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Urinary and plasma metabolite differences detected by HPLC-ESI-QTOF-MS in systemic sclerosis patients

Álvaro Fernández-Ochoa[a,b], Rosa Quirantes-Piné[a], Isabel Borrás-Linares[a,*], David Gemperline[c], PRECISESADS Clinical Consortium , Marta E. Alarcón Riquelme[d], Lorenzo Beretta[e,1], Antonio Segura-Carretero[a,b,1]

[a] Research and Development of Functional Food Centre (CIDAF), Health Science Technological Park, Granada, Spain
[b] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Granada, Spain
[c] Eli Lilly and Company, Indianapolis, United States
[d] Centre for Genomics and Oncological Research (GENYO) Pfizer, University of Granada, Andalusian Government, Health Science Technological Park, Granada, Spain
[e] Scleroderma Unit, Referral Center for Systemic Autoimmune Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy

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ABSTRACT

Systemic Sclerosis (SSc) is a chronic autoimmune disease whose origin and pathogenesis are not yet well known. Recent studies are allowing a better definition of the disease. However, few studies have been performed based on metabolomics. In this way, this study aims to find altered metabolites in SSc patients in order to improve their diagnosis, prognosis and treatment. For that, 59 SSc patients and 28 healthy volunteers participated in this study. Urine and plasma samples were analysed by a fingerprinting metabolomic approach based on HPLC-ESI-QTOF-MS. We observed larger differences in urine than plasma metabolites. The main deregulated metabolic families in urine were acylcarnitines, acylglycines and metabolites derived from amino acids, specifically from proline, histidine and glutamine. These results indicate perturbations in fatty acid beta oxidation and amino acid pathways in scleroderma patients. On the other hand, the main plasma biomarker candidate was 2-arachidonoylglycerol, which is involved in the endocannabinoid system with potential implications in the induction and propagation of systemic sclerosis and autoimmunity.

1. Introduction

Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by immune system activation, endothelial damage and widespread vasculopathy, fibrosis of the skin and of internal organs [1]. Despite recent progress, the pathogenesis of SSc remains elusive and so its treatment, with most patients experiencing long term disability, severe morbidity and increased mortality ratios compared to the general population or to other systemic autoimmune diseases [2].

A better understanding of biological pathways involved in SSc development is mandatory to tackle the processes that lead to disease progression and for the discovery of effective therapies. Ideally this process should be carried on an individual basis and considering the broad spectrum of alterations that may happen in the organism. Recent technical developments together with the increasing availability of high-throughput methodologies have enabled the detailed description of multiple molecular alterations that coexist in sick individuals [3]. Large scale biology techniques are commonly referred to as "-omics" and include genomics, epigenomics, transcriptomics, proteomics and metabolomics. The study of –omics has gained much attention in many fields of medicine, including systemic autoimmune diseases [4] and SSc is no exception [5]. Nonetheless, among the different -omics, metabolomics has not yet extensively been studied in SSc and to our knowledge, just one study with a limited number of scleroderma samples has been published so far [6]. Here 19 SSc patients were used as a comparison group of 30 systemic lupus erythematosus (SLE) patients along with 20 primary Sjogren's syndrome (SjS) subjects.

Metabolomics can be considered the final step of the biological processes described by –omics techniques, and it concerns the

* Corresponding author at: Research and Development of Functional Food Centre (CIDAF), Health Science Technological Park, Avda. del Conocimiento 37, 18016, Granada, Spain.
E-mail address: iborras@cidaf.es (I. Borrás-Linares).
[1] These authors share co-senior authorship.

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study of the complete set of small molecules intermediates in a biofluid [7]. The description of metabolome provides a portrait of the metabolic state of individuals at a certain point in time and the analysis of the metabolic profile may give insight on the biochemical consequences of disease. The metabolic characterization of patients and the description of metabolite profiling in relation to clinical features and sub-setting may have relevant consequences in understanding disease pathogenesis, in discovering biomarkers and in suggesting individualized therapies [8].

This work aims at characterizing metabolic alterations associated with SSc. To this end, a metabolic fingerprinting strategy based on high performance LC coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) is used for the analysis of plasma and urine samples. Metabolic differences between different subtypes of the disease and in relation to major organ involvement are also explored to describe a set of potential metabolic biomarkers.

2. Material and Methods

2.1. Patients and controls

A total of 59 Italian SSc patients were included in the study. All the patients fulfilled the 2013 ACR/EULAR criteria [9] and were categorized into the limited (lcSSc, n = 43) or the diffuse cutaneous (dcSSc, n = 10) subsets. Patients with definite SSc without skin fibrosis yet with puffy fingers were categorized in the lcSSc subset; the remaining patients with definite disease without fibrosis were retained as a separate group (defSSc, n = 6). Interstitial lung disease (ILD) was defined as in Vigone et al. [10], that is involvement of lung parenchyma > 5% on high resolution computed tomography accompanied by a reduced forced vital capacity (FVC) <80% of predicted values or by a reduced diffusing capacity for carbon monoxide (DLco) < 80% of predicted values.

Twenty-eight age- and sex-matched Italian healthy volunteers were included as control group.

Blood samples were collected into tubes with dipotassium ethylenediaminetetraacetic (K_2EDTA) acid and immediately centrifuged at 1500 g for 10 min at room temperature. Plasma were obtained from the supernatant and then the samples were frozen and stored at -80 °C until sample processing. Random single urine-plot samples were collected and then centrifuged at 2500 g for 10 min at 4 °C. Urine samples were also frozen and stored at -80 °C until sample processing. This metabolomic analysis is ancillary to the PRECISESADS project (www.precisesads.eu) that was approved by the local ethic committee (comitato etico Area B), and written consent was obtained from each participant.

2.2. Chemicals

All chemicals were of analytical reagent grade and used as received. Formic acid and LC–MS grade methanol for mobile phases were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively. Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). For plasma treatment, ethanol and methanol (Fisher Scientific Madrid, Spain) were used.

2.3. Plasma analysis

Plasma samples, which were stored at −80 °C, were thawed on ice. A plasma aliquot of 100 μl was mixed with 200 μl methanol:ethanol (50:50, v/v) in order to remove the protein content. To achieve an efficient protein precipitation, the mixture was kept at −20 °C during 30 min. Next, the sample was centrifuged during 10 min at 14,800 r.p.m. and 4 °C, and the supernatant was evaporated to dryness under vacuum in a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany) during 2 h. Afterwards, the dry residue was reconstituted in 100 μl of 0.1% aqueous formic acid:methanol (95:5, v/v) and centrifuged at the same conditions in order to remove solid particles. Finally, a 40 μl aliquot was transferred into HPLC vials and stored at −80 °C prior to analysis. A quality control sample (QC) was prepared by mixing equal volumes (20 μl) from each sample. This sample was treated as described above.

Analyses were performed using an Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF equipped with a Jet Stream dual ESI interface.

The compounds were separated using a reversed-phase C18 analytical column (Agilent Zorbax Eclipse Plus, 1.8 μm, 4.6 × 150 mm) protected by a guard cartridge of the same packing. The mobile phases used in the analysis were water containing 0.1% of formic acid (Mobile Phase A) and methanol (Mobile Phase B). The following gradient of these mobile phases was used in order to obtain an efficient separation: 0 min (A:B, 95/5), 5 min (A:B, 90/10), 15 min (A:B, 15/85), 32–40 min (A:B, 0/100), and 45 min (A:B, 95/5). Finally, initial conditions were kept for 5 min at the end of each analysis to equilibrate the analytical column before the next analysis. The column and autosampler compartment temperatures were set at 25 and 4 °C, respectively, whereas the flow rate and the injection volume were 0.4 ml/min and 5 μl.

Detection was performed in positive-ion mode over a range from 50 to 1700 *m/z*. All spectra were corrected by means of continuous infusion of two reference masses: purine (*m/z* 121.050873) and hexakis (^{1}H, ^{1}H, ^{3}H-tetrafluoropropoxy) phosphazine or HP-921 (*m/z* 922.009798). Both reference ions provided accurate mass measurement typically better than 2 ppm.

Ultrahigh pure nitrogen was used as drying and nebulizer gas at temperatures of 200 and 350 °C and flows of 10 and 12 L/min, respectively. Other optimized parameters were as follows: capillary voltage, +4000 V; nebuliser, 20 psi; fragmentor, 130 V; nozzle voltage, 500 V; skimmer, 45 V and octopole 1 RF Vpp, 750 V.

The samples were analysed following this sequence: 2 blanks, 5 QCs, 5 randomized samples, 1 blank, 2 QC, 5 randomized samples, etc. Finally, a MS/MS analysis of the QC sample was performed in order to facilitate the identification of potential biomarkers. This experiment was performed using nitrogen as the collision gas with the following collision energy values: 10 eV, 20 eV and 40 eV.

2.4. Urine analysis

Urine samples, which were stored at −80 °C until treatment, were thawed on ice. In order to correct the concentration variation between samples due to individual's hydration status, a pre-analysis normalization step was performed in these samples by means of osmolality measure [11]. The measurement of urine osmolality was determined by freezing point depression using an OSMOMAT 3000 osmometer (Gonotec, Berlin, Germany). The samples were diluted with water in order to achieve a final osmolality value of 100 mOsm/Kg.

Afterwards, the samples were centrifuged during 10 min at 14,800 r.p.m. and 4 °C in order to remove solid particles, and 40 μl of the supernatant was transferred into HPLC vials and stored at −80 °C prior to analysis. A urine QC sample was also prepared by mixing 20 μl from each sample.

Regarding the HPLC-ESI-QTOF-MS methodology, all conditions were the same as plasma samples with the exception of the mobile phases gradient and injection volume. In this case, the following gradient was performed: 0 min (A:B, 95/5), 30 min (A:B, 70/30),