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Amino acids in a targeted *versus* a non-targeted metabolomics LC-MS/MS assay. Are the results consistent?

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ABSTRACT

Background: The results of plasma amino acid patterns in samples from kidney transplant patients with good and impaired renal function using a targeted LC-MS/MS amino acid assay and a non-targeted metabolomics assay were compared.

Methods: EDTA plasma samples were prospectively collected at baseline, 1, 2, 4 and 6 months post-transplant (n = 116 patients, n = 398 samples). Each sample was analyzed using both a commercial amino acid LC-MS/MS assay and a non-targeted metabolomics assay also based on MS/MS ion transitions. The results of both assays were independently statistically analyzed to identify amino acids associated with estimated glomerular filtration rates using correlation and partial least squares-discriminant analysis.

Results: Although there was overlap between the results of the targeted and non-targeted metabolomics assays (tryptophan, 1-methyl histidine), there were also substantial inconsistencies, with the non-targeted assay resulting in more "hits" than the targeted assay. Without further verification of the hits detected by the non-targeted discovery assay, this would have led to different interpretation of the results. There were also false negative results when the non-targeted assay was used (hydroxy proline). Several of said discrepancies could be explained by loss of sensitivity during analytical runs for selected amino acids (serine and threonine), retention time shifts, signals above the range of linear detector response and integration of peaks not separated from background and interferences (aspartate) when the non-targeted metabolomics assay was used.

Conclusions: Whenever assessment of a specific pathway such as amino acids is the focus of interest, a targeted seems preferable to a non-targeted metabolomics assay.

1. Introduction

Metabolomics assays can be separated into two distinct approaches, targeted and non-targeted. Non-targeted metabolomics is also often described as unbiased, due to the fact that non-selective assay strategies are employed [1], whereas these strategies monitor a large group of unrelated metabolites. Targeted metabolomics, on the other hand, involves the quantification of a specific set of often related metabolites [2]. Although several analytical platforms can be used for both strategies, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), including ion traps and high-resolution mass spectrometers, are frequently the preferred analytical tools due to their high sensitivity and specificity [1–4]. While both targeted and non-targeted metabolomics utilize LC-MS/MS, there are often key differences in terms of sample preparation and separation techniques. While non-targeted strategies typically employ non-specific and rather non-selective sample preparation techniques to ensure that metabolites with a wide range of physicochemical properties can be monitored, targeted strategies use specific and optimized sample extraction techniques customized based on the physiochemical properties of the analytes in question to achieve the best possible specificity and sensitivity for the specific metabolites of interest [5]. Moreover, such physiochemical properties are also taken into account during analytical

Abbreviations: CAD, Collision-Activated Dissociation; CKD, chronic kidney disease; cps, counts per second; EDTA, ethylenediaminetetraacetic acid; eGFR, estimated glomerular filtration rate; ESI, electrospray ionization; GCP, good clinical practices; HILIC, hydrophilic interaction liquid chromatography; MDRD, Modification of Diet in Renal Disease; MMF, mycophenolate mofetil; MRM, multiple-reaction monitoring; PLS-DA, partial least squares-discriminant analysis; TIC, total ion chromatogram; VIP, variable importance for projection; v/v, volume by volume.

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method development including mobile phase and column selection as well as design of the elution gradient. In contrast, as aforementioned, to achieve their purpose, non-targeted, non-biased strategies have to use a "one size fits all" analytical approach, which results in less specificity and sensitivity than more targeted approaches, negatively affecting the quality of data. Targeted metabolomics assays are often quantitative and can be validated to a much larger extent whereas non-targeted assays typically generate semi-quantitative data. Nevertheless, non-targeted assays are often used as a first screening assay in clinical biomarker discovery studies and only those metabolites that show significant differences or changes are further confirmed using targeted, quantitative assays [1].

The kidney plays a central role in the metabolism of amino acids and control of plasma concentrations [6]. As a result, samples with varying degrees of kidney function were analyzed. Here we present a direct comparison of circulating amino acid concentrations in 398 EDTA plasma samples prospectively, longitudinally collected from 116 patients with good and impaired kidney function before and until 6 months after *de novo* kidney transplantation as measured in parallel with a semi-quantitative, non-targeted metabolomics LC-MS/MS assay [7] and a targeted, commercial LC-MS/MS assay kit specifically developed to quantify amino acids in human plasma.

2. Materials and methods

2.1. Study population

Samples were collected during a prospective, phase 3b interventional, multi-center, parallel group, randomized, open-label clinical trial to compare the efficacy and safety of concentration-controlled everolimus with low-dose tacrolimus *versus* mycophenolate mofetil (MMF) with standard-dose tacrolimus to establish clinical non-inferiority (CRAD001AUS92, Novartis Pharmaceuticals, East Hanover, NJ). One-hundred and twenty of the subjects enrolled agreed to participate in the present biomarker sub-study. Four of these patients were excluded from the analysis due to incomplete sample sets. All clinical study protocols and their amendments were reviewed and approved by the study centers' appropriate institutional review board. All patients gave their written informed consent. The study followed all applicable regulatory, institutional, national and international rules and regulations of good clinical practices (GCP) and was in compliance with the Declaration of Helsinki and its amendments. This clinical trial was monitored by a Drug Safety Monitoring Board and was registered at *clinicaltrials.gov* (NCT01025817).

After informed consent, samples were collected from each patient prior to transplantation (baseline), as well as 28 days, 2, 4 and 6 months post-transplant. 8 mL of K_2-EDTA blood was collected and plasma was isolated by standard procedures. As stability of plasma samples is of concern, handling procedures to preserve integrity of the samples were employed [8–10] and all clinical personnel handling these samples were trained accordingly. Plasma was aliquoted and stored at −80 °C within 4 h after collection. In the meantime, samples were kept on ice or in a refrigerator at +4 °C. Samples were shipped to the bioanalytical laboratory overnight on dry ice, where they were also kept at −80 °C until analysis.

2.2. Targeted analysis of twenty-four amino acids in EDTA plasma

Amino acid analysis in EDTA plasma samples was performed using the EZ: faast kit purchased from Phenomenex (Torrance, CA) following the manufacturer's instructions. The EZ: faast amino acid analysis procedure involves a solid phase extraction step, derivatization and subsequent liquid/liquid extraction of the derivatized amino acids. The derivatized samples were then analyzed using LC-MS/MS.

2.2.1. Preparation of standards

The EZ: faast kit contained an internal standard mix consisting of homo-arginine, methionine-d3 and homo-phenylalanine at concentrations of 200 μmol/L and was added to each sample during extraction. In addition, two calibrator solutions were also received. Standard solution one contained: alpha-aminoadipic acid, cystine, 4-hydroxy proline, 1-methyl histidine, threonine, lysine, proline, aspartic acid, glycine, methionine, sarcosine, histidine, citrulline, isoleucine, ornithine, tyrosine, alanine, leucine, phenylalanine, valine, arginine, glutamic acid, and serine at concentrations of 200 μmol/L. Standard solution two contained: asparagine, glutamine and tryptophan, also at a concentration of 200 μmol/L, and was provided separately due to differences in stability. Using these standard mixes, calibrators were prepared at the following concentrations: 0.001, 0.01, 0.1, 0.5, 1, 5, 25, 100 and 200 μmol/L. Calibration curves were constructed by plotting the peak area ratios of the corresponding analyte and internal standard against nominal analyte concentrations of the aforementioned calibrators.

2.2.2. Extraction procedure

Samples were first diluted 1/5 (v/v) with HPLC-grade water (20 μL of plasma into 80 μL of water) and combined with 100 μL of internal standard solution. Solid phase extraction was carried out using sorbent packed tips that bind amino acids while interfering compounds are not retained. Following a wash step with 200 μL of *N*-propanol, amino acids were eluted with 200 μL of NaOH/*N*-propanol, 3/2, v/v) into a sample vial. Samples were then derivatized with 50 μL of propyl chloroformate at room temperature in aqueous solution for 1 min. The derivatization reaction is shown in Supplementary Fig. 1. Hereafter, 100 μL isooctane was added, the sample was vortexed and allowed to react for an additional minute, during which the derivatized amino acids migrated into the organic layer for additional separation from interfering compounds. The organic layer was then removed, evaporated, and re-dissolved in aqueous mobile phase and analyzed on an LC/MS-MS system.

2.2.3. LC-MS/MS analysis

Samples were analyzed using an Agilent 1100 series HPLC system consisting of a G1312 binary pump, a G1322A vacuum degasser, and a G1316A thermostated column compartment (Agilent Technologies, Palo Alto, CA) in combination with a Leap CTC PAL auto sampler (Carrboro, NC). The HPLC system was interfaced with an ABSciex 4000 triple quadrupole mass spectrometer (Foster City, CA) operating with an electrospray ionization source (ESI) using nitrogen (purity: 99.99%). 5 μL of the extracted sample were injected onto a 3.0 × 250 mm EZ: faast AAA-MS column 4 μm particle size, Phenomenex (Torrance, CA). The starting mobile phase concentrations were 68% 10 mM ammonium formate in methanol and 32% 10 mM ammonium formate buffer (aqueous) with a flow of 0.5 mL/min. The analytical column was kept at 25 °C. A gradient from 32% to 83% organic mobile phase was run over 13.0 min. The column was then re-equilibrated for 4 min to starting conditions. The mass spectrometer was run in the multiple reaction monitoring (MRM) mode with the interface heated to 425 °C. Nitrogen of >99.999% purity was used as Collision Activated Dissociation (CAD) and curtain gas. A list of the MRM transitions for each amino acid as well as a representative total ion chromatogram (TIC) is shown in Supplementary Table 1 and Supplementary Fig. 2, respectively.

2.3. Non-targeted metabolomics in EDTA plasma

Plasma metabolomics profiles were assessed using a previously described semi-quantitative LC-MS/MS assay platform [7].

2.3.1. Extraction procedure

50 μL of plasma were combined with 30 μL of internal standard solution and 270 μL of methanol. Samples were vortexed for 10 min and