

Metabolomics: The Cornerstone for Discovering New Biomarkers in Atherosclerotic Cardiovascular Disease

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ABSTRACT

For metabolomics researchers it is challenging to predict how much the metabolomic study of biological fluids will transform the diagnostic methods currently used in medicine. Alongside analytical techniques achieving enhanced sensitivity, our knowledge of the collateral effects of diseases will exponentially increase as well. Unquestionably, this development will bring greater diagnostic capacity with patients being diagnosed at earlier stages and therefore increasing survival rates. Furthermore, a deeper understanding of the pathophysiology of disease would allow us to develop a personalized medicine based on the particular situation of each patient, optimizing the effectiveness of pharmacological treatments. Bearing in mind that these are only two of many possibilities that this knowledge will result in, it is essential to establish a solid basis in metabolomic theory to achieve all their expected benefits.

INTRODUCTION

Atherosclerosis is a heterogeneous disease generally supported by other pathologies including hypertension, hyperlipidemia and diabetes. Along them, multiple factors of different nature such as smoking or a sedentary lifestyle will combine each other to catalyze an irreversible process that will develop over decades and that will eventually trigger an Acute Coronary Syndrome

(ACS). Even though the mechanisms underlying the formation of the atherosclerotic plaque and its progression are not entirely identified, a progressive thickening of the arterial wall and a consequent accumulation of fatty materials, macrophages and muscle cells into the intima can be safely considered as the origin of the atherosclerotic process [1-4]. During this migration, smooth muscle cells will secrete growth factors and will produce matrix proteins i.e. collagen, elastin and proteoglycans, which will form the fibrous cap of the plaque.

Subsequently to this process, the occlusion of the vessel lumen will occur and the first ischemic manifestations, depending on the presence or absence of collateral circulation, may appear. The most advanced and complicated plaques will also show fibro calcified degeneration with intracellular and extracellular lipids, calcium, necrotic debris, extravasated blood and a layer of fibrous tissue [5]. Their instability will be partly determined by local and systemic factors such as infection, inflammation and autoimmune or genetic influences [6]. In their latest stages, this instability could result in wall weakening, cracking or breaking, bleeding, thrombosis and ulceration. This phenomenon will produce the rupture of the atherosclerotic plaque and the onset of the ACS [7].

From a clinical angle, this silently and steady progress constitutes the greatest trouble for its early diagnosis, with symptoms only becoming evident when it has already reached an irreversible stage. This limitation clearly remarks the need for new biomarkers with real clinical value to both, predict the disease and establish a stratification of individual cardiovascular risk.

METABOLOMICS

A Promising Approach for Cardiovascular Diseases

Metabolomic studies are recently emerging as one of the most interesting ‘-omics’ technologies that complement genomics, but especially proteomics, in the search of new clinical biomarkers [8,9].

Definitely, one of the most interesting features of this technology is that we can use different approaches depending on our interest. For example, if we want to have a global picture of the metabolome of a particular biological sample, the metabolic fingerprinting would be the most appropriate choice. It describes the unbiased analysis of the metabolome by examination of metabolite patterns in different experimental groups and their subsequent classification. In this approach, metabolites are typically described by m/z values and retention times, and the using of an adequate database can be very useful to their correct identifications [10].

On the other hand, if we have already identified a selected number of metabolites and intend to quantify them in different samples, we should use metabolic profiling. It constitutes a widespread tool to study different aspects of metabolism and the individual contribution of metabolites in biochemical pathways. It is also particularly attractive when our interests focus on low-abundance metabolites, which would not be detected by the fingerprinting approach [10].

Whatever the selected technique, it is beyond question the massive benefits that we will get from a deep and adequate knowledge of the composition of body fluids. However, despite major advances achieved in the recent years, acquiring this information still depends on the sensibility of the analytical method used, and it currently constitutes the greatest obstacle with metabolomic research still being at its early childhood. This opening period emphasizes the need to establish a solid knowledge with regard to the technologies used to ensure the success of future experiments.

Biological Samples

Human biofluids are excreted or secreted fluids including blood, urine, saliva and extravascular fluids. Among them, plasma/serum and urine are considered the most promising sources for the discovery of novel diagnosis and prognosis biomarkers of disease.

Plasma/Serum

Plasma represents the most interesting biological sample for discovering candidate metabolites due to its direct communication with almost all body cells, which release at least a part of their content into them upon damage or death [11,12]. It converts serum/plasma in the richest metabolite-containing sample, with metabolites and proteins originated from circulating blood cells and other tissues.

In fact, several diseases as cancer [13], respiratory disorders [14] or infections [15] have already been studied using metabolomics approaches.

Definitely, a comprehensive and systematic characterization of the serum/plasma metabolome will greatly facilitate the finding of new biomarkers for early detection and diagnosis of these and other diseases [16,17].

Urine

Urine is along with plasma, the biological sample of easier collection. It contains the final products of several metabolic pathways, many of which appear only under disease conditions. Unfortunately, the important influence of lifestyle factors such as diet, physical exertion, and stress on their profiles constitutes an important source of variability that must be carefully evaluated when using these samples for biomarker discovery [18]. Additionally, the high content of contaminants that may be present (microorganisms, cells, urinary crystals, etc.) requires a previous extraction with organic solvents before the analysis. This step also permits to modify the experimental conditions (pH, solvent polarity, etc.) to obtain a particular fraction of desired metabolites in order to optimize the identification process. An illustrative example of the potential of urine in cardiovascular metabolomics is the implication of the arginine-proline metabolism recently revealed in atherosclerosis [19].

Extravascular fluids

Most of metabolomic studies are restricted to plasma and urine [11,12], however the using of extravascular fluids (cerebrospinal, synovial, ascitic and pericardial fluid), can be indicated in

some particular diseases. For example, the study of cerebrospinal fluid has focused great interest in the case of neurological diseases [20,21]. In the case of Atherosclerotic Coronary Diseases (**ACD**), despite being a sample of difficult access, metabolomic analysis of pericardial effusion may be of great interest to elucidate the concomitant inflammatory process. Since extravascular fluids are ultra-filtrates of plasma, their study could be developed with the experimental conditions used for plasma.

Tissue and cell culture

The metabolomic analysis of a tissue represents a major challenge for clinical research. The possibility of carrying out a direct study of damage tissues will exponentially increase our understanding of the pathophysiology of diseases. Unfortunately, the needed process for its proper study is tedious and clearly undeveloped therefore cannot be sure if the extraction of the metabolites has been completely carried out. Some possibilities for this extraction are 1) osmotic lysis via hypotonic medium, 2) repeated freeze-thaw cycles, 3) the using of a detergent solution and 4) sonication.

Whereas these mentioned methods improve, the metabolite analysis of cell cultures can be of extraordinary interest to characterize both, extracellular and intracellular metabolic profiles [22,23]. Extracellular profile only requires prior centrifugation to separate culture media and cells before the analysis. Once it has been separated, the necessary protocol is not different from plasma. Definitely, the intracellular profile will provide more complete information about potentially altered pathways and a pre-treatment step will be necessary for metabolite extraction from cells as previously explained in tissue.

Current Strategies

Comprehensive identification and quantification of metabolites from complex biological matrices remains a challenging task for analytical chemistry [24]. No single method can achieve this goal. For example, highly volatile compounds like terpenes cannot be assessed with Liquid Chromatography-base methods (**LC**) and Gas Chromatography (**GC**) must be used. Similarly, LC is clearly better suited for separation of polyphenols than Capillary Electrophoresis (**CE**), whereas bis- and trisphosphates might be most suitable for CE-separations. Nevertheless, each of these three major separation methods allows the detection and quantification of a range of different compounds in a multiparallel way, especially when mass spectrometry is used for unambiguous and sensitive detection.

Nuclear magnetic resonance

Nuclear Magnetic Resonance (**NMR**) is a spectroscopic technique with interesting advantages for metabolomic analysis, such as great ability to detect multiple metabolites in a single experiment [25], being a not destroying-sample technique and not requiring previous pre-treatments. It also offers structural information that can facilitate the identification of unknown metabolites.

Between its disadvantages are poorer sensitivity and resolution when compared with MS, which limits its ability to identify low-concentration metabolites [26].

Liquid chromatography-mass spectrometry

Liquid Chromatography coupled to Mass Spectrometry (**LC/MS**) using Electrospray Ionization (**ESI**) is the most currently used platform for metabolomic analysis [27,28]. Its extended utilization is essentially due to its high versatility, which allows the analysis of a wider variety of molecules than GC/MS, being therefore considered as the platform of choice when it is necessary to analyse a wide range of metabolites with very different physicochemical properties.

Gas chromatography-mass spectrometry

Gas Chromatography coupled to Mass Spectrometry (**GC/MS**) is a platform commonly used in metabolomics and constitutes an excellent choice for the analysis of volatile compounds such as fatty acids and organic acids.

Since GC separation occurs according to the different boiling points of the metabolites, it is necessary them to be volatile and thermally stable. In most cases, the set of metabolites in a sample will not meet these conditions, thus it is necessary a previous derivatization step with appropriate reagents before the analysis [10,29,30]. This pre-treatment of the sample may produce the formation of side products, which will result in qualitative and quantitative errors. In fact, a large overlap of identified compounds is usually observed when comparing results between the major separation methods, despite the large differences in separation mechanisms. The underlying reason for this high degree of overlap is the conserved nature of the most abundant metabolic intermediates [31].

On the positive side, one of the most useful features when using GC/MS for metabolomics studies is the existence of mass spectral libraries, which hugely facilitates the identification process. Additionally, the GC has superior resolving power than LC and their retention times are more robust and reproducible [32].

Consequently, the physicochemical characteristics of the interesting metabolites will be the most important criteria to choose the most suitable platform. In the case of carrying out the study of a set of metabolites with a wider range of physicochemical properties, LC/MS will permit to determine a larger number of metabolites. On the contrary, for the study of highly hydrophobic compounds such as fatty acids, GC/MS will be the platform of choice because of its excellent sensitivity and resolution. NMR is not particularly influenced by the physicochemical characteristics of the metabolites and, despite its lower sensitivity and resolution, it could be considered as a supporting technique for determining the structure of unknown metabolites.

Workflow

Once the best platform has been carefully selected according to the characteristics of the study, the analysis and identification processes will be developed in three different stages:

1) Sample preparation 2) Separation and identification of metabolites 3) Data collection and statistical analysis.

Sample preparation

This step involves the extraction and enrichment of metabolites in an organic solvent with the secondary objective of removing the existing proteins. In general, any previous step included before analysis will result in some loss of sample, thus it is necessary to carefully select the appropriate preparation protocol for limiting these pre-treatment steps and to minimize the losses [10,33].

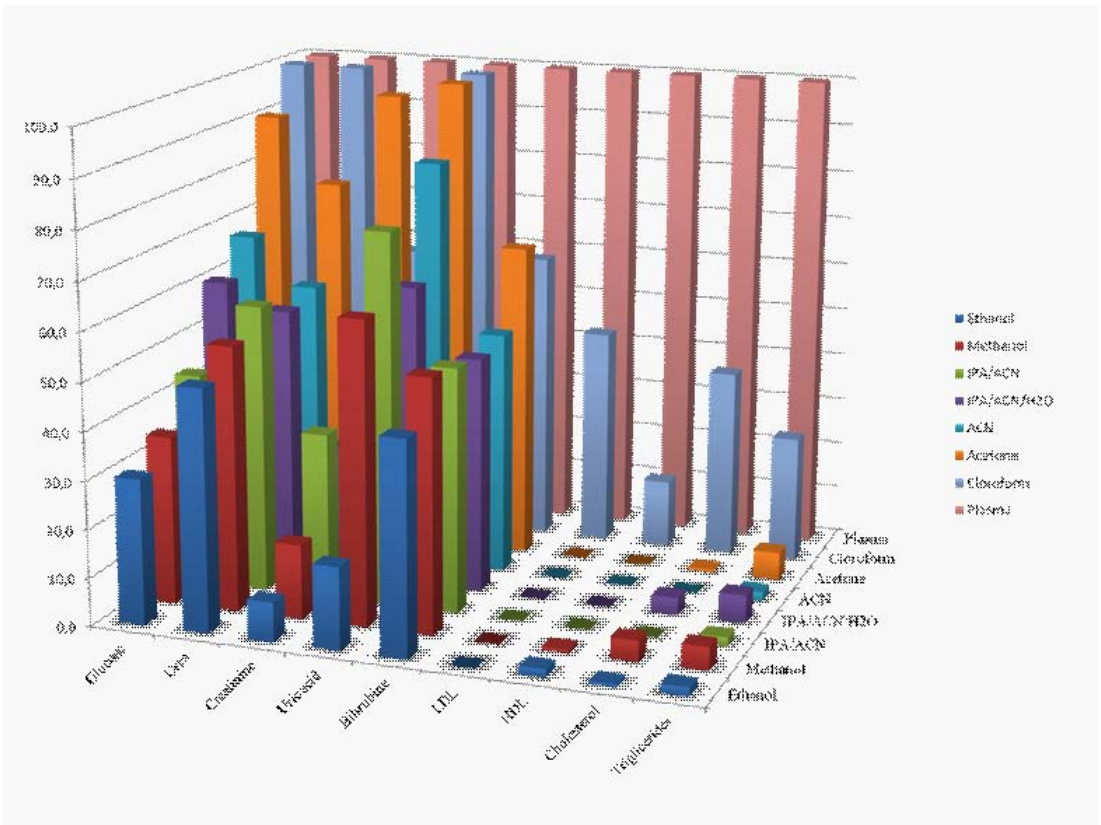


Figure 1: Recovery percentage of several commonly used metabolites in clinical practice using different organic solvents.

Figure 1 shows the recovery percentages of nine common biomarkers of clinical use depending on the organic solvent used. This study was carried out by our laboratory in order to find the best solvent for our subsequent metabolomic studies. Note the direct relationship existing between the polarity of the pair solvent/metabolite and the recovery obtained.

Separation and identification of metabolites

As previously explained, the great variability in the physicochemical properties of metabolites in terms of polarity, solubility and volatility, as well as its wide dynamic range of concentrations requires the combined use of various techniques. The joint use of NMR and MS will allow to reproducibly measure the largest number of metabolites in a given sample [34,35].

Data collection and statistical analysis

Metabolomic analyses generate large amounts of data that require correct interpretation. Ideally, data analysis software must be able to remove noise from the spectra, properly identify which metabolite generates every chromatographic peak and make a correct alignment of the peaks corresponding to the same compound in several successive samples [36,37]. In fact, most companies usually include their own programs to generate data and undergo the statistical analysis [38]. When the identification and quantification of the metabolites have been completed, a multivariate statistical analysis must be performed to determine which metabolites show significant differences between experimental groups.

At this point, it is important to underline the error that can be introduced due to small variations in the experimental conditions or the preparation of different samples. An easy way to overcome this shortcoming consists in using an internal standard to normalize the data.

A PRACTICE CASE: PLASMA METABOLOMICS USING GC/MS IN PATIENTS WITH ACUTE CORONARY SYNDROME

As it has been previously explained the discovery of new biomarkers is critical for early diagnosis of atherosclerotic cardiovascular diseases. This chapter is focused on metabolomics and the possibilities that it offers to improve our knowledge of the physiopathology of a disease through the identification of altered metabolic pathways.

For this reason, and with the objective of providing a better understanding of the metabolomics workflow, an example of our studies in Acute Coronary Syndrome (**ACS**) is shown with a resumed protocol and the results obtained [31].

Materials and Reagents

Blood samples were collected in sterile EDTA containers from Non-ST Segment Elevation ACS (**NSTEACS**) patients and sex and age matched healthy subjects. They were immediately taken to our laboratory to prevent sample degradation (<2 h) and centrifuged. The resulting plasma was aliquoted and stored at -80°C until analysis [31].

Methods

To perform the metabolomic analysis by GC/MS, the protocol described by Jiye et al. showed in (Figure 2) was used with minor modifications [39]. Briefly, 50 µl of thawed plasma were

vortex-mixed with 50 μ l of acetonitrile to precipitate the plasma proteins and centrifuged. 75 μ l of supernatant was carefully separated and dried out in a heating block at 70°C for 1 h. This dried aliquot was derivatized by methoximation adding 40 μ l of methoxiamine hydrochloride in pyridine and kept for 24 h at room temperature. Later, 10 μ l of BSTFA + 1 %TMCS was added and kept for 1 h in a heating block at 70°C for 60 min for trimethylsilylation. Finally, 5 μ l of methyl pentadecanoate (as internal standar, IS) was added and the mixture was centrifuged. Supernatant was separated for analysis [31].

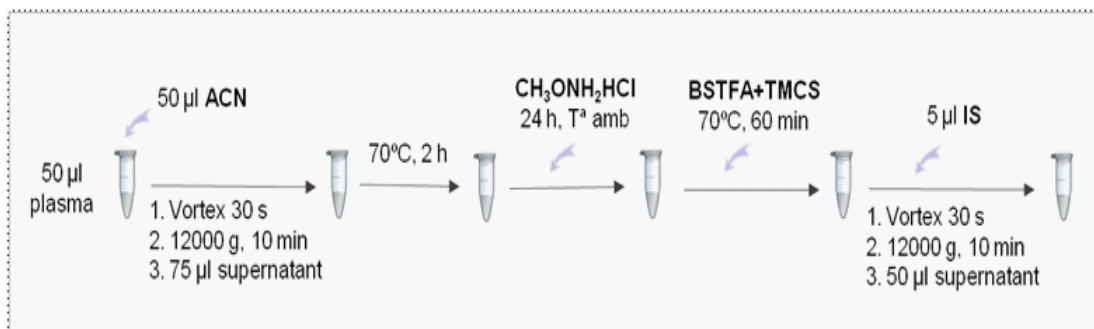


Figure 2: Protocol for plasma metabolomic analysis using GC/MS31.

Experimental Conditions

Derivatized samples (1 μ l) were analyzed using an Agilent 6890N GC system, coupled to an Agilent 5975c single quadrupole MSD [31]. All samples were injected in triplicate and the average normalized peak area for every metabolite was used for further analysis. The GC oven was programmed with an initial temperature of 60°C and then increased to 285°C at 5°C/min and held at the final temperature for 2 min. High purity helium was used for the carrier gas at a flow rate of 1.5 mL/min. The injection port temperature was 230°C and the interface temperature was set at 290°C. The EI source temperature was set at 230°C with energy of 70 eV. The selected mass range was 50-600 m/z and the selected scan speed was 0.99 scans per second. Identification of GC/MS detected peaks was performed by direct comparison of their mass spectral with the NIST02 MS library.

Results

A total of 69 metabolites (including IS) were identified in healthy subjects and NSTACS patients. Principal Component Analysis (**PCA**) was used as multivariate non-targeted analysis. Two well-separated groups were obtained in the score plot suggesting that the plasma metabolomic profiling of the NSTACS patients was significantly different from that of the healthy subjects (Figure 3) [31].

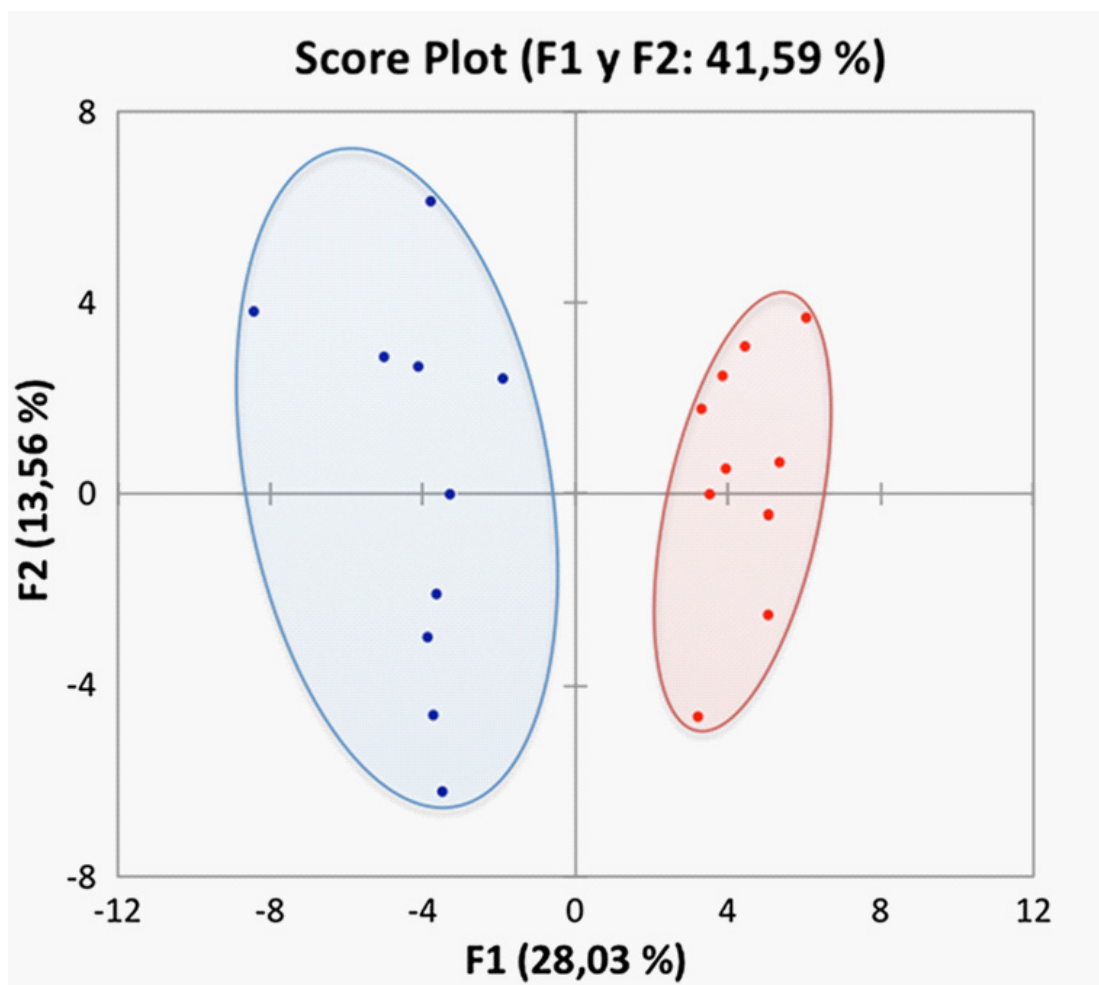


Figure 3: PCA. In the score plot, healthy controls (left, blue) and NSTEMI patients (right, red) are represented.

Statistical analysis using a Student's t-test of the normalized areas of every chromatographic peak resulted in the existence of 18 metabolites whose normalized peak areas significantly varied ($p < 0.05$) between both experimental groups. Of these, 10 metabolites (2-OH butyric acid, 3-OH butyric acid, 2-OH benzoic acid, palmitic acid, citrate, cis-vaccenic acid, lactate, alpha-OH valeric acid, 5-oxo-proline and stearic acid) were increased in patients and 8 metabolites (alanine, 2-keto-3-methylvaleric acid, serine, leucine, threonine, glycine, 5-OH tryptophan and linoleic acid) were significantly decreased (Figure 4) [31].

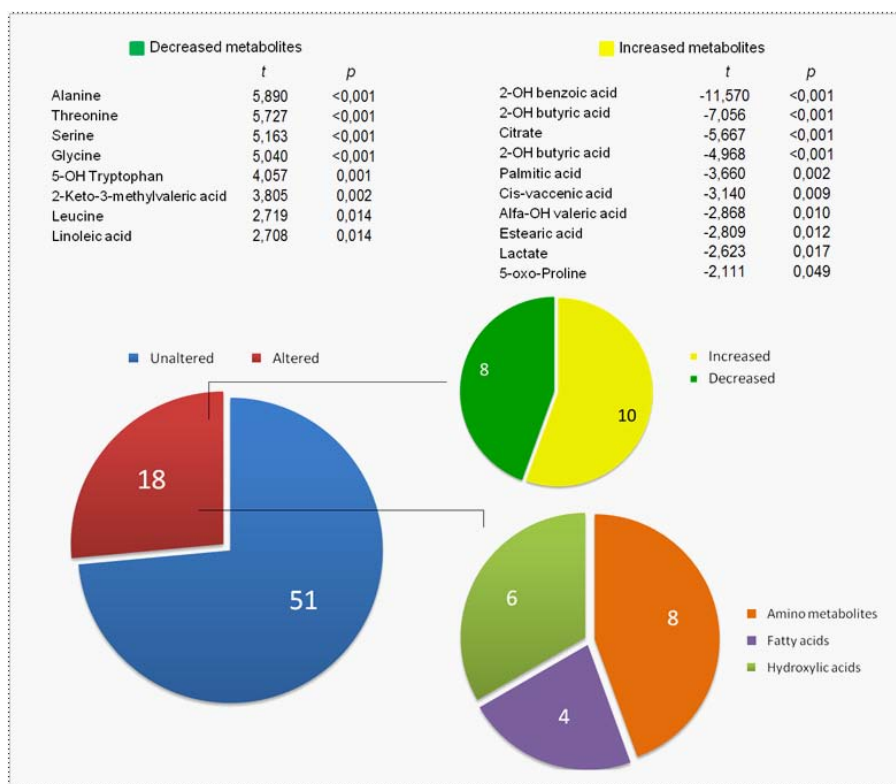


Figure 4: Results obtained in the plasma metabolomic analysis using GC/MS31.

Potential Panel of Biomarkers

The results of our analysis revealed the complex network of metabolic pathways altered in the course of ACS. In our case, we considered that hypoxia and oxidative stress are the basis to understand and explain how the complex metabolism of every cell must adapt to the situation of lack of oxygen.

We also validated the altered concentrations observed in three metabolites, 5-OH tryptophan, 2-OH butyric acid and 3-OH butyric acid by GC/MS and LC/MS.

5-OH tryptophan

The existence of quantitative alterations in plasma levels of amino acids and their metabolites can be explained considering either a decrease in anabolic activity of these metabolites or an increase in the catabolic pathways [40,41].

The 5-OH tryptophan is the first metabolite in the biochemical pathway for serotonin synthesis. We suggest that under hypoxia conditions, the diminution of plasmatic levels of 5-OH tryptophan may reflect a diminished synthesis of serotonin since cells prioritize its use as a precursor of glucose versus serotonin production.

2-OH butyric acid

Glutathione is the main endogenous antioxidant produced by cells. It neutralizes free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants like vitamins C and E in their reduced forms. Oxidative stress conditions dramatically increase the rate of hepatic synthesis of glutathione [42]. It is synthesized from the amino acids cysteine, glutamic acid and glycine. Under these conditions of metabolic stress, the supply of cysteine for glutathione synthesis become limiting and homocysteine is used to form cystathionine, which is cleaved to cysteine and 2-OH butyric acid. Thus elevated plasma levels of 2-OH butyric acid can be a good biomarker of cellular oxidative stress for the early diagnosis of ACS.

3-OH butyric acid

Another altered metabolite of similar structure was 3-OH butyric acid, a ketone body together with the aceto acetate, and acetone. Elevated levels of ketone bodies in blood and urine mainly occur in diabetic ketoacidosis [43]. Type 1 Diabetes Mellitus (**DMI**) patients have decreased levels of insulin in the blood that prevent glucose enter cells so these cells use the catabolism of fats as energy source that produce ketones as final products. We hypothesize that the high plasma levels observed in NSTEACS patients can be due to hypoxia more than to DMI, since just 16% of our NSTEACS patients had been diagnosed with DMI. However, pre-diabetic conditions in patients such as impaired glucose tolerance and insulin resistance should also be taken into account in terms of 3-OH butyric acid blood levels. In this theoretical scenario, we postulate that the hypoxia situation comes to “mimic” the physiological situation that occurs in DMI. In this case, the low energy yield of glucose metabolism “forces” these cells to use fat as energy source (through catabolism independent of aerobic/anaerobic conditions) occurring ketones as final products. In our experiment, the 3-OH butyric acid was strongly elevated in NSTEACS patients compared to healthy controls which would reflect the situation of hypoxia that are suffering the heart cells.

Finally, this exciting theoretical basis is supported by our experimental results obtained through two different chromatographic techniques (GC/MS and LC/MS). Despite these interesting results, further studies are necessary to achieve a stronger evidence of its validity. If these confirm our results, we strongly believe that the panel of biomarkers consisting of 5-OH tryptophan, 2-OH butyric acid and 3-OH butyric acid will have a great potential for early diagnosis of ACS [31].

CONCLUSION

Every day, metabolomics is increasing its prominent role as one of the most potential techniques in finding new biomarkers of disease. In the case of cardiovascular diseases, inflammation, oxidative stress and hypoxia at advanced stages, are three phenomena that significantly modify the balance of many metabolic pathways and subsequently the concentrations of their metabolites. These changes provide us with a golden opportunity to identify those disorders caused by the atherosclerotic process through a comprehensive and detailed analysis of biological fluids.

Of all them, it is clear that plasma stands out as the sample of greatest interest, thanks to its direct contact with all body tissues and its easy access emerging as the centre of our present and future works.

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