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Untargeted urinary metabolomic profiling in post-kidney transplant with different levels of kidney function

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ABSTRACT: The ability to monitor patients plays a major role in the success of kidney transplants. However, transplant monitoring still depends on relatively outdated, inadequate technologies. The aim of this study was to reveal the metabolomic profile of the kidney allograft using the metabolomic screening technique and to identify specific eGFR-based biomarkers to monitor individuals with different levels of post-transplantation graft dysfunction. In the current study, urine samples from 131 unique kidney transplant recipients were collected and analyzed by ultra-high performance liquid chromatography and benchtop QTof mass spectrometer (Xevo G2 XS QTof). Acquired data were first pre-processed by Progenesis QI 2.3 (Nonlinear Dynamics, Waters, UK). Putative annotation was performed against the HMDB database following multivariate statistical analysis. Post-transplant biomarker panels that can distinguish stages of renal dysfunction were created by combining the significant markers and taking their ratios. Overall, 8 metabolites were significantly altered within three groups of kidney transplant recipients:4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0), QYNAD and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate as biomarker candidates ($p<0.05$). The ratio of 4,5-Dihydroorotic acid to Pantothenic acid (panel-1) can be used to monitor kidney function. Specifically, these metabolite ratios were found to be more sensitive to changes in kidney function than panel-2, which consisted of 7 metabolites, excluding QYNAD, of the 8 major metabolites. Our results may contribute to the monitoring of kidney transplant patients based on post-transplant eGFR-based kidney function stages, thus providing a method for the early evaluation and monitoring of the kidney transplant recipient after transplantation for kidney transplant patient management.

KEYWORDS: Urine Metabolites; metabolome profiling; metabolomics; UPLC/ESI/QTOF-MS/MS; Kidney Transplantation

1. INTRODUCTION

Chronic kidney disease (CKD), defined by a progressive loss in kidney function for more than a few months, is a recently recognized global public health problem[1–4] Complications such as diabetes, hypertension and dyslipidemia can cause CKD, as well as increase cancer risk in end-stage kidney disease (ESKD) and kidney transplant populations [5]. Patients with ESRD must receive dialysis treatment or a kidney transplant to survive, however, transplantation is always the best treatment of *choice* when compared to hemodialysis or peritoneal dialysis, in view of morbidity and mortaliteyi, life quality, and cost efficiency. The number of people receiving kidney replacement therapy was more than two million worldwide in 2015 and this number is expected to increase significantly by 2030 [6].

Current treatment decisions and monitoring of kidney function of patients after kidney transplantation are generally based on serum creatinine (Scr) originating from muscle mass, which is a breakdown product of

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creatine phosphate in muscle. This is an estimated glomerular filtration rate (eGFR) recommended by The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines and proteinuria[1].

These diagnostic tests are used in daily clinical practice because they are a stable compound and very inexpensive in routine clinical use. Besides the advantages of being easy and inexpensive to perform these diagnostic tests, there are also some disadvantages including creatinine-based estimates of Scr concentration are affected by biological confounders such as age, gender, and particularly muscle mass and causes an overestimation of kidney function due to the fact that Glomerulus capture both which are produced at a fairly constant rate depending on muscle mass and secreted creatinine actively secreted in the proximal tubule[7,8]. Alternative tests of creatinine clearance and serum creatinine are not suitable in most clinical situations. Inulin clearance is difficult to perform due to technical difficulties in testing and a lack of adequate sources of inulin for clinical use. [51Cr] EDTA, [125I] iothalamate and [99Tcm] DTPA are radiolabeled compounds to measure the eGFR. These compounds are not useful owing to fact that some radiation is exposed, radiopharmaceuticals are more expensive, and skilled personnel and the use of a gamma camera are needed [9]. Monitoring of diagnostic tests used currently for patients who need therapy may provide inaccurate results and may be insufficient in monitoring kidney function. Diagnostic tests that are currently used show poor sensitivity and specificity for the detection of kidney damage.

OMICS refers to the fields of study in biology that ends in "omics" such as genomics, proteomics, transcriptomics, or metabolomics. These are the branches of science that study molecules' structure and functions at the gene level, the protein level, and the metabolic level in a biological organism, organ, tissue, or cell [10,11]. Metabolomics defined an omnibus analytical approach to study all small molecular weight species typically defined as <1500 Da, in easily accessible biofluids present such as urine, blood, feces, or tissues [12]. The primary purpose of metabolomics is to conduct an all-inclusive study on metabolites, which are intermediate products of living organisms' biochemical processes [13].

Metabolomics study includes the use of high-throughput technologies such as capillary electrophoresis mass spectrometry, Raman spectroscopies, nuclear magnetic resonance, ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry, infrared, gas-chromatography and mass-spectrometry to comprehensively identify and quantify untargeted and targeted small molecule metabolites [14–16].

In this study, we enrolled 131 participants that had a renal transplant and subdivided them into three groups based on their estimated GFR: 53 in eGFR >60 ml/min/1.73 m2(S1), 56 in 30<eGFR <60 ml/min/1.73 m2 (S2) and 22 in eGFR <30 ml/min/1.73 m2 (S3). (S1, S2, and S3 indicate patient groups with eGFR>60, 30<eGFR<60, and eGFR<30, respectively). Using Xevo G2 XS QTof with enhanced selectivity, sensitivity, and reproducibility we aimed to reveal the metabolomic profile of the kidney allograft using the metabolomic screening technique and to identify specific eGFR-based biomarkers to monitor individuals with varying degrees of post-transplantation graft dysfunction.

2. RESULTS

2.1 Patient baseline characteristics

The clinical and demographic characteristics of the subjects are described in Table 1. A cohort of 131 kidney transplant individuals were enrolled in our cross-sectional study and stratified according to tertiles of eGFR distribution as follows: 53 in eGFR >60 ml/min/1.73 m^2(S1), 56 in 30<eGFR <60 ml/min/1.73 m^2 (S2) and 22 in eGFR <30 ml/min/1.73 m^2 (S3) (S1, S2, and S3 indicate patient groups with eGFR>60, 30<eGFR<60, and eGFR<30, respectively). There were no statistically significant differences between groups in terms of age, lipid profile (cholesterol, LDL and triglyceride), *Body mass index* (*BMI*), hypertension and cigarette smoking. Diabetes Mellitus (DM) and gender differed significantly between these groups.

Table 1. Basic characteristics of the participants in this study. All values are expressed as mean ± SD, median (25th, 75th percentiles) or counts n and n (%). M, Male; F, Female; eGFR, Glomerular Filtration Rate; BMI, Body Mass Index; BUN, Blood Urea Nitrogen; NS, not significant.

Patient characteristics	S1 (n=53)	S2 (n=56)	S3 (n=22)	P value
Age	47.50±13.26	46.6±10.51	52.0±14.31	NS
Gender (F/M) (n)	18/35	16/40	13/9	0.038
BMI (kg/m2)	27.16±5.13	27.02±4.41	27.16±4.98	NS
eGFR(ml/min/1.73m2)	79.00±15.54	46.64±7.98	19.64±5.80	<0.001
Smoking status				
Never (%)	62.3	62.3	76.2	NS
Former (%)	17	9.4	9.5	NS
Smoking occasionally (%)	16.7	24.5	9.5	NS
Smoking Regularly Every (%)	3.8	3.8	4.8	NS
Serum concentrations				
BUN (mg/dL) (%)	24.0 (16.5-37)	21.5 (14.75-28.0)	25.5 (18.5-57.25)	NS
Cholesterol, LDL (mg/dL) (%)	113.56±48.26	110.11±36.68	110.77±40.99	NS
Triglyceride (mg/dL) (%)	143.0 (96.75-233.75)	140.0 (105.0-181.0)	144.0 (104.0-254.0)	NS
Diabetes Mellitus (%)	7.4	9.1	39.1	0.01
Hypertension (%)	9.3	7.3	18.2	NS

2.2 Assessing The Technical Reproducibility of An Analytical Method

As seen in Figure 1, the QC samples were tightly clustered on the PCA score plot, thus confirming the stability of the device's testing and the reliability of the metabolomics data.

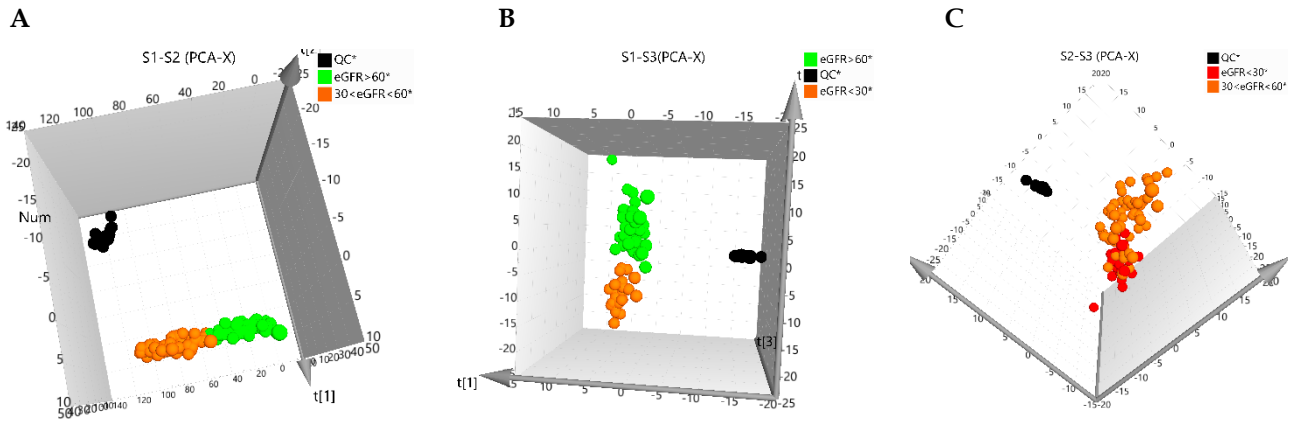


Figure 1. PCA analyses of) analysis of the data generated from the ESI-positive mode for S1-S2 group (A), S1-S3 group (B) and S2-S3 group (C). An PCA was constructed using samples from individuals with eGFR>60 (green circles), 30<eGFR>60 (orange circles), eGFR<30 (red circles) and QC (black circles) after kidney transplantation. QC; Quality Control Sample.

2.3 The Determination of Biomarker Candidates, Performing Combination of Panel from The Eight Significant Metabolites and Their Ratios and ROC Analysis

To evaluate the differences of urine metabolites between S1, S2 and S3 groups, initially, metabolites were measured in all urine samples and were then assessed by comparing values among groups (S1, S2, and S3).

Paired comparisons revealed that 311 features exhibited significant differences between the S1 and S3 groups, whereas 371 and 589 features showed obvious differences between S1 versus the S2 and S3 group, respectively ($P < 0.05$, QC<30, Max fold change (MFC) >1.2, variable importance in the projection (VIP) >1). Of these significantly changed metabolites, we screened out eight metabolites, that candidate to major graft monitoring molecules, with significant differences in expression between the three groups.

The spectral features that contribute most to variation or separation are identified for further analysis through multivariate analysis (MVA) methods such as principal component analysis (PCA), *Partial Least-Squares* Discriminant Analysis (PLS-DA) and *Orthogonal Partial Least-Squares* Discriminant Analysis (OPLS-DA)[17]. A two-dimensional OPLS-DA was created to find potential biomarker candidates that could genuinely reveal individual differences between groups. OPLS-DA score plots highlighted the difference between the patients in the S1-S2, S1-S3 and S2-S3 groups, and displayed metabolic profiles in groups that were separated clearly (respectively, (**A**) $R^2Y = 0.64$, $Q^2 = 0.50$ (S1-S2); (**B**) $R^2Y = 0.98$, $Q^2 = 0.82$ (S1-S3) ;(**C**) $R^2Y = 0.70$ $Q^2 = 0.55$ (S2-S3)) (Figure 2-A,B,C).

VIP is an estimate of the importance of each variable in the projection used in the OPLS-DA model as a quantitative estimate of the discriminatory power of each feature. Variables with a VIP score of ≥1 were considered significant in the OPLS-DA model[18]. In this study, VIP plots of the OPLS-DA were built to verify the differentiated metabolites between the groups VIP and were calculated to identify distinguishing variables in the data set. Significant variables (VIP value) were selected as potential markers (Figure 2-D, E, F).

Test of variance analysis of cross-validated predictive residuals (CV-ANOVA) and a permutation test (with n = 200) were used to evaluate and validate the predictive ability and reliability of the models obtained (Table 2).

Table 2. (A) Display CV-ANOVA TEST for S1/S2 Group; (B) CV-ANOVA TEST for S1/S3 Group (C) CV-ANOVA TEST for S2/S3 Group.

A

M40(Untitled)	SS	DF	MS	F	p	SD
Total corr.	107	107	1			1
Regression	53.841	4	13.46	26.08	6.08E-15	3.67
Residual	53.159	103	0.52			0.72

B

M9(Untitled)	SS	DF	MS	F	p	SD
Total corr.	68	68	1			1
Regression	56.26	8	7.03	35.93	4.15E-20	2.65
Residual	11.74	60	0.196			0.44

C

M4(Untitled)	SS	DF	MS	F	p	SD
Total corr.	76	76	1			1
Regression	42.42	4	10.60	22.74	3.59E-12	3.26
Residual	33.58	72	0.466			0.68

The p-value of cross-validation ANOVA test at 6.082E-15 (S1-S2), 4.15137E-20 (S1-S3) and 3.59E-12 (S2-S3) showed the strong predictive power of the model.

In the permutation test, this is performed by randomly assigning two different groups, after which the OPLS-DA models are fitted to each permuted class variable. Then, the values of R2 and Q2 for the permuted models are calculated and compared with the values of the true model, and therefore the calculated real OPLS-DA models are statistically much better than the 200 permutation models for each dataset (Figure 2-G,H,I).

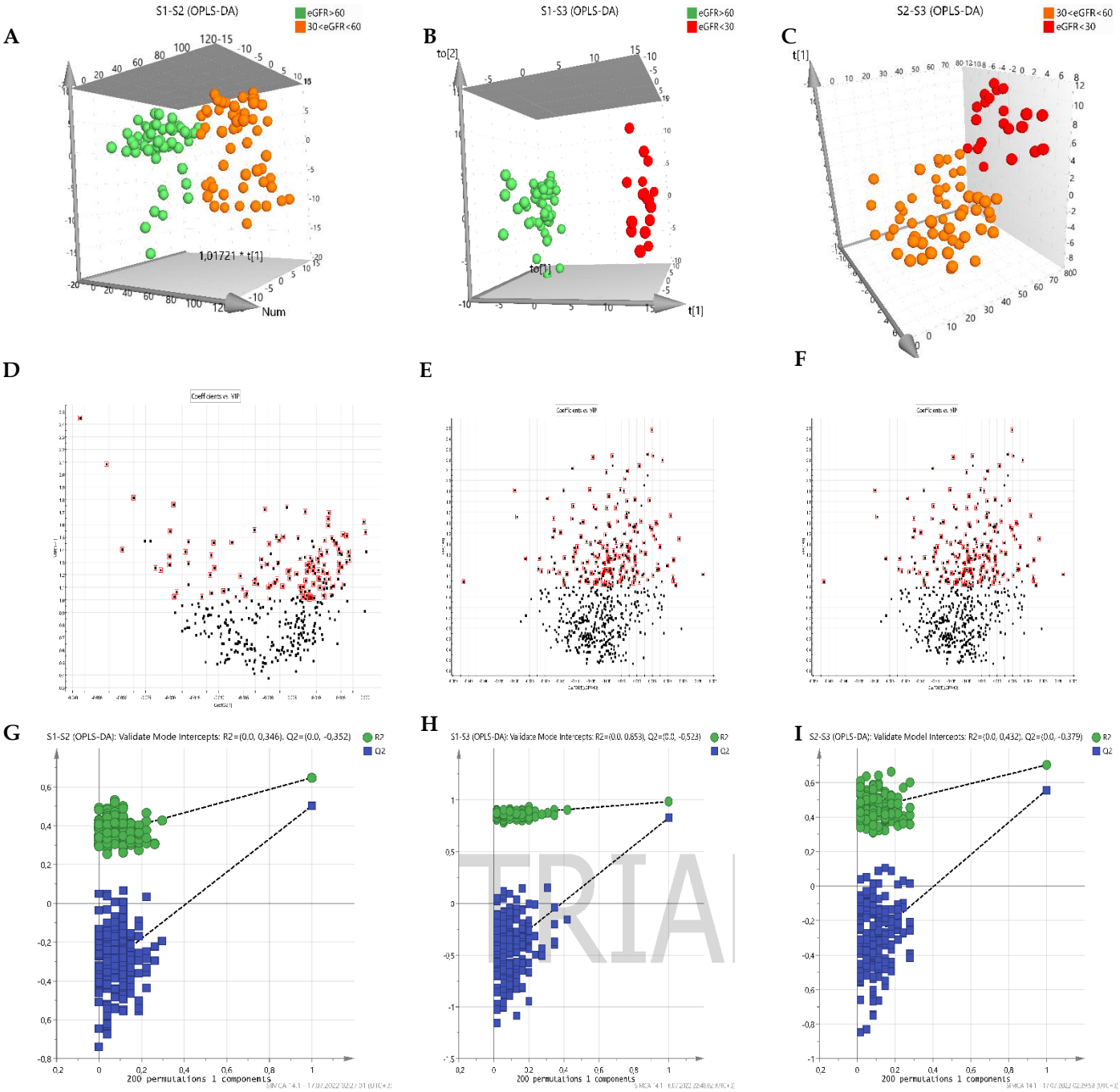


Figure 2. Orthogonal partial least squares-discriminant analysis (OPLS-DA) analysis of the data generated from the ESI-positive mode. An OPLS-DA model was constructed using samples from individuals with eGFR>60 (green circles), 30<eGFR>60 (orange circles), and eGFR<30 (red circles) after kidney transplantation. (A,B,C) Displays the result of OPLS-DA model using the data from the S1/S2, S1/S3 and S2/S3 groups in ESI-mode. (D,E,F) Displays

the response permutation test plot (n = 200) for the OPLS-DA model in (D), the R2 and Q2 values of the permutated model are represented on the left-hand side of the plot, corresponding to y-axis intercepts: R2 = (0.0, 0.346) and Q2 = (0.0, −0.352) (D), R2 = (0.0, 0.853) and Q2 = (0.0, −0.523) (E), R2 = (0.0, 0.432) and Q2 = (0.0, −0.379) (F). VIP plots constructed from the supervised OPLS analysis of urine (G,H,I), Metabolite ions with variable influence on the projection (VIP) value >1 were marked with a red square.

Using multivariate statistical analysis, we detected 4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0), QYNAD and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate among the three groups as biomarker candidates (Supplement Table S1)

As seen in Figure 3, an unsupervised Principal Component Analysis (PCA) was used to examine statistically these significant eight metabolic changes in urine between groups. A clear separation in metabolic states was observed between the groups.

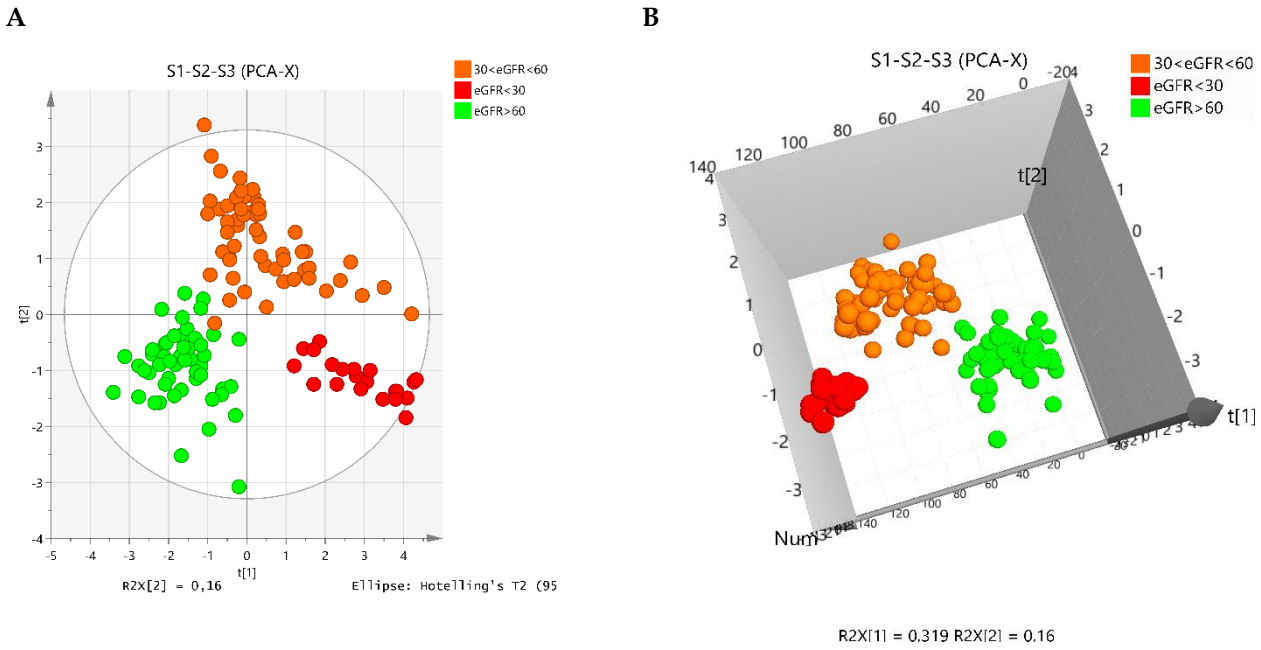


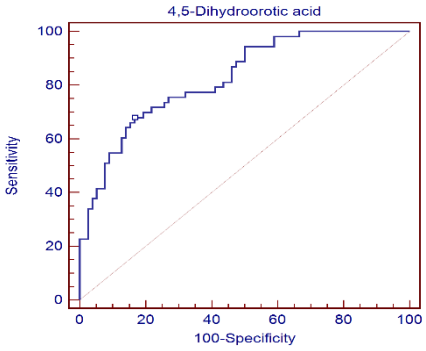
Figure 3. PCA analyses of metabolic marker panels generated based on the eight metabolites, identified against the HMDB database (using an untargeted profiling approach), (ESI+ mode). Each spot represents one sample, and a different color indicates each group. (B) Displays three-dimensional PCA score plots based on the data from UHPLC-Q-TOFMS separation.

To further characterize both the predictive value of these individual metabolites independently and the combination of these detected potential candidates, we performed ROC analysis. The ROC curves of these detected individual potential candidates including 4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0), QYNAD, 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate among the two groups (eGFR <60 vs eGFR>60) had an AUC: 0.827 (95% Confidence interval 0.751 to 0.887, pvalue : 0.0001); 0.725 (95% Confidence interval 0.640 to 0.799, pvalue : 0.0001), 0.691 (95% Confidence interval 0.605 to 0.769, pvalue : 0.0001), 0.778 (95% Confidence interval 0.698 to 0.846, pvalue : 0.0001), 0.757 (95% Confidence interval 0.675 to 0.828, pvalue : 0.0001), 0.694 (95% Confidence interval 0.607 to 0.771, pvalue : 0.0001), 0.659 (95% Confidence interval 0.571 to 0.740, pvalue : 0.0013) and 0.764 (95% Confidence interval 0.682 to 0.834, pvalue : 0.0001), respectively (Figure 4).

An AUC of 0.5 suggests no discrimination, 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding [19]. Among the individual metabolites, we found with highest significant the 4,5-Dihydroorotic acid. The results of ROC curve analyses indicated that in the urine samples, 5 metabolites with high AUC above 0.70 were: 4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid

5-semialdehyde, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate (p value <0.01).

A



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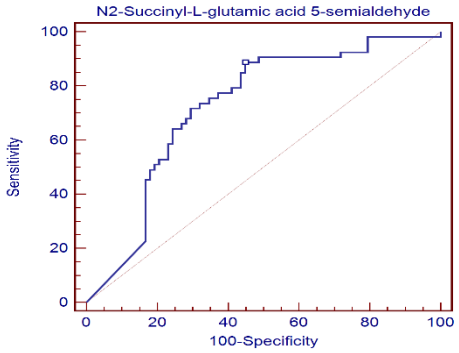

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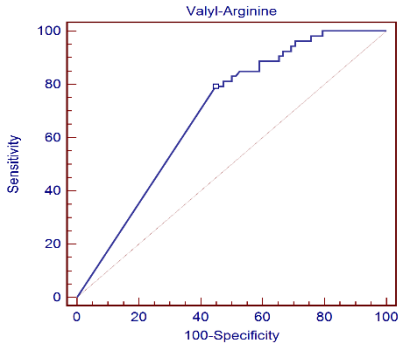

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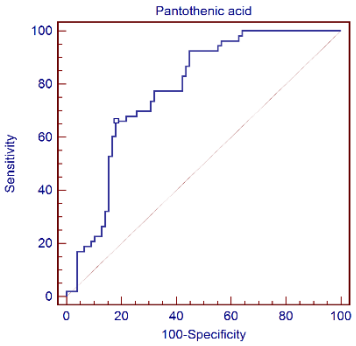

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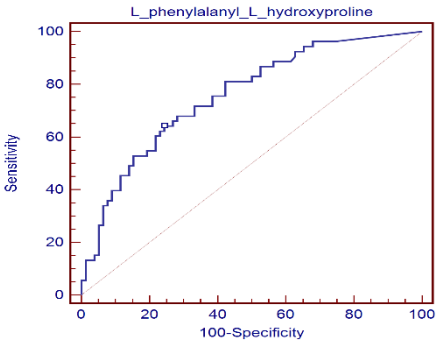

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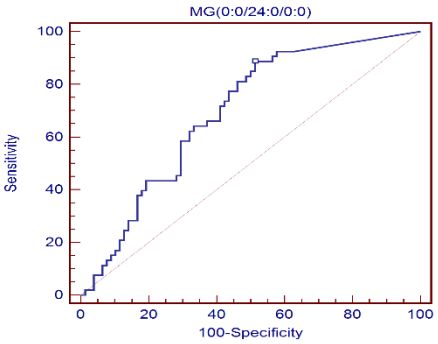

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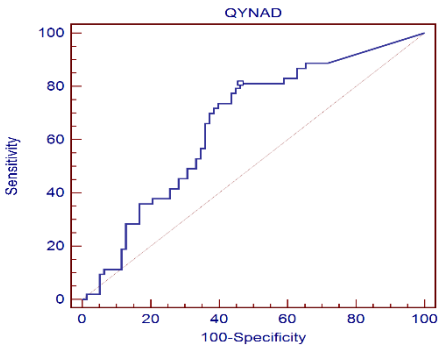

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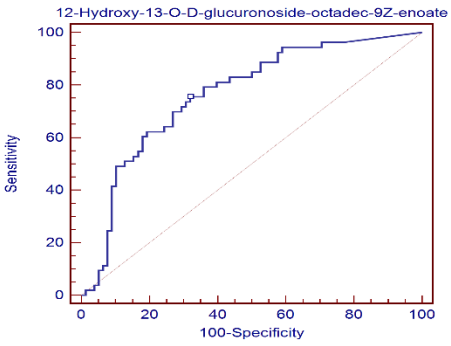

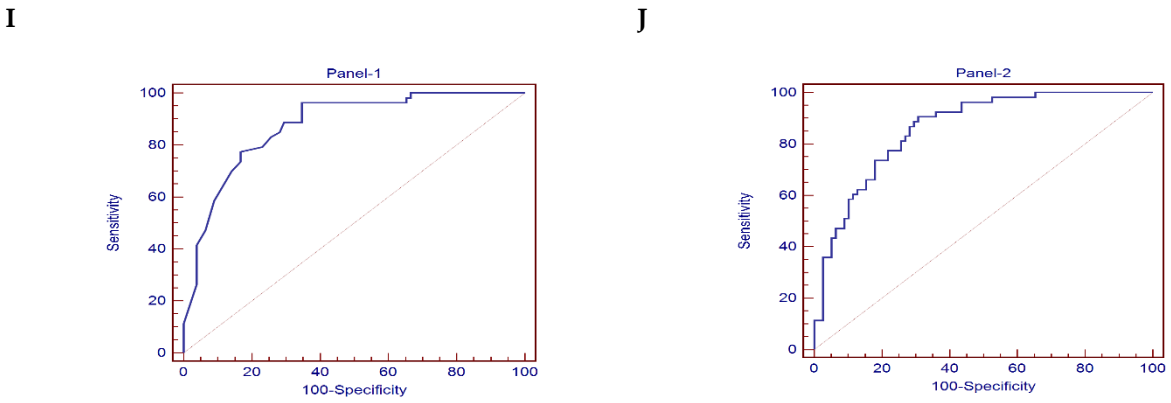

Figure 4. Shows of ROC curves analysis. ROC curves of biomarker candidates for 4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0), QYNAD and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate panel-1 and panel-2.

In a biological system there may be multiple marker candidates that work in tandem with their own individual discriminating capability. In a biological system, there may be more than one candidate working together with their individual discrimination abilities. If a panel of markers is used instead of a single marker, overall discrimination can be improved. *We* investigated the discriminating capability of these eight detected individual potential candidates, *their 57 ratios (*Supplement Table S2*) and* 245 different combinations of metabolite panels, which were generated based on the eight metabolites (Supplement Table S3), to monitor post-transplant patients.

245 different combinations of metabolite panels achieved AUCs that ranged from 0.695 to 0.862 while the 57 ratios of eight potential *markers* achieved AUCs that ranged from 0.463 to 0.875. While *4,5-Dihydroorotic acid/Pantothenic acid had the highest AUC of 0.875 (panel-1)* ($p < 0.05$ in the 57 ratios of eight potential *markers*, the panel-2 consisted of 7 potential *markers* (4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0) and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate) *had the highest AUC of 0.862* ($p < 0.05$) in 245 different combinations of metabolite panels (Supplement Table S3).

2.4 Correlation Analysis of Individual Significant Metabolites, Their Metabolite Ratios, Combinations of Metabolite Panels Generated Based on The Eight Metabolites and Kidney Function Indicators

Creatinine-based estimates of Scr concentration do have some disadvantages, including the cause of an overestimation of kidney function, as they are influenced by biological confounders such as age, gender, and particularly muscle mass. We analyzed Panel-1, panel-2, eight significant individual metabolites by Spearman's rank bivariate correlation analysis for their association with glomerular filtration rate (eGFR) (Supplement Table S4), estimated separately from creatinine by CKD-EPI (CKD Epidemiology Collaboration) equations, age, gender, body surface and BMI. eGFR was significantly correlated with panel-1 (Spearman r=-0.786, P<0.001), panel-2 (Spearman r=-0.746, P<0.001), 4,5-Dihydroorotic acid (Spearman r=-0.716, P<0.001), N2-Succinyl-L-glutamic acid 5-semialdehyde (Spearman r=-0.530, P<0.001), Valyl-Arginine (Spearman r=-.400, P<0.001), Pantothenic acid (Spearman r=0.586, P<0.001), L-phenylalanyl-L-hydroxyproline (Spearman r=0.521, P<0.001), MG(0:0/24:0/0:0) (Spearman r=0.389, P<0.001, QYNAD (Spearman r=0.358, P<0.001) and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate (Spearman r=0.590, P<0.001). No panels and metabolites were associated with age, gender, BMI, and body surface.

3. DISCUSSION

KTx is the best renal replacement therapy for patients with ESKD. In addition to identifying signs of kidney dysfunction, close post-transplant monitoring is important to localize organ damage or detect early stages of acute rejection. It can allow preventive or corrective measures to be taken without the organ being irreparably damaged. This study reports the biomarker panel to identify metabolic urinary biomarkers of

reduced kidney function in kidney transplant individuals divided into groups based on post-transplant eGFR-based kidney function stages with a metabolomics screening technique, further revealing metabolic abnormalities that contribute to renal dysfunction after kidney transplant. The staging and diagnosis of CKD and the monitoring of patients after kidney transplantation withstand creatinine as a biomarker to predict GFR. In this study, we collected urine samples from 131 unique kidney transplant recipients and analyzed by Xevo G2 XS QTof. Followed by pre-processed and multivariate statistical analysis, putative annotation was performed against the HMDB database. VIP, fold change and p-values from OPLS-DA revealed that 8 metabolites were significantly altered within three groups of kidney transplant recipients: 4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0), QYNAD and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate as biomarker candidates ($P < 0.05$, QC<30, Max fold change (MFC) >1.2). Firstly, we created the post-transplant biomarker panels that can distinguish stages of renal dysfunction by combining the significant markers and taking their ratios and then performed ROC analysis of both these individual metabolites separately and of the generated panels to predict worsening kidney graft function. Evaluation of biomarkers by ROC analysis showed that there are 5 metabolites with AUC over 0.70, also the ratio of 4,5-Dihydroorotic acid to Pantothenic acid (pantothenic acid) can be used to monitor kidney function. Numerous findings have been published in recent years that the use of marker candidates can potentially improve diagnosis and aid in the monitoring of kidney transplants [20–23] Urine may be a perfect fluid for discovering biomarkers related to disease because collection is a non-invasive, inexpensive, large volume, non-complex, lower protein ingredient, and low cost [24]. To the best of our knowledge, this study is the first in the literature to describe specific eGFR-based noninvasive biomarkers for monitoring individuals with post-transplant graft dysfunction using mass spectrometry-based untargeted metabolomics, representing the prediction of worsening kidney graft function. This study might offer insight into pathophysiologic mechanisms.

Metabolic biomarkers may provide useful information to contribute to monitoring kidney function. Pantothenic acid (PA), also known as pantothenate or vitamin B5, is, in addition to its antioxidant properties[25], mostly involved in lipid, protein and sugar metabolism in the form of CoenzymeA. It is also an important member of pantothenate and CoA biosynthesis [26]. For lipid metabolism, acyl group activation is determined as the primary function of pantothenic acid., CoA is required to modulate transport properties or functions for the oxidation of pyruvate, fatty acids, and oxogutarate, acetylation of other molecules metabolism of sterols. β-oxidation, is the principal process of fatty acid catabolism in peroxisomes, is CoA dependent and down-regulated by pantothenate deficiency. Fatty acid oxidation-mediated lipid accumulation is thought to contribute to kidney disease, including chronic kidney disease, acute kidney injury, and diabetic nephropathy [27]. Gao and et al. demonstrated that the decreased pantothenic acid and the increased succinic acid are associated with Nephrolithiasis which is a common urinary tract disease found in the study of rats. Our results show pantothenic acid decreases, indicating that energy metabolism and antioxidant capacity are reduced.

Succinic acid, a key intermediate in the tricarboxylic acid cycle, is associated with some kidney disease. Several researchers have shown that in vivo in, 4-hydroxybutyric acid (also known as gamma-hydroxybutyric acid or GHB) is converted to succinic acid via succinic acid and the TCA cycle in rat liver and kidney as well as rat and mouse brain [28–30]. Chambliss et al. were studied on succinate semialdehyde dehydrogenase (SSADH) deficiency, known as a disorder of Gaba metabolism, in brain and SSADH expression in human liver and kidney. In this study, they demonstrated that SSADH exists in liver and kidney as well as brain. SSADH deficiency causes an elevation in GHB [31]. N2-succinyl-l-glutamic acid 5-semialdehyde are intermediates involved in arginine and proline metabolism as well as a substrate for SSADH [32]. L-Arginine is catabolized into various products initiated by arginase, nitric oxide synthase. One of these pathways is nitric oxide synthetic pathway. An impaired NO synthetic pathway may play a key role in many physiologic processes that influence kidney function associated with the progression of kidney diseases [33]. Our study showed that N2-succinyl-L-glutamic acid 5-semialdehyde, one part of Arginine and proline metabolism, showed progressive growth with decreased kidney function.

4,5-Dihydroorotic acid is an orotic acid derivative that acts as an intermediate in pyrimidine biosynthesis and is also a substrate for the enzyme known as dihydroorotate dehydrogenase [34]. It is known in the literature that impaired purine nucleotide metabolism is a risk factor in chronic kidney disease. Impaired purine metabolism and renal excretion contribute to prevalence of hyperuricemia is associated with a risk factor for CKD [35]. In our study, among the ROC curves of the individual potential candidates identified between the three groups, the candidate with the largest AUC was 4,5-Dihydro-Orotic acid. When the correlations of individual potential candidates with eGFR were investigated, 4,5-Dihydro-Orotic acid had the

highest correlation with eGFR among the identified individual potential candidates (Spearman r=-0.716, P<0.001). Increase in 4,5-Dihydroorotic acid, which is part of Pyrimidine metabolism, indicates that this metabolite may have a major role in impaired renal dysfunction.

Lipids are the fundamental components of biological membranes. Experiments over the years have shown that abnormality in lipids and lipid-derived metabolites is not only involved in oxidative stress and inflammatory processes, but also contributes to the progression of kidney disease [36]. 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate is one of the organic compounds known as saccharolipids. In our study, 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate was the second metabolite with the highest correlation with eGFR, and with the second largest AUC after 4,5-Dihydroorotic acid. MG(0:0/24:0/0:0) is a glycerolipid, which is one of the eight categories of lipids. This chemical structure is a glyceride consisting of one fatty acid chain covalently bonded to a glycerol molecule through an ester linkage [37]. This study showed that MG(0:0/24:0/0:0) progressively decreased with decreased kidney function. 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate and MG(0:0/24:0/0:0) with the other significant metabolites may help to understand the pathways that contribute to the kidney transplant *pathophysiology*.

Collagen, widely distributed among all kidney tissues, is an important structural component of the kidney. In addition, it plays a vital role in normal physiology. Numerous studies have demonstrated an association between urinary collagen peptides and various CKD etiologies [38,39]. In these studies, col1a1 peptides were identified as negatively correlated with obesity-related nephropathy and fibrosis as well as positively correlated with both mild-to-moderate and advanced CKD [39]. L-phenylalanyl-L-hydroxyproline is a dipeptide in urine, which is produced by a proteolytic breakdown of collagen. In our study, L-phenylalanyl-L-hydroxyproline decreased with decreased kidney function.

Peptides have a role in the inflammatory response, tumor biology, and endocrine processes, presenting them as appealing biomarker candidates. Peptide profiles are most used in clinical diagnosis to successfully distinguish prostate, bladder, and breast cancer patients from healthy persons [40,41]. Among the detected significant metabolites; Valylarginine is a dipeptide composed of valine and arginine and QYNAD is an endogenous pentapeptide with the sequence Gln-Tyr-Asn-Ala-Asp, isolated from the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) and Guillain-Barre syndrome (GBS). This was detected at elevated levels in the cerebrospinal fluid of patients with Immune mediated inflammatory neurological disorders like with multiple sclerosis and Guillain-Barré syndrome [42]. To the best of our knowledge, there is no study that has found an association QYNAD with kidney dysfunction. Our study showed that QYNAD and Valylarginine had a weak correlation with eGFR.

Metabolomics is a sensitive and powerful diagnostic tool to detect metabolite profiles that is useful for understanding biochemical functions and changes in related diseases. Early diagnosis plays a key role in successful treatment of the disease. Detection of disease biomarkers has become not only part of this key role, but also an important one for monitoring the status of biological organisms [43].

4. CONCLUSION

Our results showed that biomarker panels that include these significant biomarker metabolites could be used to monitor renal function after graft surgery. The minimum number of metabolites that can be used to monitor kidney function includes the ratio of 4,5-Dihydroorotic acid to Pantothenic acid (panel-1). Specifically, these metabolite ratios were found to be more sensitive to changes in kidney function than panel-2 which consisted of 7 potential *markers (*4,5-Dihydroorotic acid, N2-Succinyl-L-glutamic acid 5-semialdehyde, Valyl-Arginine, Pantothenic acid, L-phenylalanyl-L-hydroxyproline, MG(0:0/24:0/0:0) and 12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate) . These metabolic marker panels, which can play an important role in the follow-up of patients after transplantation and can be promising targets for treatment, can be used as useful urinary biomarkers due to their high predictive performance, high specificity and sensitivity. Future studies in the *largest prospective cohort* of *kidney transplant* recipients are needed to identify individuals at high risk for kidney post-transplant graft dysfunction of kidney transplants and to test the potential contribution of fingerprinting of *kidney transplant* recipients to early identification of kidney damage.

5. MATERIALS AND METHODS

5.1 Study Design and Population

The study recruited outpatients with post transplantation according to the following inclusion criteria: adult (age ≥18 years) kidney transplant with a living or cadaver kidney transplant.; willingness to comply in

all aspects of the study procedures and to provide serum and urine 24 hours samples. Patients with the following conditions were excluded: a history of infection, pregnancy, cancer, acute cardiovascular event or blood diseases. The estimated glomerular filtration rate (eGFR) level was calculated from serum creatinine using the CKD-EPI 2021 formula for post transplantation patients. An overview of the study design is shown in Figure 5, urine and blood samples were collected from outpatients in a specific timeframe at the Kidney Transplant Center at Bursa Acıbadem Hospital. The discovery phase was approached by UPLC-MS-MS, followed by data processing and statistical analysis. This study was approved by Acıbadem Mehmet Ali Aydınlar University (Turkey). Informed written consent was obtained from all participants (Approval ID: 2020-08/14). More details are explained in the supplementary file.

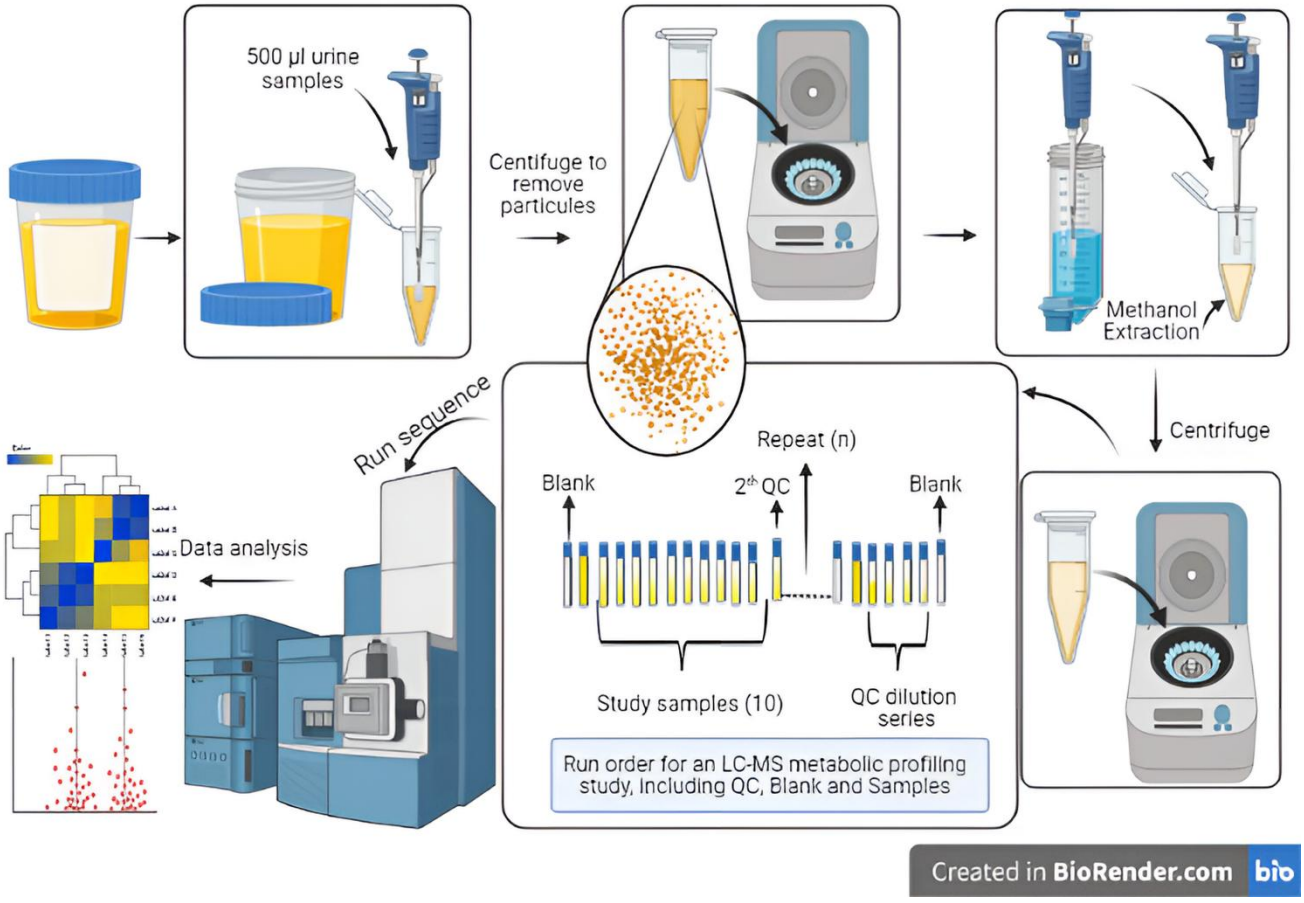

Figure 5. The workflow in the present study. Following sample preparation procedures, each sample was run according to the run order mentioned above; the last step was to discover biomarker candidates by data processing and statistical analysis. QC: Quality control.

5.2 Data Acquistion

Liquid chromatography-mass spectrometry-based metabolomics using a Xevo G2 XS QTof) was applied for metabolite identification (see the Supplementary File for details).

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Conflict of interest statement: The authors declare that they have no competing interests.

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