^{-1} to reach a final temperature of 260 °C. The mass spectrometer detector was a Saturn 2110T (Varian) with ion trap. The mass spectrometer was operated in the full-scan mode only (scanning from m/z 50 to m/z 650).

FAME were identified in the profiles by retention time and by comparison with the mass spectra in NIST library and with an in house library built using the pure standards. The method was developed and validated in our laboratory.

3. Results

3.1. Metabolomic studies

The initial hypothesis of the present study proposed that ACS disrupts metabolism, and therefore will be reflected in changes to the metabolite composition that can be detected with GC–MS when comparing diseased plasma profiles with the corresponding controls using a non-targeted approach. These changes will evolve over time, as the acute biological processes which trigger the ACS disappear. Chemometric tools can be used to highlight changes in plasma composition and their evolution over time.

Firstly, a non-supervised principal component analysis (PCA) was applied; however no clustering was observed when variables were not selected. The final data set comprised of 82 observations (patients = 19 in four time points, controls = 6).

Next, a partial least square-discriminant analysis (PLS-DA) was performed. When studying the score plot for only the 1st component it was possible to observe series of characteristic time-dependent changes, which arose during the recovery process (Fig. 1), resulting in a time-trajectory appearing on the plot.

The group of patient samples from time zero was separated from the control group. This group was used for obtaining reference values, although it can be true that the physiological situation of such people can be not exactly comparable to the patients whose evolution was subsequently studied.

Following this, a comparison of each possible pair of groups, performing orthogonal partial least square discriminate analysis (OPLS-DA) was carried out and found metabolites that differentiated both groups. OPLS [20] is a modification of PLS developed to improve interpretation of the resulting models. The difference between PLS and OPLS is that the latter splits the variation in X, needed to predict Y in PLS, into two parts; the Y-related variation and the Y-orthogonal variation. OPLS facilitates interpretation since these two sources of variation are separated, providing the opportunity to interpret them independently. This also makes it possible to estimate the size of the variation in X that is related to Y. S-plot combines the contribution/covariance (Cov(t,X)) and reliability/correlation (Corr(t,X)) from OPLS-DA model and helps to identify differential metabolites between classes and in addition it permits to obtain a jack-knifed based confidence interval for the variables. OPLS-DA was applied in order to improve the separation between the groups and to obtain the significance of the correlation between the variable and the scores on the predictive vector. Classifying metabolites were grouped according to their biochemical classes and in order to prove their statistical significance the Jack knife interval with level of confidence 95% was calculated (Fig. 2). Those compounds including zero can be considered with a random probability of being correlated, for that reason only compounds not including zero in at least one of the comparisons were considered. Statistical significance was estimated as ±0.02 on Y axis of the OPLS-DA score plot.

Finally a colour coded representation was created which included significant metabolites and their percentage of abundance through time as compared to control, which was considered as a reference (Fig. 3).

3.2. Fatty acids

Among these metabolites several fatty acids appeared and therefore targeted analysis was applied with a specifically developed method. In the chromatogram of fatty acid methyl esters, 21 fatty acids were identified by the retention time of the standards and the spectrum (C14:0, C16:0, C18:0, C20:0, C22:0, C23:0, C24:0, C16:1, C16:1ω7 (Z), C18:1, C18:1ω9 (Z), C24:1ω9, C18:3ω3, C20:5ω3, C22:5ω3, C22:6ω3, C18:2ω6 (E), C18:2ω6 (Z), C18:3ω6, C20:2ω6, C20:3ω6, and C20:4ω6). After correcting the signal for that of the internal standard (C17:0 methyl ester), 5 out of 21 fatty acids comprised for approximately 85% of the corrected signal in all the chromatograms (C18:2ω6 (Z) –linoleic acid, C16:0 – palmitic acid, C18:1ω9 (Z) – oleic acid, C18:0 – stearic acid, and C20:4ω6 – arachidonic acid). In Fig. 4A the values for the corrected signal of these five fatty acids are depicted. As can be seen, patients with ACS at day 0 have the highest values for all the fatty acids. After 4 days, values have decreased, and they maintain these values during the 6 months.

For evaluating changes in the profile of individual fatty acids, intensity values were normalised for each fatty acid, to the maximum level attained after ACS or control. Therefore the trends of all the fatty acids can be observed independently from their relative amount in the profile. In Fig. 4 these results can be seen, grouped by type of fatty acid (saturated, monounsaturated, polyunsaturated ω3, and polyunsaturated ω6 in Panels B, C, D and E, respectively).

4. Discussion

Patients suffering ACS present alterations in metabolism that can be attributed to at least two different metabolic challenges, with different impact on metabolic changes: metabolic stress under hypoxia and, to a lesser extent, insulin resistance.

At the onset of ACS, catecholamines (epinephrine and norepinephrine) increase, and remain high for several hours, activating the flight-or-fight response [21]. The global result is that, as higher energy is urgently needed for facing a situation of stress, carbohydrates become the preferential substrate of myocardium (by glycolysis) because less oxygen is needed for very quick ATP production.

Besides this situation, insulin resistance could be present even though patients had not been previously diagnosed as diabetics. Insulin resistance has been reported in patients of myocardial infarction and unstable angina pectoris on admission [21]. In cases of insulin resistance lipogenesis is reduced and hormone sensitive lipase (HSL) activity is increased, whereas lipoprotein lipase (LPL) is reduced, leading to accumulation of lipids in plasma (higher free fatty acids in circulation).

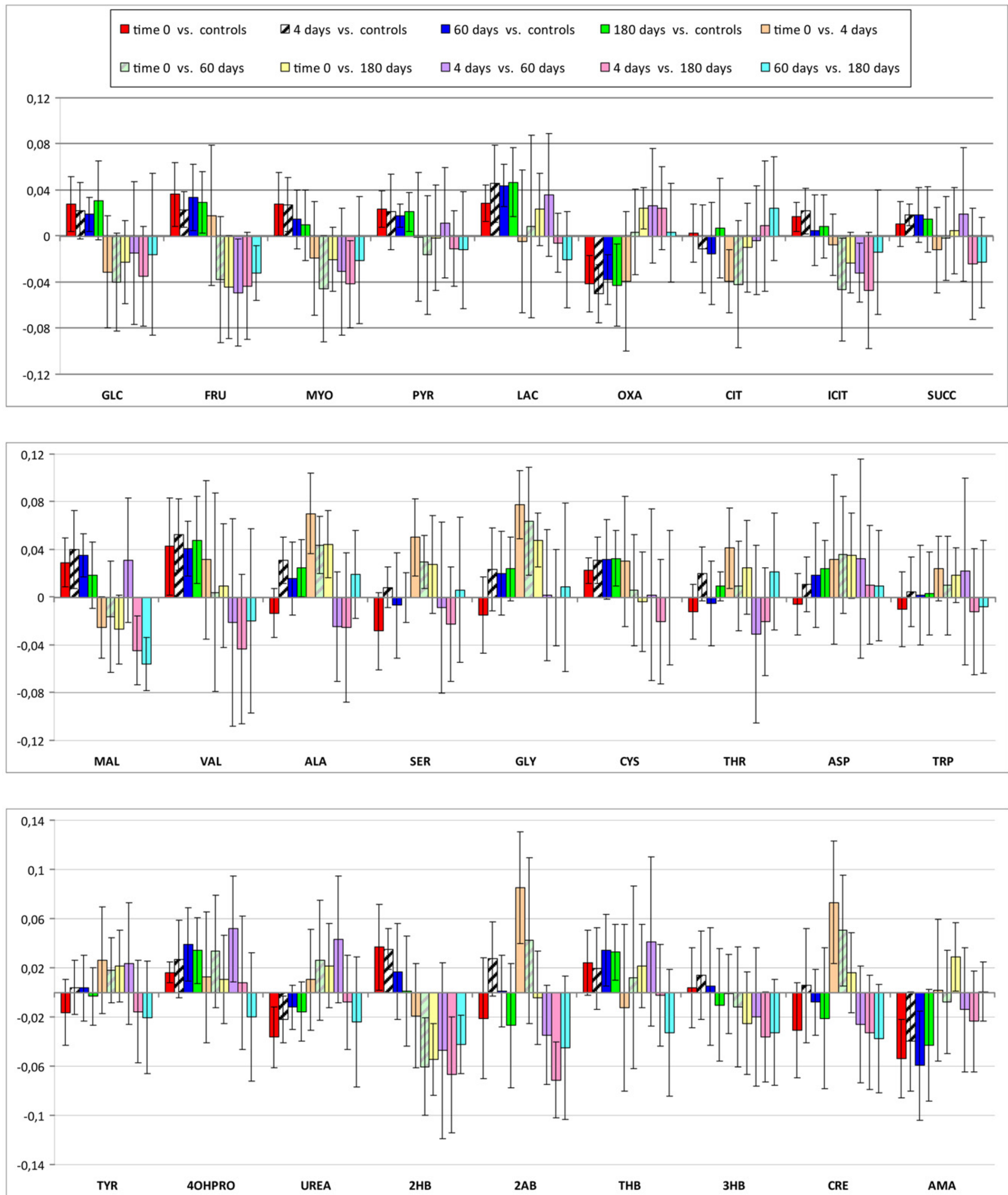

Fig. 2. Correlations of the metabolites and their scores on the predictive vector in the OPLS-DA plots of each possible pair. The Jack-knife standard errors of prediction for OPLS models were calculated (the level of confidence was 95%). First 4 bars: positive bar – increase in patient compared to controls. The rest of the bars: positive bar – increase with time. Negative bars – decrease with time. *Abbreviations*: GLC, glucose; FRU, fructose; MYO, myoinositol; PYR, pyruvate; LAC, lactate; OXA, oxalate; CIT, citrate; ICIT, isocitrate; SUC, succinate; MAL, malate; VAL, valine. ALA, alanine; SER, serine; GLY, glycine; CYS, cysteine; THR, threonine; ASP, aspartate; TRP, tryptophan; TYR, tyrosine; 4OHPRO, 4-hydroxyproline; 2HB, 2-hydroxybutyrate; 2AB, 2-aminobutyrate; THB, 2,3,4-trihydroxybutyrate; 3HB, 3-hydroxybutyrate; CRE, creatinine; and AMA, aminomalonate.

Patients at day 4 had started a treatment, based mainly on beta-blockers and statins. Beside the clinical goals that must be achieved in the management of their complications, these drugs induce changes in the metabolic profile.

Under treatment with beta-blockers myocardium glycolysis is inhibited, but not liver gluconeogenesis. Beta-blockers increase insulin release and will induce the reverse switch of myocardium metabolism to catabolism of free fatty acids, as a consequence of

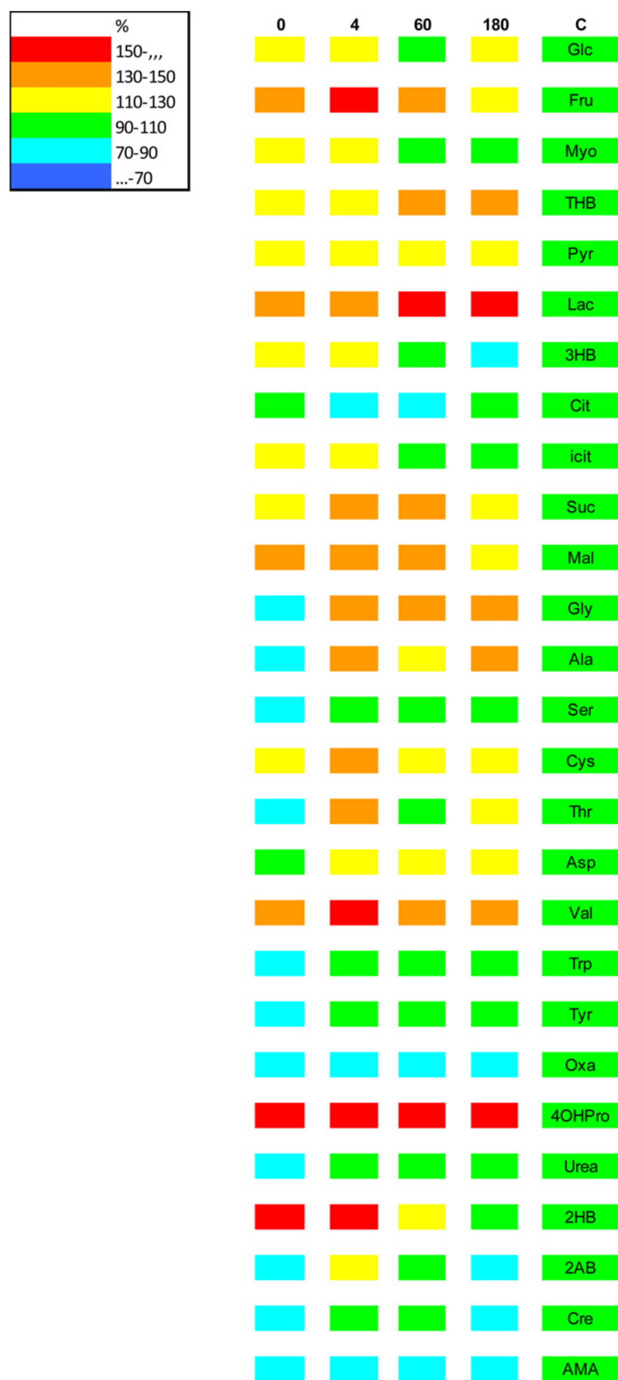

Fig. 3. Time trajectory of selected metabolites, calculated as relative changes of intensity compared to controls. Abbreviations as in Fig. 2.

less conversion of pyruvate to acetyl CoA. Moreover, these agents partially block the catecholamine-induced release of fatty acids from adipose depots.

During the first hours of acute myocardium infarction, plasma free fatty acids increase and can be double or triple that of resting values [12,22] and this investigation found, as expected, higher fatty acid concentration in the plasma of patients at onset of ACS, therefore suggesting an increase in free fatty acids. After ACS, fatty acids were greatly reduced, and this change can be mainly attributed to the reduction in circulating free fatty acids, which should have been accomplished by the treatment with statins (reduction in LDL-cholesterol) [23] and beta blockers [24]

The fatty acid profile of all the patients at each sampling time point was evaluated, in order to highlight possible differences in the proportion of fatty acids, together with the changes in concentration of circulating lipids. There was no significant change in the overall profile (percentage of each fatty acid); because the action of drugs from the treatment must modify activities of lipases and other lipogenetic enzymes (such as hydroxymethylglutaryl-CoA reductase), and not those responsible for elongation or desaturation reactions.

Together with hyperlipidemia, the main changes associated to catecholamine challenge are hyperglycemia and hyperlactatemia. Increase in glucose production comes first from glycogenolysis, followed quickly afterwards by liver gluconeogenesis which is then maintained. In the patient samples from our study variations of metabolites that support this situation have been identified: glucose and lactate were found to be higher than in controls, together with pyruvate, the end product of glycolysis. Although six months after ACS glucose and pyruvate levels were not different to those at onset, lactate was higher after 2 months, and was high even 6 months later.

Fructose was also found to exist at higher levels. In a previous study with stable atherosclerosis, lower levels of fructose were found in patients, which were attributed to higher consumption because glucose uptake could be compromised due to insulin resistance [9]. Under hypoxia, the biological condition of patients differs, with both glycolysis and gluconeogenesis enhanced. Therefore increased production of glycolytic intermediates such as fructose 1,6-diphosphate and fructose 6-phosphate, easily convertible to fructose, may be the origin of the increased plasma fructose, which shows the highest levels 4 days after ACS, and then decreases.

Regarding carbohydrate metabolites, trihydroxybutyrate, a metabolite of heterosugars, was also found increased in patients. Trihydroxybutyrate comes from the metabolic breakdown of heterosugars (N-acetylglucosamine and N-acetylgalactosamine) found in conjunctive tissue and/or glycoproteins. Their degradation can be increased when patients suffer from shock. Moreover, degradation of the connective tissue of the atherosclerotic plaques is of key importance predisposing to plaque rupture and thrombosis [25]. Nevertheless, after 6 months of treatment, values are even higher than at onset. The reason why these changes have not been reverted should consider that convalescence of the patients includes a repair of the damage caused by plaque rupture and vascular occlusion. However, independent of the (local) repair process atherosclerosis typically is a general disease. In consequence, the changes observed after 6 months include both, local repair and ongoing systemic atherosclerosis.

In addition, myoinositol levels also can increase as a secondary result of higher glucose concentrations. This may also be caused by deficiencies in insulin response, as insulin administered to diabetic patients can reduce plasma myoinositol to levels similar to controls [26].

Among the metabolites related to oxidative catabolism of acetyl CoA (Krebs or tricarboxylic acids cycle), patients on admission presented higher signals for isocitrate, malate, and succinate, with no differences in citrate. It is difficult to assign variations in these metabolites to one single factor, because they are involved in many reactions, in different tissues and intracellular compartments.

The first point of regulation of Krebs cycle is citrate synthase. Sabatine et al. found that, in patients with inducible ischemia, citrate signals decreased when submitted to a stress test [4]; whereas if they did not present symptoms of ischemia variation in citrate levels remained non significant. In our study with patients after ACS we have found no differences in citrate signals between patients and controls at onset and after six months, but lower levels in the samples taken 4 days after. Isocitrate, malate and succinate levels were found to be higher, with high levels during the period of study,

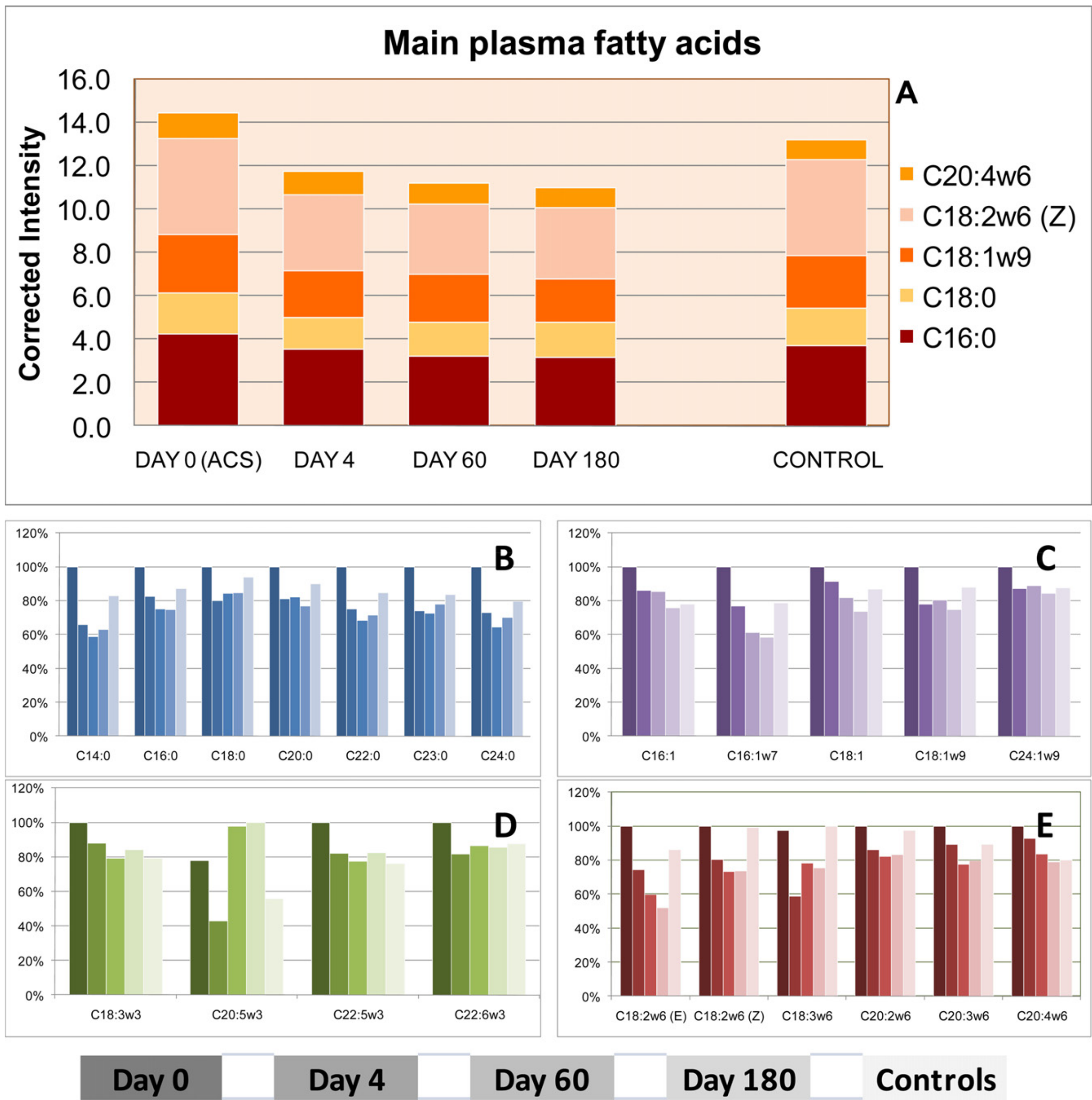

Fig. 4. Fatty acids in plasma. Panel (A) corrected intensity of the fatty acids that account for more than 85% of the sum of all the signals. Panels (B–E) time-trajectory of the signal for each fatty acid, grouped by type of fatty acid (B = saturated, C = monounsaturated; D = ω-3 polyunsaturated; E = ω-6 polyunsaturated). Maximum signal attained = 100%.

but with different patterns: isocitrate was at two months lower than at onset, whereas it took 6 months for malate and succinate. This may be related to the lower activity of the cycle together with lower activities of other enzymes, leading to an accumulation of intermediates. For instance, the activity of malate decarboxylase, a key enzyme in lipogenesis, is reduced under stress and/or impaired insulin response, therefore conversion of malate to pyruvate could be decreased. It is also noteworthy that oxalate, a metabolite from glyoxylate and enhancer of malate decarboxylase activity, was found to be also lower in patients at onset.

An effect of epinephrine and norepinephrine is to increase cortisol concentration, which in turn, increases the level of amino acid catabolism; in addition, lack of insulin or the presence of insulin resistance also increases proteolysis and catabolism of amino acids as substrates for glucose and/or ATP synthesis.

Nevertheless, these effects do not influence all the amino acids metabolic routes in the same way: 4 out of 7 amino acids that were lower than in controls at the onset (glycine, alanine, threonine, and aspartate) presented higher signals than controls 4 days after, whereas the remaining three (serine, tryptophan, and tyrosine), presented signals that were not different than controls. Cysteine, valine, and 4-hydroxyproline, presented data higher than the controls at onset, and even higher at day 4. At 6 months, the signals for glycine, alanine, cysteine, threonine, aspartate, valine and 4-hydroxyproline were higher than in controls; only serine, tryptophan and tyrosine (among the 10 studied amino acids) were comparable to controls. It could be hypothesised that proteolysis was not decreased as a consequence of treatment, although subsequent catabolism of amino acids did not proceed to the same extent.

2-Hydroxybutyrate and 2-aminobutyrate are metabolites of 2-oxobutyrate (product of catabolism of threonine), converted respectively by dehydrogenases (either lactate dehydrogenase or α-hydroxybutyrate dehydrogenase, an isoform of lactate dehydrogenase present in the heart [27]), or by transaminases. Although this suggests that both activities must be enhanced on admission, compared with controls, it could be hypothesised that the efficiency of the dehydrogenases is higher, as 2-aminobutyrate levels were identified at lower values, whereas 2-hydroxybutyrate levels were found to be higher in patients at onset.

As threonine levels increased, signals for 2-hydroxybutyrate decreased, and a "peak" at day 4 for 2-aminobutyrate was found. There was a net reduction in these two metabolites, which should correspond to higher 2-oxobutyrate conjugation with cysteine for generation of cystathionine, because activities of lactate dehydrogenase and transaminases are known to be decreased under beta-blocker treatment.

Furthermore, oxidative stress or detoxification demands can dramatically increase the rate of hepatic glutathione synthesis. Under such metabolic stress glutathione can be *de novo* synthesised from cystathionine, releasing 2-hydroxybutyrate as a by-product [28]. This synthesis of reduced glutathione can be increased in patients, because under stress conditions the pentoses-phosphate metabolic pathway (the main source for $NADPH+H^+$), is reduced, and it is essential for obtaining reduced glutathione from its oxidised form. Other studies have found increased 2-hydroxybutyrate [8,9], and it has been proposed as an early biomarker of insulin resistance [29].

4-Hydroxyproline also was found to exist at increased concentrations post ACS, and remained high six months later. As previously commented, proteolysis induces collagen breakdown and may predispose to plaque rupture [25]. In fact, macrophages have been demonstrated *in vitro* to degrade collagen from atherosclerotic plaques resulting in release of hydroxyproline into the medium [30]. In addition, retro-inhibition by the substrate of the last aldolase of this pathway can also be considered.

Hyperhydroxyprolinemias are known only as result of inherited metabolic diseases. Although they are considered to be benign, as 4-hydroxyproline may account for 10–13% of collagen, and collagen may account for 30% of all the proteins in human organism, catabolism of 4-hydroxyproline becomes a significant source for glyoxylate and pyruvate. It has been previously described in rat mitochondria that oxalate formation from 4-hydroxyproline was significantly reduced when serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT) had been induced by prior injection of glucagon, because glyoxylate from 4-hydroxyproline can be very efficiently metabolised to glycine, reducing the harmful formation of oxalate [31]. In this study, an increase in glycine was not observed at onset, maybe because the catabolism of glycine into NH_3 and CO_2 was very active due to epinephrine and norepinephrine. Nevertheless, during the treatment with beta-blockers for 6 months (lower glucagon expected), 4-hydroxyproline was not reduced, oxalate remained lower than in controls, and glycine showed a tendency to be increased; this could be a result of decreased glucagon and therefore reduced conversion of 4-hydroxyproline into glyoxylate and oxalate, as well as reduced glycine catabolism.

Aminomalonate has been unequivocally identified as one of the most important signals for the classification of samples. Nevertheless, the biochemical meaning of this significance is still a matter of research, because its role in metabolism is unknown.

After 6 months under treatment, it can be seen that levels of metabolites are not comparable to controls. Among the 27 metabolites presented herein, only 8 showed signals comparable to controls. Moreover, only 4 among the identified metabolites did not change throughout the 6 months studied, justifying the time trajectory observed in the score plot of the PLS-DA (Fig. 1).

The lower availability of fatty acids and lower activity of transaminases could have been induced by beta-blocker treatment. Therefore, carbohydrate metabolism may have become more important as a source of energy, although hyperglycemia and hyperlactatemia could be still present because insulin resistance (if present) had not been treated.

Many of these changes have previously been described in independent contexts; however the main novelty in this work is the capability of modelling different metabolite changes in a simultaneous way.

5. Conclusion

In summary, this study showed for the first time in patients that have suffered from ACS, the time line changes in metabolite concentrations throughout the 6 months of the investigation compared to the controls. Thus with these new ideas that are emerging more detailed knowledge of the changes in the metabolism can lead to support new diagnosis strategies (biomarkers) and/or therapies, including those treatments that are designed to specifically address changes in the metabolism induced by ACS, such as glucose-insulin-potassium or 3-ketoacyl CoA thiolase inhibitors [32].

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