Original Research

UROLOGY

Identification of urinary metabolites correlated with tacrolimus levels through high-precision liquid chromatography-mass spectrometry and machine learning algorithms in kidney transplant patients

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Abstract

Background and aim. Tacrolimus, a widely used immunosuppressive drug in kidney transplant recipients, exhibits a narrow therapeutic window necessitating careful monitoring of its concentration to balance efficacy and minimize dose-related toxic effects. Although essential, this approach is not optimal, and tacrolinemia, even in the therapeutic interval, might be associated with toxicity and rejection within range. This study aimed to identify specific urinary metabolites associated with tacrolimus levels in kidney transplant patients using a combination of serum high-precision liquid chromatography-mass spectrometry (HPLC-MS) and machine learning algorithms.

Methods. A cohort of 42 kidney transplant patients, comprising 19 individuals with high tacrolimus levels (>8 ng/mL) and 23 individuals with low tacrolimus levels (<5 ng/mL), were included in the analysis. Urinary samples were subjected to HPLC-MS analysis, enabling comprehensive metabolite profiling across the study cohort. Additionally, tacrolimus concentrations were quantified using established clinical assays.

Results. Through an extensive analysis of the HPLC-MS data, a panel of five metabolites were identified that exhibited a significant correlation with tacrolimus levels (Valeryl carnitine, Glycyl-tyrosine, Adrenosterone, LPC 18:3 and 6-methylprednisolone). Machine learning algorithms were then employed to develop a predictive model utilizing the identified metabolites as features. The logistic regression model achieved an area under the curve of 0.810, indicating good discriminatory power and classification accuracy of 0.690.

Conclusions. This study demonstrates the potential of integrating HPLC-MS metabolomics with machine learning algorithms to identify urinary metabolites associated with tacrolimus levels. The identified metabolites are promising biomarkers for monitoring tacrolimus therapy, aiding in dose optimization and personalized treatment approaches.

Keywords: kidney transplant, Tacrolimus, therapeutic drug monitoring, high-precision liquid chromatography-mass spectometry, machine learning algorithms, serum metabolites, personalized medicine

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Backround and aims

Kidney transplant (KTx) is the gold standard treatment for patients with end-stage renal disease, providing a superior quality of life and increased life expectancy compared to chronic dialysis [1,2].

Despite significant advancements in surgical techniques, graft allocation systems, and pharmacological approaches over the past decade, the clinical management of KTx patients remains challenging [3,4].

Tacrolimus (TAC) based regimens, combined with mycophenolate and steroids, have been introduced as the first-line chronic immunosuppression treatment to prolong graft survival [5]. However, initial clinical trials reported high rates of acute rejection within the first year (14-31%) and significant toxicity events, including nephrotoxicity (39%), neuropathy (6%), paresthesia (10%), diabetes mellitus (13%), and hyperglycemia (36%) [6,7].

Due to the high pharmacodynamic and pharmacokinetic variability of TAC, achieving optimal blood concentration is critical. Nevertheless, even within the therapeutic range, both toxicity and rejection can occur, highlighting the need for further characterization of optimal TAC levels [8,9].

Current follow-up methods for KTx patients rely on traditional, non-specific technologies such as clinical examination, serum creatinine levels, and serum TAC levels [10]. These methods do not effectively differentiate between nephrotoxicity caused by immunosuppression and graft rejection in patients with average TAC and elevated creatinine levels. Consequently, more invasive protocols, such as graft biopsies, are often recommended, exposing patients to potential secondary complications [10-12].

Metabolomics, the study of small molecules or metabolic products weighing less than 1500 Daltons, offers a close representation of an organism's phenotype. This omics science investigates metabolites in cells, biofluids, or tissues, known collectively as the metabolome. Metabolomics studies using urine and plasma have identified many candidate small molecules that could serve as biomarkers for kidney disease and help elucidate mechanisms of disease progression and treatment response [13].

Recent studies have focused on identifying non-invasive alternatives with high specificity, such as urine and serum protein or metabolite biomarkers [14,15].

Non-targeted metabolomics assays using ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) can characterize the metabolomic signature and reveal specific metabolic networks in TAC-induced nephrotoxicity patients [14]. Urine is particularly valuable in this research due to its high availability and non-invasive, cost-effective collection.

This study aims to describe the urinary metabolomic profile of kidney graft recipients with varying levels of TAC (<5 ng/mL vs. >8 ng/mL) using untargeted metabolomic investigation by UHPLC-MS and machine learning algorithms. This approach may help distinguish between TAC toxicity (due to high TAC levels) and acute rejection (due to insufficient TAC levels) [16].

Methods

In this prospective transversal study, we included 135 consecutive patients who underwent KTx in our hospital and had a stable creatinine level (defined as a level variation below 25% of the mean creatinine value) for whom we performed standard follow-up between May 2020 and July 2020 at the Clinical Institute of Urology and Renal Transplantation Cluj-Napoca. The enrolled patients presented a TAC-based immunosuppressive therapy protocol (Advagraf 0.075-0.3 mg/kg/day) at least six months after the surgery alongside Prednisone 5 mg/day. We excluded the patients who developed autoimmune diseases or lymphoproliferative disorders after the Ktx procedure.

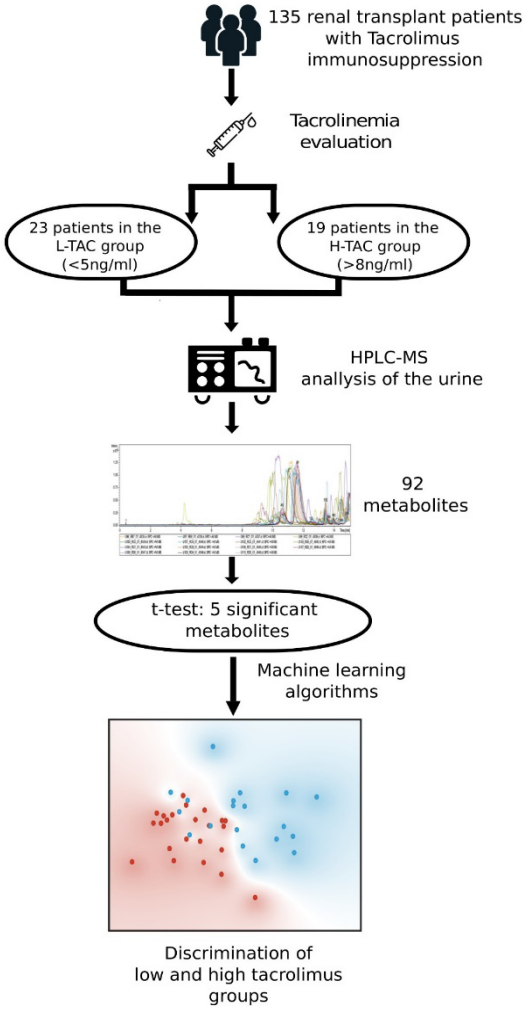

Figure 1. Workflow of the study. Abbreviations: L-TAC—low tacrolinemia group; H-TAC—high tacrolinemia group; HPLC–MS—high-precision liquid chromatography mass spectrometry analysis.

Routine follow-ups were performed on all patients, including clinical examinations, standard haematology, biochemistry panels, and tacrolinemia level. The patients were divided into two groups according to TAC levels (<5 ng/mL vs>8 ng/mL), and urinary metabolomic profiling was performed, as shown in the workflow of the study (Figure 1).

Thus, we defined the patients with outranged TAC as low TAC level group (<5 ng/mL) and high TAC level group (>8 ng/mL).

Sample processing

Mid-stream, first voided urine samples (approximately 10 mL) alongside standard haematology, biochemistry panels and serum tacrolinemia level were collected from patients after a minimum of eight hours of fasting, at 24 hours after administering the TAC dose.

The urine samples were obtained by centrifugation at 1500 rpm for 10 minutes to separate the sediment, and the supernatant was collected by decantation. For each 5mL supernatant, 0.5 mL sodium azide 1% was added. Aliquots of 2 ml were frozen and stored at −80 °C until analysis. To precipitate proteins, 0.4 ml of a methanol and acetonitrile mixture (1:1) was added to 0.3 ml of urine. The mixture was vortexed for 1 minute, kept at 4 °C overnight, and then vortexed again for 1 minute. After thorough mixing, the vials were centrifuged at 12,500 rpm for 5 minutes, and the supernatant was collected and filtered through 0.2 µm nylon filters.

Laboratory tests

Tacrolinemia was assessed using semi-automated electrochemiluminescence immunoassays using the ArchitectPlus CI4100 automatic analyzer [17,18].

Prior to starting the automated Architect sequence, a manual pretreatment step was carried out. In this step, the whole blood sample was treated with a precipitation reagent and then centrifuged. The resulting supernatant was carefully transferred into a Transplant Pretreatment Tube and then loaded onto the Architect iSystem for further analysis.

The UHPLC-MS analysis was conducted using a Bruker Daltonics MaXis Impact system (Bruker GmbH, Bremen, Germany), which included a Thermo Scientific HPLC UltiMate 3000 system equipped with a Dionex Ultimate quaternary pump and ESI+-QTOF-MS detection. The analysis was performed on a C18 reverse-phase column (Acquity, UPLC C18 BEH, Dionex) with dimensions of 5 µm and 2.1 x 75 mm, maintained at 25 °C with a flow rate of 0.3 ml/min. The injection volume was set to 5.0 µl. The mobile phase utilized a gradient of eluents, with eluent A being water containing 0.1% formic acid, and eluent B comprising a 1:1 mixture of methanol and acetonitrile, also containing 0.1% formic acid. The gradient system progressed from 99% A at minute 0, to 70% A at minute 1, 40% A at minute 2, 20% A at minute 6, 100% B from minutes 9 to 10, followed by a return to 99% A for the final 5 minutes. The total runtime was 15 minutes.

The MS parameters were set for a mass range between 50-1000 Da. The nebulizing gas pressure was set at 2.8 bar, the drying gas flow at 12 L/min, and the drying gas temperature at 300 °C. Before each chromatographic run, a calibration with sodium formiate was done. The instrument control and data processing used the specific software provided by Bruker Daltonics, namely TofControl 3.2, Hystar 3.2, and Data Analysis 4.2.

Statistical methods

We ranked all the metabolites identified through UHPLC–MS by their potential to differentiate between the two groups using the t-test feature selection method. The significance level for the t-test was set at $p < 0.05$, and a Student's t-test was performed for each metabolite. The five metabolites that were statistically significant in the t-test were selected for further analysis. To assess the classification accuracy of high and low tacrolinemia based on each significant metabolite, a receiver operating characteristic (ROC) curve was used to calculate the area under the curve (AUC).

Logistic regression, an independent machine learning model, was trained to differentiate between the two groups in order to quantitatively assess the multivariate classification effectiveness of the five significant metabolites. Logistic regression was implemented using the Quasar-Orange Software, with Ridge (L2) regularization and a regularization strength of C=1. Cross-validation was performed using the leave-one-out method.

The inputs for the machine learning algorithm consisted of either the individual five selected metabolites or a combination of all five. Prior to classification using the combined metabolites, the data were normalized to unity. The classification performance was evaluated based on metrics such as the AUC obtained from ROC analysis, classification accuracy, F1 score, precision, and recall. These performance metrics were reported as the average values from each iteration of the cross-validation process.

Subsequently, a principal component analysis (PCA) was conducted to examine the dataset; the five metabolites were used as inputs. PCA was utilized to reduce the data dimensionality to represent better the capacity to differentiate the TAC level of the experimental model, choosing the PCs that can offer the best visual differentiation of the two groups. The relationship between the number of PCs and the explained variability in the original dataset is presented in figure 2c.

The Pearson correlation coefficient was utilized for the correlation analysis.

All statistical analyses were conducted using the Quasar-Orange software from the Bioinformatics

Laboratory at the University of Ljubljana [19,20].

The study received approval from the Ethics Committee of the Clinical Institute of Urology and Kidney Transplantation in Cluj-Napoca (No. 2/2020) and the Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy in Cluj-Napoca (No. 285/2020). All patients provided written informed consent in accordance with the guidelines and principles outlined in the Helsinki Declaration.

Results

After selecting patients with outranged tacrolinemia, our database comprised 42 patients. Thus, 23 patients were included in the low-TAC group (under five ng/mL) and 19 in the high-TAC group (over eight ng/mL).

The UHPLC-MS assay detected a total of 92 urinary metabolites. Among them, we chose the top 5 metabolites that exhibited the highest capability to effectively differentiate between the two groups: Valerylcarnitine, Glycyl-tyrosine, Adrenosterone, LPC18:3 (lysophosphatidylcholine) and 6-methylprednisolone.

The statistical method to select the five statistically relevant metabolites was the Student's t-test and ROC analysis. The mean level of each metabolite was calculated and presented in table I. Thus, we can observe higher levels of Adrenosterone and 6-methylprednisolone and lower levels of Valerylcarnitine, Glycyl tyrosine and LPC18:3 in the H-TAC compared to the L-TAC group.

A logistic regression machine learning algorithm was applied to discriminate between the two groups, utilizing the five metabolites mentioned above. Thus, we obtained an area under the curve of 0.810 and classification accuracy of 0.690 (Table II).

PCA reduces the dimensionality of the data by transforming it to a new set of variables (principal components) that capture the most important information. The principal components (PC) chosen were used to visualize high-dimensional data in 2D plot. PC2 and PC10 had the best visual differentiation of the two groups.

The distribution of score values after performing PCA on the metabolic profiles of the H-TAC and L-TAC groups (PC2 and PC10) is illustrated in figure 2a, highlighting the clustering tendency of the H-TAC and L-TAC groups. Figure 2b presents the loading plot of the most relevant 20 metabolites for PC2 and PC10, showing that Glycyl tyrosine, 12-HETE (12-Hydroxyeicosatetraenoic acid), Carboxy-alpha-tocotrienol and LPC18:3 are the variables that contribute the most to PC2, and Glycyl tyrosine, 12-HETE, and Diatrizoate to PC10. Moreover, a negative correlation of Glycyl tyrosine and 12-HETElevels between PC2 and PC10 is observed. We can also notice a higher fluctuation of metabolites level in the PC2 plot than in PC10. The screen plot presenting the variance between PC1 and PC10 is depicted in figure 2c. Thus, we can observe that PC1 and PC2 cover almost 70% of the variation, and the top 4 PCs over approximately 90% of the total variation.

Figure 3 depicts the heatmap of the entire urinary metabolome identified by our UHPLC–MS analysis, showing the clustering of metabolites according to the TAC levels of the patients.

Table I. Student's t-test and the area under the curve were applied to identify the significantly different metabolites used to distinguish between patients with low and high tacrolinemia. The average levels of these metabolites correspond to the peak UHPLC–MS intensities.

Metabolite (counts)	High Group Mean ± SD	Low Group Mean ± SD	*t- test p-Value*	AUC	95% CI	*p*-Value
Valerylcarnitine	86540 ± 25838	101316 ± 20370	0.04	0.68	0.52 to 0.85	0.04
Glycyl-tyrosine	302652 ± 133091	403461 ± 152812	0.03	0.70	0.54 to 0.87	0.03
Adrenosterone	801799 ± 166910	673840 ± 115878	0.04	0.72	0.52 to 0.92	0.06
Lysophosphatidylcholine 18:3	1072290 ± 207222	1205877 ± 158682	0.05	0.68	0.49 to 0.87	0.08
6-methylprednisolone	63924 ± 21717	36326 ± 21599	0.03	0.87	0.69 to 1.0	0.02

Table II. Representation of the area under the curve results for the classification accuracy provided by the five metabolites using logistic regression classification algorithms.

Statistic model	AUC	CA	F1	Precision	Recall
Logistic regression	0.810	0.690	0.682	0.695	0.690

Abbreviations: AUC-area under the curve; CA- classification accuracy; F1 score; Precision-positive predictive value; Recall-sensitivity.

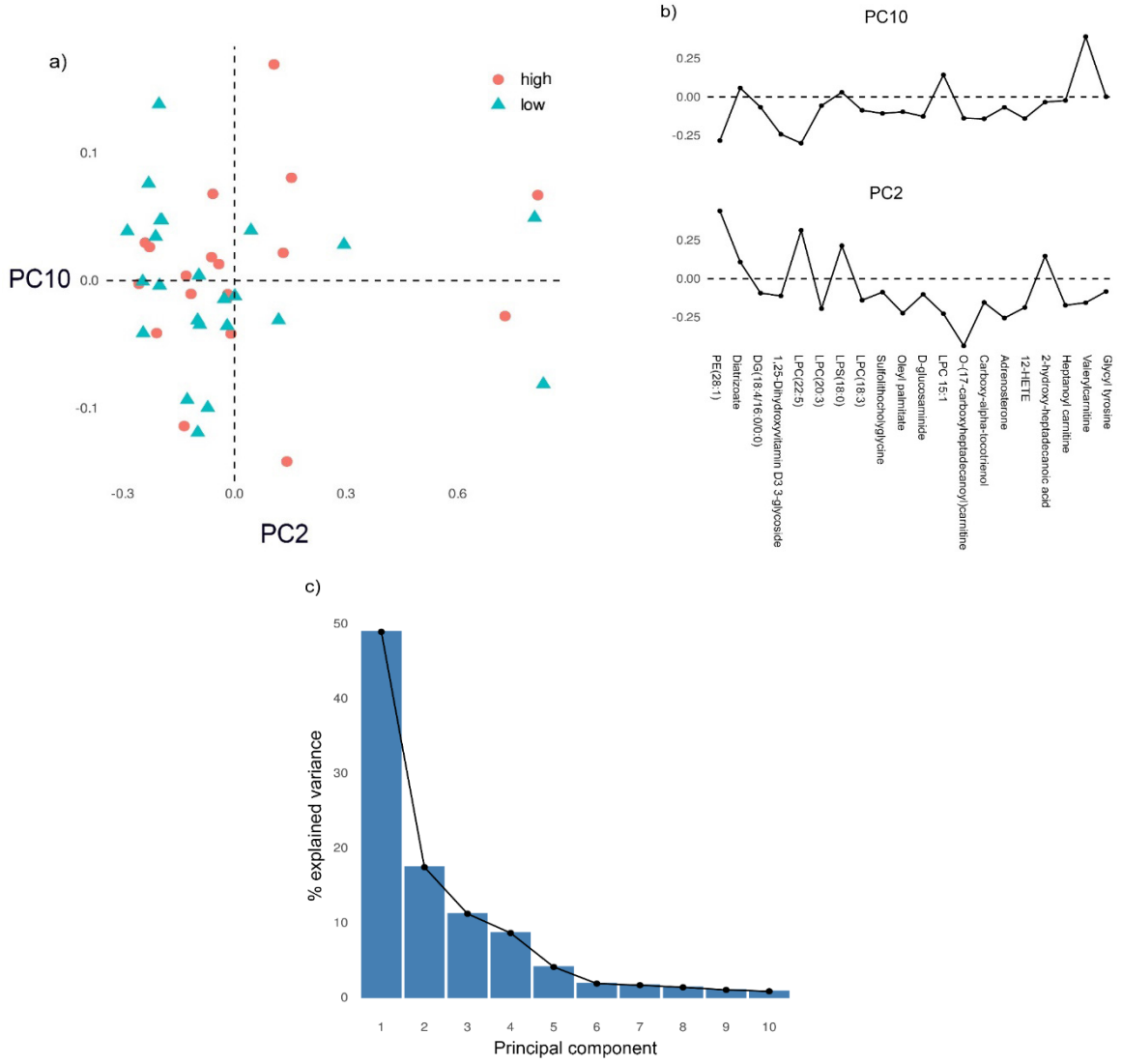

Figure 2. Principal component analysis of metabolic profiles. a) The distribution of principal component (PC) score values (PC2 and PC10) of patients with metabolic profiles associated with low and high tacrolinemia; b) Loading plots of PC 2 and PC 10; c) The percentage of explained variance for each PC.

Figure 3. Heatmap of the levels of urine metabolites. Abbreviations: Cer – Ceramide; LPC – lysophosphatidylcholine; PS – phosphatidylserine; PA – phosphatidic acid; HETE – hydroxyeicosatetraenoic acid; DG – diglyceride; PC – phosphatidylcholine; PG – phosphatidylglycerol; MG – monoacylglyceride; LPA – lysophosphatidic acid; APC – Aromatic polycyclic compounds; PE – phosphatidylethanolamine; SM – Sphingomyelin.

Discussion

In this study we aimed to discern between the metabolomic profile of kidney transplant patients who exhibit abnormal TAC levels (high and low TAC levels) while maintaining stable renal function. Thus, we have identified five metabolic molecules Valerylcarnitine, Glycyl-tyrosine, Adrenosterone, LPC18:3 (lysophosphatidylcholine) and 6-methylprednisolone capable to discriminate between the two goups. The resultant group disparities in our findings hold the potential to offer a viable approach for distinguishing between graft dysfunction arising from inadequate TAC exposure and allograft rejection and graft toxicity resulting from excessive TAC exposure. Due to the narrow therapeutic window and the need for more knowledge

about the personalization of immunosuppression treatment, improving and prolonging graft survival is challenging [21,22]. Withal, the current follow-up protocols for patients who underwent Ktx are based on the determination of serum TAC levels and creatinine levels, which are insufficient to assess optimal systemic exposure [22-24].

There is a trend in approaching multi-omics biological analysis to offer the patient the best adapted TAC exposure by obtaining the maximum immunotherapeutic effect with the minimum side effects [14,25,26]. Thus, novel biomarkers and their association may describe certain pathological events in a preclinical moment. Among the biofluids, urine is of significant interest because of its high availability and non-invasive and inexpensive collection.

Banas et al. recently published their prospective trial describing the patient recruitment protocol based on detecting the urinary metabolomic constellation. The novel biomarkers identified can predict allograft damage over time, improve graft allocation strategies and prolong graft survival [27].

Our study aims to help clarify the differentiation in metabolomic profile between TAC over and under-exposure; such information might subsequently help to develop a precise algorithm to predict the initial TAC dose and the optimal therapeutic dosage needed to enhance clinical outcomes in kidney transplant patients.

Valerylcarnitine is a short chain acylcarnitine and belongs to the body's most prevalent group of carnitines, comprising over 50% of all quantified acylcarnitines found in tissues and biofluids [28]. Its primary function is facilitating the transportation of acyl groups, including organic and fatty acids, from the cytoplasm to the mitochondria. This crucial process enables the breakdown of these acyl groups, leading to energy production [29]. It has been recognised that changes in the levels or presence of Valerylcarnitine in tissues or biofluids may offer valuable insights into different diseases or metabolic disorders, cardiovascular diseases, diabetes, depression, neurologic disorders, and certain cancers [28,30,31]. A recently published meta-analysis study investigating the metabolomics signatures in type 2 Diabetes, associated the risk of developing type 2 diabetes mellitus with higher levels of valeryl carnitine; on the other hand, lysophosphatidylcholine was negatively correlated with the risk of developing type 2 diabetes mellitus [32]. Another recent metabolomics article identified that Valerylcarnitine was found as a specific biomarker able to measure alterations in biochemical pathways related to the pathogenesis of diseases such as Alzheimer's disease [33], as well as non-alcoholic fatty liver disease (NAFLD) [34].

Related to the role of energy production, valerylcarnitine has been identified as a moderate biomarker candidate in a study that highlights the significance of investigating and classifying the kinetic patterns of dynamic metabolic biomarkers in response to physical activity [35].

Despite extensive research conducted on valerylcarnitine, when considering the technical aspects of the UHPLC–MS assay, it was observed that storing urine samples containing valerylcarnitine at +4°C carried a greater risk of bias compared to storage at -20°C or -80°C [36].

A research study on rats suggests the implication of L-carnitine in the protective role against TAC-induced renal injury by attenuating programmed cell death via PI3K/AKT/PTEN signaling. Thus, using L-carnitine in TAC-treated rats improved renal function and ameliorated histological changes [37]. Valeryl carnitine, a metabolite formed through the breakdown of amino and fatty acids from L-carnitine, holds the potential as a prognostic indicator of kidney function in individuals undergoing tacrolimus treatment.[38] According to our research findings, reduced valeryl carnitine levels in patients with elevated TAC levels may signify an inadequate protective response by the body against the aggressive effects of TAC.

Glycyl-tyrosine is a dipeptide composed of the amino acids glycine and tyrosine, and it may have yet to be extensively studied in the context of metabolomics analyses.

A previous study showed that decreased glycyl tyrosine levels correlate with reduced lower left ventricular ejection fraction. In contrast, reduced lysophosphatidylcholine (LPC) indicates diastolic dysfunction, suggesting their potential to be used as metabolic biomarkers in detecting cardiovascular risk [36].

Unfortunately, as far as our understanding goes, there is a lack of research establishing any correlations between glycyl tyrosine and other nephrological or urological conditions.

Lysophosphatidylcholine is a phospholipid representing a major component of cell membranes. It is involved in various cellular processes and is essential in biological membrane and lipid metabolism [39]. It is considered that LPC is involved in promoting inflammation and the development of diseases by stimulating the production of pro-inflammatory cytokines, inducing oxidative stress, and enhancing apoptosis [40]. In addition, elevated levels of LPC have been linked to cardiovascular complications associated with atherosclerosis, ischemia, and diabetes-inducing insulin resistance [40,41]. Other studies concluded that the concentration of LPC varies in different tumours, playing an essential role in the invasion, metastasis, and prognosis of tumours [40,42-46]. Therefore, targeting LPC and lipid metabolism might be a potential therapeutic method for inflammation-related diseases. To our knowledge, LPC has not been studied concerning TAC toxicity.

Adrenosterone is classified as a steroid hormone with a minimal androgenic impact. Its mechanism of action is believed to involve competitive selectivity as an inhibitor of 11βHSD1, an enzyme responsible for converting cortisone to cortisol. This inhibition helps prevent muscle

breakdown and significantly affects the hormone's overall effects [47].

One recent publication investigating potential specific biomarkers for thyroid cancer identified adrenosterone as one of four small molecules that could reverse the gene expression induced by thyroid cancer, potentially improving the diagnosis, prognosis, and treatment selection, facilitating personalized approaches for patients [48].

In correlation to the urinary tract, more precisely in kidney pathologies, modified levels of adrenosterone, alongside 11 other metabolites, were found in adenine-induced chronic kidney disease [49,50].

Another zebrafish model biochemical research investigated the effects of adrenosterone on suppressing epithelial-mesenchymal transition, inhibiting metastatic dissemination, and suggesting reduced tumour cell migration, invasion, and metastatic dissemination upon adrenosterone treatment [51]. Androsterone was not studied in TX pathology.

Methylprednisolone is a synthetic systemic corticosteroid derived from prednisone, which is essential in mediating the human body's anti-inflammatory and immunosuppressive activity [52]. As mentioned, Prednisone therapy is one of the primary drugs used in kidney transplant patients' regimes [5]. In our groups, all the patients recruited for this research follow a daily dose of 5mg oral Prednisone intake.

It is important to note that the extent of renal excretion of prednisone can be influenced by factors such as kidney function, glomerular filtration rate, and the presence of kidney disease or impairment. In individuals with renal dysfunction, the clearance of prednisone and prednisolone may be decreased, leading to potential drug accumulation and the need for dosage adjustments [53-55]. On the contrary, another study demonstrated that methylprednisolone clearance from the circulation did not appear to be significantly influenced by the peak plasma levels of the drug or the creatinine clearance. No drug accumulation was apparent, even in patients with profoundly reduced renal function [56].

Research was carried out to examine whether there is a clinically significant interaction between steroids and tacrolimus following kidney transplantation. The study's findings revealed that as the dosage of steroids increased, a higher dosage of tacrolimus was required to attain the desired levels in these patients. The primary mechanism for this interaction is thought to be the specific stimulation of CYP3A and/or P-gp1 enzymes [57]. A difference in prednisone dose could explain the notable difference in the quantity of methylprednisolone observed in the urine samples collected from the H-TAC and L-TAC groups.

Even though methylprednisolone was found in the patient's urine in a significant amount to statistically discriminate between the two groups, we mention a high risk of bias due to the different filtration rates of the metabolite in the kidneys in correlation with the individual glomerular filtration rate and creatinine levels [53].

To contextualize our findings, we have drawn upon the insights from our previous serum metabolomics study [58], which provided crucial groundwork for the current investigation. By integrating data from urine and serum analyses, we aimed to establish a more holistic view of the systemic metabolic changes, paving the way for potential diagnostic advancements and therapeutic interventions for TAC dose adaptation in kidney transplant patients. The synergy between these two studies underscores the significance of our research and its potential impact on personalized medicine and disease management.

The hallmark strength of our study lies in its pioneering approach, utilizing metabolomics to observe graft rejection attributed to TAC underexposure.

This study's constraints are twofold: firstly, the limited number of patients included, and secondly, the absence of essential graft biopsies required for diagnosing graft dysfunction and comprehensively characterizing its underlying cause.

Personalized kidney transplant immunosuppression is the next step for the transplant patients. This step is both necessary and realistic in future, but with a concept of using both genomics and pharmacogenomics alongside m/3-etabolomic approach. In this set up it is important to take in consideration challenges like cost, genetic data interpretation and the accessibility.

Moreover, the correlation of the urinary metabolomic signature with the serum may provide a more accurate tool for diagnosis and follow-up to provide individualized treatment for each patient.

Our results must be further validated with targeted MS on larger cohorts with biopsy-proven TAC toxicity.

Conclusions

Our research proved that by exploring the urinary metabolomic signature of the patients following a TAC-based immunosuppression regimen using HPLC–MS and machine learning algorithms, we could identify five urine metabolites that may differentiate between different TAC levels.

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