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# Comparison of Analytical Methods Of Serum Untargeted Metabolomics

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***Abstract*** **–** Metabolomics has emerged as a powerful tool in the discovery of new biomarkers for medical diagnosis and prognosis. However, there are numerous challenges, such as the methods used to characterize the system metabolome. In the present work, the comparison of two analytical platforms to acquire the serum metabolome of critically ill patients was conducted. The untargeted serum metabolome analysis by ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) enabled to identify a set of metabolites statistically different between deceased and discharged patients. This set of metabolites also enabled to develop a very good predictive model, based on linear discriminant analysis (LDA) with a sensitivity and specificity of 80% and 100%, respectively. Fourier Transform Infrared (FTIR) spectroscopy was also applied in a high-throughput, simple and rapid mode to analyze the serum metabolome. Despite this technique not enabling the identification of metabolites, it allowed to identify molecular fingerprints associated to each patient group, while leading to a good predictive model, based on principal component analysis-LDA, with a sensitivity and specificity of 100% and 90%, respectively. Therefore, both analytical techniques presented complementary characteristics, that should be further explored for metabolome characterization and application as for biomarkers discovery for medical diagnosis and prognosis.

***Keywords:*** Metabolomics; Mass Spectrometry; Liquid Chromatography; Fourier Transform Infrared Spectroscopy

## I. INTRODUCTION

Metabolomics is at the downstream of genomics, transcriptomics and proteomics and, consequently, provides a direct reflection of an organism's status and dynamic responses to various disturbances arising from genetic and environmental factors, such as microbiota, diet, stress, gender, age, lifestyle, and diseases [1]–[3]. For that, untargeted biofluids metabolomics has been applied in order to discover biomarkers for medical diagnosis and prognosis. Biofluids, e.g., plasma, serum, and urine, reflect the organism's pathophysiological state [4], with the advantage of being obtained by non-invasive or minimally invasive methods. Indeed, biofluids metabolomics have been applied as a starting point towards personalized medicine [5], by allowing the discovery of biomarkers for disease diagnosis such as Parkinson's disease [6], type 2 diabetes [7], and transplant rejection [8], among others. Metabolomics involves the profiling of a system metabolites, i.e., molecules with molecular weights below 1.5 kDa, including amino acids, peptides, sugars, lipids, among others, as well as the understanding of their interactions, mechanisms of action, and functions within the metabolic pathway network. The Human Metabolome Database (HMDB) reports that the human body contains over 250,000 metabolites [9].

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Biofluids metabolomics, is usually retrieved by ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). An alternative technique to retrieve the system metabolic state is Fourier Transform Infrared (FTIR) spectroscopy, that, despite not being used to identify the set of metabolites, is applied in a more holistic mode to acquire, in a highly sensitive and specific mode the molecular fingerprint associated to a defined metabolic state [10]. Biofluids' FTIR spectroscopic analysis associated to multivariate data analysis, including machine learning algorithms, have been applied, for medical diagnosis [11], [12]. The study's aim was to analyze the serum metabolome of critically ill patients using untargeted UPLC-MS/MS and FTIR spectroscopy. The focus was on developing mortality prediction models and identifying other molecular data that could enhance the management of critically ill patients admitted to the intensive care unit (ICU).

## II. MATERIALS AND METHODS

### *A. Biological Samples*

Serum samples from 6 male Coronavirus Disease 2019 (COVID-19) patients (3 discharged and 3 deceased), admitted to the ICU of *Hospital São José, Centro Hospitalar Universitário Lisboa Central*, were considered, according to all legal requirements and ethics approval from the Hospital's Ethics Committee. The two groups of patients (deceased and discharged from ICU) were not statistically different ($p>0.05$) concerning age and comorbidities. The serum samples were deproteinated by mixing with methanol at 75:265 (v/v), followed by centrifugation at 18,000×g for 15 minutes at 4 ºC according to [13]. Each serum sample was submitted to this procedure in triplicates and kept at -80ºC till analysis.

### *B. UPLC-MS/MS*

Each sample was analyzed in triplicate using an UPLC system coupled with a QqTOF Impact II mass spectrometer and an electrospray ion source (Bruker Daltonics GmbH & Co.). Reverse-phase (RP) and Hydrophilic interaction liquid chromatography (HILIC) were used. A 25 µM solution containing quercetin, L-tryptophan (indole-d5), L-valine (d8), sulfolene (d4), and N, N-dimethyl-d6 glycine HCl was prepared and used as quality control (QC). These QC samples were analyzed every six hours to ensure that chromatographic resolution and spectrometer detection weren't changed throughout time. MS data was acquired using Data Analysis (Bruker Daltoniks), converted to mzXML with ProteoWizard MSConvert and uploaded to the XCMS server, were data processing, including feature detection, retention time correction, peak alignment, METLIN annotation, pairwise sample comparison, multimodal analysis (independent of separation and acquisition modes), and global metanalysis were conducted [14]–[18].

### *C. FTIR spectroscopy*

Each sample was analyzed in quadruplicate in a 384-well silicon microplate. Spectra were acquired with a FTIR spectrometer (Vertex 70) associated to a HTS-XT (Bruker Optics GmbH & Co. KG). Each spectrum resulted from 64 scans with a 2 $cm^{-1}$ resolution on mid-infrared (MIR). Atmospheric and baseline correction of the spectra was performed on OPUS software (version 6.5, Bruker Optics GmbH & Co. KG.).

### *D. Data Analysis*

Principal component analysis (PCA) and Linear Discriminant analysis (LDA) were conducted with ORANGE Data Mining 3.32, IBM SPSS Statistics version 26 and The Unscrambler® X (CAMO software AS, version 10.4, Oslo, Norway), respectively.

## III. RESULTS AND DISCUSSION

Over 1800 features, i.e., *m/z* peaks, were detected by UPLC-MS/MS. From those features, 12 metabolites were identified as significantly different between the deceased and discharged patients ($p<0.01$) (Fig.1A).

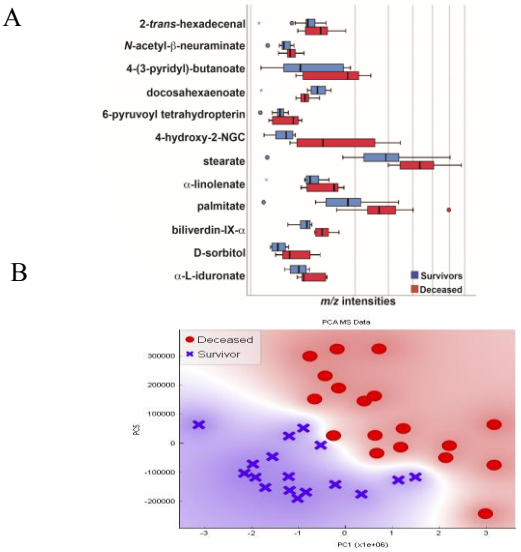


**Fig. 1.** Metabolites significantly different ($p<0.01$) between deceased and discharged patients' serum samples (A), and the corresponding PCA considering the most intense *m/z* peak of those metabolites (B).



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Some of these metabolites are associated with SARS-CoV-2 pathogenesis, as the up-regulation in deceased patients of 2-trans-hexadecenal, palmitate, iduronate and sorbitol are associated to the increase of apoptotic events and immunogenic responses by the host [19]–[22]. The up-regulation of stearate, α-linolenate and 4-hydroxy-2-nonenal-glutathione conjugate can be associated with oxidative damage and mitochondrial insult [23], [24]. The up-regulation of N-acetyl-β-neuraminate and biliverdin in deceased patients are associated to renal and hepatic failure [25]–[28]. The down-regulation in deceased patients of (4Z,7Z,10Z,13Z,16Z,19Z)-docosahexaenoate is interesting as this metabolite has been associated to neuroprotection, inflammatory and immune response modulation [29], [30].

PCA of the most intense *m/z* peak of each 12 metabolites (Fig. 1B) points to a separation between scores of the two patients' groups. Interestingly, the loading vector of PC1 highlighted the following molecules with a high contribution for sample separation according to the patients' group: palmitate, stearate, biliverdin, iduronate, sorbitol and 2-trans-hexadecenal.

LDA was conducted, based on the most intense *m/z* peak of the 12 metabolites, where 80% of data was used for model training and 20% as an independent data set for model validation. As previously pointed out by the PCA score-plot, a very good LDA model enabled to predict mortality in the validation model with a sensitivity and specificity of 80% and 100%, respectively.

It was also evaluated the FTIR spectra of serum metabolome. The average spectra of deceased patients presented different wavenumber bands in comparison to discharged patients (Fig. 2A). Indeed, the spectra PCA pointed a separation between scores of the two groups of patients (Fig. 2B). PC3 loading vector pointed the following wavenumbers as relevant for sample separation between the two patients' groups: ≈ 1650 $cm^{-1}$ due to N-H vibrations and ≈2920 $cm^{-1}$ due to $CH_2$ vibrations in lipids (Fig. 2C). This was according to previously identified metabolites by UPLC-MS/MS platform, including the up-regulation in deceased patients of diverse lipidic metabolites (e.g., hexadecenal, butanoate, stearate, linolenate, palmitate, and biliverdin at ≈ 1650 $cm^{-1}$).

The mortality predicting model based on FTIR spectra PCA-LDA was also trained on 80% of data and validated on 20% independent data set. The validation model, lead to very high sensitivity and specificity of 100% and 90% respectively.

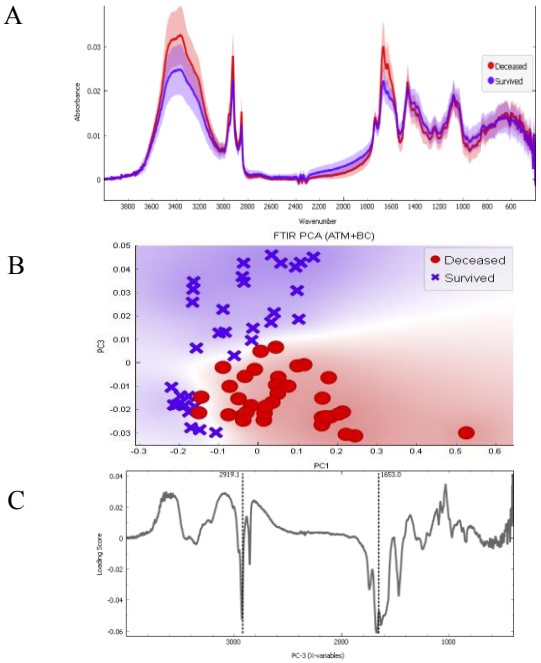


**Fig. 2.** FTIR spectra of serum metabolome (A) and its corresponding PCA (B), and the PC3 loading vector (C).

As observed, with both MS and FTIR spectroscopic data, it was possible to separate the samples according to the patients' outcome. Mortality predicting models based on LDA or PCA-LDA, were also developed based on both techniques, leading to sensitivities and specificities higher than 80%. A better predictive model was achieved with FTIR spectroscopy as a 100% sensitivity was achieved against 80% obtained with the metabolite set identified by UPLC-MS/MS. This is a preliminary result, since a very small study sample was used.

The UPLC-MS/MS technique is more laborious, complex and time consuming than FTIR spectroscopy. However, its advantages include the identification of metabolites, that can facilitate biomarkers validation and acceptance by the community. Furthermore, the identification of metabolites also helps to identify dysregulated metabolic pathways, and consequently the understanding of the underlined pathophysiologic mechanisms, which can be used to improve and develop new therapies. In the future, the study sample will be increased to retrieve more robust classification models, enabling its application in real ICUs context. FTIR spectroscopy can be applied in a much simpler, rapid, and economic mode. It can also be conducted in high-throughput, as the one used in the current study,



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based on a microplate with 384 wells. Therefore, FTIR-spectroscopy represents a very interesting and complementary technique for the metabolome characterization and for the development of predicting models for medical diagnosis and prognosis.

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