ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

LONGITUDINAL NON-TARGETED METABOLOMIC PROFILING OF URINE SAMPLES FOR MONITORING OF KIDNEY TRANSPLANTATION PATIENTS

İHSAN YOZGAT

PH.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethicel rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

15.04.2024

İhsan YOZGAT

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation	Explanation
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
CIT	Cold ischemia time
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CV	Coefficient of variation
CV-ANOVA	Cross-validated predictive residuals
DCD	Donation after-cardiac death
DGF	Delayed graft function
DM	Diabetes mellitus
ECDs	Expanded criteria donors
eGFR	Estimated glomerular filtration rate
ESI	Electrospray ionization
ESI+	Positive ionization
ESRD	End-stage renal disease
FA	Formic acid
GC-MS	Gas chromatography-mass spectrometry
HILIC	Hydrophilic interaction liquid chromatography
HLA	Human leukocyte antigens
HLA-DSA	Donor-specific hla antibodies
HMDB	Human metabolome database
HPLC	High-performance liquid chromatography
HREGL	Highest risk of early graft loss
hs-CRP	C-reactive protein
KEGG	Kyoto encyclopedia of genes and genomes
KTx	Kidney transplantation
LC-MS	Liquid chromatography-mass spectrometry
LMSD	Lıpıd maps structure database

LTGS	Long-term renal graft survival
LTO	Long-term outcomes
m/z	Mass-to-charge ratios
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PEKT	Pre-emptive kidney transplantation
PRA	Panel reactive antibody
QC	Quality control
ROC	Receiver operating characteristic
RP	Reversed-phase
RP-HPLC-MS	Reverse-phase high-performance liquid chromatography-mass spectrometry
RRT	Renal replacement therapies
RT	Retention time
SCD	Standard criteria donor
THF	Tetrahydrofuran
TUBITAK	The Scientific and Technological Research Council of Turkey
UHPLC	Ultra-high-pressure liquid chromatography
UPLC/ESI/QToF- MS	Ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry
VIP	Variable influence on projection
WIT	Warm ischemia time
Xevo G2 XS QTof	Ultra-high-performance liquid chromatography coupled with a benchtop qtof mass spectrometer

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ÖZET

Böbrek Nakli Hastalarının İzlenmesi İçin İdrar Örneklerinin Boylamsal Hedeflenmemiş Metabolomik Profili

Transplantasyon sonrasındaki ilk yıl içinde böbrek fonksiyonlarının değerlendirilmesi, uzun vadeli greft sağkalımını tahmin etmek açısından önemlidir. Bu çalışma, erken greft kaybı riski taşıyan hasta gruplarında metabolit profillerini kullanarak erken uzun vadeli sonuçları öngörmek için sağlam ve doğru bir model geliştirmeyi amaçlamaktadır. Altmış bir böbrek transplantasyonu alıcısı, bir haftalık hastanede kalışı ve üç ve altı ayda yapılan takip değerlendirmelerini içeren bir yıl süresince kapsamlı bir izleme sürecine tabi tutuldu. Onikinci ay takip serum kreatinin seviyelerine göre: Grup 2'nin seviyeleri 1.5 mg/dl'nin üzerindeyken, Grup 1'in seviyeleri 1.5 mg/dl'nin altındaydı. Metabolitler kütle spektrometresi ile tespit edildi ve ilk olarak ön işlemden geçirildi. İki grup arasındaki önemli farklılıkları belirlemek için tek değişkenli ve çok değişkenli istatistiksel analizler kullanıldı. On dokuz metabolitin 1. haftada ve on yedi metabolitin 3. ayda önemli ölçüde farklılık gösterdiği bulundu (ayarlanmış p-değeri < 0.05, kalite kontrolü < 30, kat değişim > 1.1 veya kat değişim < 0.91, projeksiyondaki değişkenin önemi > 1). Ancak, 6. ayda önemli farklılıklar gözlemlenmedi. Bu özgün metabolitler genellikle lipid, yağ asidi ve amino asit kategorilerine aitti. On model, geriye doğru bir koşullu yaklaşım kullanılarak oluşturuldu ve en iyi performans, 1. haftada Grup 2 için model 5'te (AUC 0.900) ve 3. ayda model 3'te (AUC 0.924) görüldü. Sonuç olarak, erken aşamalarda geliştirilen modeller, böbrek transplantasyon hastalarının yönetiminde potansiyel faydalar sunabilir.

Anahtar Sözcükler: Biyobelirteç, Böbrek nakli, Uzun süreli böbrek grefti sağkalımı, Hedeflenmemiş metabolomikler, Boylamsal metabolit profili.

ABSTRACT

Longitudinal Non-Targeted Metabolomic Profiling of Urine Samples for Monitoring of Kidney Transplantation Patients

Assessing kidney function within the first year after transplantation is crucial for predicting the long-term survival of the graft. This study aimed to create a robust and precise model using metabolite profiles to forecast early long-term outcomes in patient groups at high risk of early graft loss. A total of 61 kidney transplant recipients underwent comprehensive monitoring over a one-year follow-up period, including a one-week hospital stay and evaluations at three and six months. Based on their serum creatinine levels at the 12-month follow-up, Group 2 had levels exceeding 1.5 mg/dl, while Group 1 had levels below 1.5 mg/dl. Metabolites were identified using mass spectrometry and underwent initial preprocessing. Univariate and multivariate statistical analyses were conducted to identify significant differences between the two groups. Nineteen metabolites showed significant differences in the first week, and seventeen metabolites at the three-month mark (adjusted p-value < 0.05, quality control (QC) < 30, fold change (FC) > 1.1 or FC<0.91, Variable Influence on Projection (VIP) > 1). However, no significant differences were observed at the six-month mark. These distinct metabolites primarily belonged to lipid, fatty acid, and amino acid categories. Ten models were developed using a backward conditional approach, with model 5 performing best for Group 2 at the first-week mark (AUC 0.900) and model 3 at the three-month mark (AUC 0.924). In summary, The findings highlight the advantages of early-stage models for enhancing the care of kidney transplant recipients, offering valuable predictive and management tools for long-term patient outcomes.

Keywords: Biomarker, Kidney transplantation, Long-term renal graft survival, Untargeted metabolomics, Longitudinal metabolite profiling.

1 INTRODUCTION AND AIM

Renal transplantation (KTx) is widely recognized as the preferred treatment option for a considerable number of patients facing end-stage kidney disease, owing to a multitude of benefits, including enhanced quality of life, diminished risk of mortality, and alleviated financial burdens (1,2). However, the treatment of kidney transplantation does come with certain drawbacks, including a higher risk of infection (3), bleeding (4), potential damage to surrounding organs (4), the development of posttransplant diabetes (5), and an increased likelihood of posttransplant malignancy (6). Numerous studies have consistently demonstrated that kidney function within the initial year following transplantation plays a pivotal role in influencing LTGS in eligible individuals undergoing KTx. Remarkably, the survival rate of transplanted kidneys has been reported to reach as high as 97% after a minimum of one year (7). Retrospective studies have consistently emphasized the significance of specific time points, evaluating renal function based on serum creatinine levels at 6 months and 1 year post-transplantation. The assessment of serum creatinine levels at both 6 and 12 months post-transplantation, along with the examination of variations between these two time points, has emerged as pivotal in determining graft survival and demonstrating a robust association with long-term graft survival (8–10). Various factors have been pinpointed as potential contributors influencing the outcomes of kidney transplants. These variables encompass incidents of survival rejection, exposure to nephrotoxic agents, acute effects on grafts, the age of both recipients and donors, racial considerations, matching in Human Leukocyte Antigens (HLA), the existence of diabetes, occurrences of delayed graft function, episodes of Cytomegalovirus infection (CMV), warm ischemia time (CIT) and cold ischemia time (WIT) (10–13). These factors contribute to the complexity and variability of graft survival, highlighting crucial aspects that require careful management and monitoring of kidney transplant patients. Evaluating kidney function during the first year post-transplantation emerges as a pivotal indicator of LTGS, significantly influencing the overall success of the transplant. It offers a useful tool for estimating LTO in kidney transplant recipients. By closely monitoring and evaluating kidney function within the initial year post-transplantation, healthcare professionals can gain insights into the

potential success and durability of the graft. This information aids in making informed decisions regarding patient management and optimizing transplant outcomes. The pivotal role of creatinine clearance and serum creatinine levels, functioning as crucial markers, lies in their central contribution to the identification of individuals at a heightened risk of early graft loss within the HREGL (8). Comprising a spectrum of approaches, conventional monitoring practices for assessing graft dysfunction encompass an examination of serum and urine parameters associated with kidney function, clinical evaluation, conducting renal allograft biopsy, and monitoring levels of immunosuppressive drugs (14,15). However, relying solely on serum creatinine levels may not offer sufficient specificity, sensitivity, or accuracy, as they can be influenced by biological factors such as age, gender, and muscle mass. Consequently, estimations of kidney function based on creatinine levels may lead to overestimations (16). In situations demanding a conclusive diagnosis, subsequent biopsies become essential. Despite their diagnostic utility, renal biopsies are burdened by various limitations, encompassing potential risks of bleeding, invasiveness, elevated blood pressure, as well as impracticality in clinical settings, and the prospect of renal malformation (17). The introduction of innovative techniques, like "omics" technology, has the capacity to address the constraints of conventional diagnostic tools and revolutionize patient management with a groundbreaking approach. Leveraging a sophisticated and multifaceted methodology, the field of metabolomics systematically investigates and analyzes metabolites present in diverse biological matrices, including but not limited to urine, blood, feces, and tissues. The overarching objectives of this analytical approach are twofold: first, to identify potential biomarkers that hold diagnostic or prognostic significance, and second, to gain a nuanced and profound insight into the intricate mechanisms governing metabolism within biological systems. Significant progress has been observed in this field, driven by notable advancements in bioinformatics and the seamless integration of high-throughput methodologies. These cutting-edge techniques encompass a diverse range of analytical tools, including nuclear magnetic resonance (NMR), capillary electrophoresis mass spectrometry, gas chromatography-mass spectrometry (GC-MS), ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry (UPLC/ESI/QToF-MS), and infrared spectroscopy. These techniques

enable comprehensive identification and quantification of both targeted and untargeted small molecule metabolites (18–20) While NMR exhibits advantages in terms of reproducibility, sample preparation, and sample recovery, it does have limitations in selectivity and sensitivity. In contrast, mass spectrometry (MS) technology provides markedly greater selectivity and sensitivity when compared with NMR, making it a more economical choice as well (21,22). Urine offers distinct benefits as a sample type. Firstly, it allows for non-invasive collection, offering a convenient and pain-free sampling method. This continuous collection feature is especially advantageous for tracking changes over time. Secondly, urine is cost-effective, making it accessible for research and diagnostics. Furthermore, urine has lower protein levels, reducing interference in analyses and enabling clearer assessment of metabolite profiles. Additionally, using urine for biomarker research helps avoid potential complications and side effects associated with other sample types (21,23). After a kidney transplant, it's vital to follow medication instructions and undergo regular check-ups to lower the risk of organ rejection. This dedication is key to keeping the transplanted kidney working well. Kidney health is closely linked to overall well-being and quality of life (24). To enhance results for kidney transplant recipients and facilitate timely therapeutic measures, the early identification of kidney disease is paramount. This research engaged 61 participants who had undergone kidney transplantation (KTx) and employed cutting-edge technology, notably ultra-high-performance liquid chromatography coupled with a benchtop Qtof mass spectrometer (Xevo G2 XS Qtof), renowned for its heightened selectivity, sensitivity, and reproducibility. Our overarching objective involved the identification of metabolite biomarkers that could serve as early predictors for anticipating LTO in patient subsets characterized by HREGL, particularly those exhibiting serum creatinine levels surpassing 1.5 mg/dl at the 12-month mark. Through the formulation of a sensitive and dependable model centered on these biomarkers, our objective is to equip physicians with a valuable instrument 5ort he early anticipation and intervention in the care of kidney transplant patients. This approach carries the potential to be life-saving and substantially enhance patient outcomes.

2 BACKGROUND

2.1 Kidney Physiology and Chronic Kidney Disease

The kidneys, being one of the most essential organs, play a crucial role in maintaining overall health. Performing diverse essential roles, these entities play a role in synthesizing an active form of vitamin D, maintaining body fluid balance, secreting erythropoietin (a hormone that governs red blood cell production in the bone marrow) (25), elimination of waste products from the body, excretion of drugs and toxins (26), and regulation of blood pressure (27). Impairment of these kidney functions can lead to various health issues, such as the formation of kidney stones (28), polycystic kidney disease(29), glomerulonephritis (30), chronic kidney disease (31), and urinary tract infections (32).

Chronic kidney disease (CKD) stands as a globally recognized public health challenge characterized by a gradual deterioration in kidney function over an extended period. The classification of CKD is structured into five stages, with these stages determined by specific parameters such as estimated glomerular filtration rate (Egfr) or measured glomerular filtration rate (GFR) (33). A detailed description of the five stages of CKD is presented in Table 1. The diagnosis and staging of CKD typically rely on various parameters including serum creatinine, Egfr (according to the guidelines of the National Kidney Foundation Kidney Disease Outcomes Quality Initiative), blood urea nitrogen, and kidney histopathology (immunohistochemistry) (33,34). Mineral and bone disorder emerges as a noteworthy complication of CKD, characterized by disruptions in calcium and phosphate metabolism. Diminished levels of 1,25-(OH)2D lead to a decline in intestinal calcium reabsorption, triggering increased secretion of parathyroid hormone and subsequent development of secondary hyperparathyroidism (35).

Table 1. The five stages of CKD

Glomerular Filtration rate (GFR) Stages	G1	Normal	≥ 90ml/min/1.73m^2
	G2	Mildly decreased	89–60 ml/min/1.73m^2
	G3a	Mildly to moderately decreased	45–59 Ml/min/1.73 m
	G3b	Moderately to severely decreased	30–44 Ml/min/1.73 m
	G4	Severely decreased	15–29 Ml/min/1.73 m
	G5	Kidney Failure	<15 ml/min/1.73m^2

2.2 Kidney Transplantation

In the advanced stage of end-stage renal disease (ESRD), as kidney failure progresses, patients necessitate either dialysis therapy or kidney transplantation to prolong life. While facing end-stage kidney disease, many individuals opt for kidney transplantation (KTx) due to its associated benefits, including improved quality of life, decreased mortality risk, and lowered overall costs. Moreover, recipients of kidney transplants are relieved from the need for regular dialysis sessions. However, this treatment approach also carries certain disadvantages, such as increased susceptibility to infections (36), risk of bleeding (37), potential damage to surrounding organs (37), development of posttransplant diabetes (38), and higher chances of posttransplant malignancies (6). After undergoing a kidney transplant, patients are closely monitored as they are placed on complex immunosuppressive regimens, which make them more vulnerable to infections, malignancies, and cardiovascular disease (39–41).

The year 1954 holds great significance in the history of kidney transplantation. While it was not the first successful transplant, it marked a major milestone as Joseph Murray became the first person to successfully perform a kidney transplant between two identical twins. This particular transplant gained immense recognition and is considered one of the most famous organ transplantations (42,43). Joseph Murray's

groundbreaking contributions to the field of organ transplantation earned him the prestigious Nobel Prize in Physiology or Medicine (42). The realization of the first kidney transplant from an unrelated donor in 1962 was made possible by a crucial development during the period of 1960 to 1970. This breakthrough involved the introduction of several immunosuppressive drugs, which played a vital role in suppressing the immune system and preventing rejection of the transplanted kidney. These advancements in immunosuppression opened up the possibility of organ donation from individuals who were not genetically identical to the recipient, expanding the pool of potential donors (44).

Currently, kidney transplantation has emerged as the standard and preferred treatment for patients with ESRD. It is the most commonly performed organ transplant procedure compared to other types of organ transplants. The high demand for kidney transplants is driven by the relatively higher availability of kidneys from both living and deceased donors, as well as the significant impact of kidney transplantation on improving the quality of life and overall survival of patients with ESRD (45).

Between 1 January 1988 and 11 May 2023, a total of 935,972 organ transplants were recorded in the USA. Among these, kidney transplantation accounted for the highest number, with 551,502 transplants, representing 58.9% of all organ transplants. Liver transplantation ranked second with 204,150 transplants, accounting for 21.6% of the total (45). In 2023 alone, there were 8,754 kidney transplants performed in the USA, out of which 6,833 were from deceased donors and 1,921 were from living donors. These transplants were carried out for 96,084 individuals who were actively waiting on the kidney transplant list. This number was higher compared to the 25,500 individuals on the list as of 31 April 2022. The year 2022 saw 42,431 new enrollments on the kidney transplant waiting list, which was a significant increase compared to the number of new enrollments in 1999, indicating a growing demand for kidney transplantation as a treatment option (45). The survival rates for both grafts and patients after kidney transplants have shown continuous improvement. Among transplant recipients between 2009 and 2013, the 1-year patient survival rate was 97.4%, and the 5-year patient survival rate was 90.0% (45). These advancements in

survival rates reflect the progress made in kidney transplantation and the effectiveness of the procedure in prolonging and improving the lives of individuals with end-stage renal disease.

The first organ transplant in Turkey took place in 1968 at Ankara Turkey Yüksek İhtisas Hospital, performed by Dr. Kemal Bayazıt (46). However, it was in 1975 that the first successful organ transplant occurred at Hacettepe University Hospital, where Dr. Mehmet Haberal and his team successfully transplanted a kidney from a mother to her son (47). These milestones in organ transplantation in Turkey influenced the enactment of Law No. 2238 on Organ and Tissue Acquisition, Storage, and Transplantation in 1978, which established the legal framework for organ transplantation in the country (47). As of 2021, the number of patients waiting for organ transplantation in Turkey exceeds 26,000. In terms of organ donors over the age of 18, their proportion in Turkey represents 0.9% of the population (48). These figures indicate the ongoing need for organ transplantation in Turkey and highlight the importance of increasing organ donation rates to meet the demand and save more lives.

2.3 Factors Known to Affect Transplant Success

Numerous studies have consistently affirmed that kidney function within the initial year post-transplantation significantly influences LTGS in individuals undergoing KTx. Reportedly, the majority of transplanted kidneys, approximately 97%, exhibit survival for at least one year post-transplantation (7). Retrospective studies scrutinizing renal function in kidney transplant recipients consistently underscore the importance of serum creatinine levels at 6 months and 1 year post-transplantation. These markers, in conjunction with the alteration in serum creatinine between 6 and 12 months, play a pivotal role in determining graft survival and exhibit a robust association with LTGS (8–10). Several known factors impact the success of kidney transplantation and the prolonged viability of the transplanted organ. These risk factors encompass HLA compatibility, CMV infection, intervals of WIT, and CIT (10–13,49). The evaluation of renal function in the first year post-kidney transplantation not only emerges as a reliable predictor of LTGS but also provides

clinicians with a valuable tool during this critical period to estimate the LTO of the transplanted organ.

2.7.3 Donor-recipient hla matching

The Human Leukocyte Antigen complex plays a crucial role in determining the outcome of transplantation, with a specific focus on the degree of HLA compatibility between the donor and recipient. Matching the HLA antigens between the two individuals is a critical factor in transplant success. Exposure to non-self HLAs, meaning HLA antigens that are not present in the recipient's own immune system, can increase the risk of graft rejection and early failure of the transplanted organ. The immune system recognizes non-self HLAs as foreign and mounts an immune response against them, potentially leading to rejection of the transplanted graft. In general, achieving better HLA matching between the donor and recipient has a positive impact on the survival of the allograft (transplanted organ). By increasing the level of HLA compatibility, the risk of rejection is reduced, and the chances of long-term graft survival are improved. This emphasizes the importance of carefully assessing the HLA compatibility before proceeding with transplantation. Healthcare professionals and transplant teams consider HLA matching as a crucial aspect when selecting potential donors for organ transplantation. By identifying compatible donors who share similar HLA antigens with the recipient, the likelihood of successful transplantation and reduced risk of rejection can be significantly enhanced. Efforts are made to find the best possible HLA match between the donor and recipient, taking into account factors such as HLA class I and class II antigens. This comprehensive evaluation of HLA compatibility aids in optimizing the chances of graft acceptance and long-term transplant success (50,51).

In order to determine the presence of specific HLA antibodies in a patient, a blood test is conducted using the patient's serum. The serum is tested against lymphocytes, which are white blood cells obtained from a panel of approximately one hundred blood donors. This test is known as Panel Reactive Antibody (PRA) testing. The percent PRA (%PRA) is calculated based on the number of positive reactions observed in the

panel. PRA testing is performed prior to transplantation to identify any anti-HLA antibodies that may be present in the recipient's serum. This information is crucial in minimizing the risk of antibody-mediated rejection following transplantation. If a recipient candidate's serum does not exhibit any reactivity with the donor samples in the panel, it indicates that the candidate is not sensitized to the tested HLA antigens, and the PRA count is reported as zero. On the other hand, a PRA greater than 80% is considered highly sensitized. This means that the recipient has a high likelihood of experiencing acute rejection in approximately eight out of ten instances if a potential donor becomes available from that specific donor pool. Assessing the PRA level helps transplant teams in evaluating the degree of sensitization in a recipient and determining the potential risk of graft rejection due to pre-existing anti-HLA antibodies. By considering the PRA results along with other factors such as HLA matching, the transplant team can make more informed decisions regarding donor selection and tailor the immunosuppressive regimen to minimize the risk of rejection and optimize transplant outcomes (52).

As the PRA value is contingent on both the composition of the panel and the methodology employed for antibody detection, it suffers drawbacks, manifesting as high variability and inconsistency in reflecting the degree of sensitization indicated by PRA. The makeup of the antigen panel exhibits significant variations when employing diverse commercially available kits or locally accessible cell panels, frequently failing to accurately represent the potential donor population (53,54).

In assessing the risk of allo-sensitization and evaluating donor-recipient compatibility before renal transplantation, various quantitative measures of donor-specific HLA antibodies (HLA-DSA) relative strength are utilized by many centers. These measures include flow crossmatch median channel shifts, fluorescent antibody titers, solid-phase assays, and reactivity in complement-dependent cytotoxic crossmatches. While these quantitative measures provide valuable information, the cell-based crossmatching technique is still widely regarded as the gold standard for assessing donor/recipient compatibility and determining the risk level of acute humoral rejection. This technique involves testing the compatibility between donor

and recipient cells, allowing for a direct assessment of compatibility. However, one limitation of the cell-based crossmatching test is its inability to specifically detect pre-existing HLA-DSA. This means that the test may not identify the presence of HLA-DSA that the recipient may already have prior to transplantation. To overcome this limitation, other assays and tests, such as solid-phase assays and flow cytometry, are often used in conjunction with the cell-based crossmatching test. These additional tests provide a more comprehensive evaluation of the presence and strength of HLA-DSA, helping to better assess the risk of allo-sensitization and potential complications related to humoral rejection. By employing a combination of these different testing methods, transplant centers can obtain a more comprehensive understanding of the compatibility between donor and recipient, allowing for a more informed decision-making process in renal transplantation and reducing the risk of complications associated with pre-existing HLA-DSA (55).

2.7.4 Cytomegalovirus infection

Cytomegalovirus infection is a prevalent viral infection that can impact individuals across various age groups. Belonging to the herpesvirus family, CMV is pervasive in the general population (56,57). Most people are infected with CMV at some point in their lives, but the virus remains dormant in the body without causing noticeable symptoms in healthy individuals with a functional immune system (57). However, CMV can pose substantial health challenges for individuals with compromised immune systems, including transplant recipients (58), HIV/AIDS patients (59), and newborn infants (60). In these susceptible groups, CMV infection may result in severe complications, with the potential to be life-threatening. CMV transmission can occur through multiple pathways, encompassing close contact with bodily fluids like saliva, urine, blood, semen, and breast milk. The virus has the potential to be disseminated through avenues such as organ transplantation, blood transfusions, sexual contact, and from mother to fetus during pregnancy. Additionally, respiratory droplets generated when an infected person coughs or sneezes can serve as a mode of transmission (61,62). The symptoms of CMV infection can vary based on the individual's immune status. In healthy individuals, CMV infection may go

unnoticed or manifest with mild flu-like symptoms, including fever, fatigue, muscle aches, and swollen glands. However, in immunocompromised individuals, CMV can cause more severe symptoms, including pneumonia, hepatitis, encephalitis (inflammation of the brain), gastrointestinal complications, and retinitis (inflammation of the retina) (63). To diagnose CMV infection, healthcare providers may perform laboratory tests, such as blood tests or polymerase chain reaction tests, to detect the presence of CMV in the body. Treatment for CMV infection may involve antiviral medications to reduce viral replication and manage symptoms, particularly in immunocompromised individuals (64). Prevention of CMV infection is crucial, especially in high-risk populations. Preventive measures to thwart CMV transmission involve adhering to proper hygiene practices, such as regular handwashing, steering clear of close contact with individuals exhibiting active CMV infection, practicing safe sexual behaviors, and instituting preventive protocols in healthcare settings (65).

Cytomegalovirus is acknowledged as the predominant infectious agent among kidney allograft recipients, with its presence having the potential to result in notable morbidity and complications. In the realm of kidney transplantation, CMV infection holds particular significance as it can adversely influence the outcomes of both the transplanted kidney and the overall health of the recipient (66,67).

In kidney transplant recipients, CMV infection may manifest in diverse clinical forms, encompassing CMV syndrome, tissue-invasive disease, and disseminated CMV infection. These presentations can lead to an array of complications, including delayed graft function, acute rejection, impaired graft function, graft loss, opportunistic infections, and heightened mortality (67–73). Prevention and management of CMV in kidney transplant recipients typically involve antiviral prophylaxis or preemptive therapy. Prophylaxis involves administering antiviral medications to prevent CMV infection during the early post-transplant period. Preemptive therapy involves monitoring the patient's CMV viral load and initiating antiviral treatment when viral replication is detected (74). Monitoring and managing CMV infection in kidney transplant recipients is crucial for optimizing transplant outcomes and reducing morbidity and graft loss associated with CMV-related complications. Regular

screening, appropriate prophylactic or preemptive strategies, and close follow-up are essential in the management of CMV in kidney allograft recipients (75).

Overall, CMV infection is a significant healthcare concern, particularly for individuals with weakened immune systems. Early detection, proper management, and preventive measures play a crucial role in reducing the impact of CMV infection on vulnerable populations. In transplant recipients, CMV infection is a significant concern as it can directly impact the success of the transplant. Prophylactic antiviral medications may be prescribed to prevent CMV reactivation or infection in these individuals (75). There is existing evidence from multiple clinical studies indicating a possible association between CMV disease and an increased risk of kidney graft loss (76–78). However, it is important to note that there are also conflicting findings from other studies that have failed to establish a significant relationship (79,80).

2.7.5 Cold ischemia time

Cold ischemia, on the other hand, refers to the period of time when the kidney is preserved and transported in a cold solution before transplantation. The purpose of cold ischemia is to slow down the metabolic activity of the kidney and minimize tissue damage during the transplantation process. CIT starts from the moment the kidney is cooled down until it is transplanted into the recipient's body (81).

Delayed graft function (DGF) denotes the incapacity of a transplanted kidney to promptly function following surgery, necessitating renal replacement therapy within the initial week. Prolonged Cold Ischemia Time (CIT), denoting the duration the kidney is preserved at a cold temperature before transplantation, has been recognized as an autonomous risk factor contributing to the onset of DGF (82). Research exploring the influence of cold ischemia on Delayed Graft Function (DGF) has been conducted in studies involving deceased, living, and donation after cardiac death (DCD) donors. However, the findings have been inconclusive. Certain studies indicate that each hour of cold ischemia heightens the risk of DGF in deceased donor grafts by 10%, particularly exceeding 18 hours of cold ischemia. Conversely, they also observed that

cold ischemia lasting under 18 hours had a minimal negative impact on DGF and overall graft survival. For instance, the graft survival rates at 5 years were 91% for cold ischemia lasting less than 18 hours and 84% for cold ischemia exceeding 18 hours (83). Additional research has corroborated these results, suggesting a correlation between an extended Cold Ischemia Time (CIT) and an elevated occurrence of Delayed Graft Function (DGF) in deceased donor transplantations. The adverse consequences of prolonged CIT were notably pronounced when the duration surpassed 20 to 30 hours. These studies propose the existence of a specific threshold for cold ischemia, beyond which the risk of DGF escalates with each successive hour (84). Remarkably, when assessing the impact of Delayed Graft Function (DGF) on graft survival, certain indications propose that DGF alone does not exert a substantial influence on patient or graft survival stemming from deceased donors. However, graft survival experiences a notable decrease when acute rejection coincides with DGF. Furthermore, studies have revealed that perioperative saline loading can effectively alleviate the adverse effects of cold ischemia and diminish the occurrence of DGF (85). In general, the duration of cold ischemia contributes to the emergence of Delayed Graft Function (DGF), and extended ischemia times are linked to heightened risk. Nevertheless, the influence on graft survival might hinge on additional factors, including the occurrence of acute rejection.

The correlation among ischemia-reperfusion injury, Cold Ischemia Time (CIT), acute rejection, and the enduring survival of grafts in kidney transplantation is an area of continuous investigation, yielding inconsistent findings. Certain studies have indicated a positive connection between the intensity of ischemia-reperfusion injury and the occurrence of acute rejection episodes. For instance, in the study by Barba et al. (2011), it was observed that cold ischemia surpassing 18 hours heightened the frequency of acute rejection episodes and prompted an earlier onset of the initial rejection episode (86). Nevertheless, the connection between Delayed Graft Function (DGF) and acute rejection remains a subject of contention. In the study by Sert et al. (2014), no substantial association was reported between Cold Ischemia Time (CIT) and the occurrence of acute rejection (84). Conversely, a study conducted by Perez Valdivia et al. (2011) observed that extended CIT correlated with inferior early graft

function, irrespective of donor and recipient age. Prolonged CIT has also been associated with diminished patient and graft survival rates (87). The impact of acute rejection and suboptimal early graft function on the enduring survival of grafts is yet to be definitively established. While certain studies indicate that acute rejection alone may not significantly impede overall graft survival, recent insights propose that the combination of acute rejection with DGF might exert a more adverse influence on graft survival (85). The relationship between suboptimal early graft function and long-term graft survival exhibits inconsistency in the literature, with some studies suggesting a correlation and others finding no significant association (85,88–90).

Given this evidence, there is a reasonable hypothesis that minimizing ischemia-reperfusion injury might potentially lower the frequency of acute rejection episodes. Preventing acute rejection within the initial year could potentially postpone allograft failure. However, owing to the inconsistent findings in existing literature, additional research is imperative to gain a more comprehensive understanding of the interplay between Cold Ischemia Time (CIT), acute rejection, and the enduring survival of grafts in kidney transplantation.

2.7.6 Warm ischemia time

Warm ischemia and cold ischemia are two important concepts in kidney transplantation. Warm ischemia refers to the period of time when the blood supply to the kidney is interrupted or reduced, leading to a lack of oxygen and nutrients. This typically occurs during the process of retrieving the kidney from the donor's body. WIT starts from the moment the blood flow to the kidney is interrupted until the kidney is cooled down for preservation. Prolonged WIT can have a detrimental effect on the kidney's viability and function after transplantation (81).

Research in kidney transplantation has indeed placed a significant emphasis on enhancing preservation techniques to mitigate the adverse effects of cold ischemia, which refers to the extended period of cold storage time. Various strategies have been developed to minimize the damage caused by prolonged cold ischemia and reduce the

occurrence of delayed graft function. However, the impact of prolonged WIT, which encompass the period from removing the organ from cold storage to reperfusion with warm blood during surgery, including the surgical anastomotic time, has received comparatively less attention in research (91). Nonetheless, studies that have investigated this aspect indicate that longer WIT are associated with higher rates of delayed graft function, prolonged hospital stays, and poorer outcomes in terms of graft and patient survival (92–95). These findings highlight the importance of optimizing WIT during kidney transplantation. While efforts have been focused on minimizing cold ischemia, it is crucial to also consider and manage warm ischemia to improve transplant outcomes.

2.7.7 Donor factors

Among the numerous factors that influence the long-term outcome of kidney transplantation, donor-related factors are considered to be of utmost importance. The quality of the kidney, which plays a crucial role in transplantation success, can be assessed using various criteria (96). One significant categorization is based on whether the kidney is obtained from a living or deceased donor. Living donor kidneys generally have better outcomes compared to deceased donor kidneys due to shorter ischemia times and better organ quality (97).

For deceased donors, further stratification can be done using simple or advanced scoring systems. These scoring systems take into account various donor characteristics such as age, cause of death, medical history, and organ function. These scoring systems aim to predict the suitability and potential success of the transplanted kidney. Additionally, clinico-histopathological scores based on preimplantation biopsy data are used to assess the quality of deceased donor kidneys. These scores provide information about the histological characteristics of the kidney, including the presence of any abnormalities or damage. This information helps determine the suitability of the kidney for transplantation and predicts the risk of complications or rejection (98). By considering these donor-related factors, clinicians can make informed decisions regarding organ selection and offer the most suitable kidneys to potential recipients.

Optimizing the quality of the donor kidney is crucial for improving the long-term outcomes of kidney transplantation. The existing literature provides various insights into the determinants affecting the prolonged results of kidney transplantation:

Living donor kidneys typically exhibit superior outcomes in comparison to deceased donor kidneys, with certain exceptions like young recipients receiving a kidney from a young standard criteria donor (SCD). The enhanced outcomes of living donor kidneys can be ascribed to factors such as thorough pre-transplant assessment of donor kidney function, the absence of detrimental pre-agonal and agonal phases, shorter Cold Ischemia Time (CIT), and the proficiency of seasoned surgeons (99).

Kidneys sourced from expanded criteria donors (ECDs) commonly result in less favorable outcomes when contrasted with Standard Criteria Donor (SCD) kidneys (100,101). ECDs are typically categorized as donors aged ≥60 years or aged 50-59 years with specific risk factors like cerebrovascular death, a history of hypertension, or elevated terminal serum creatinine levels (102). Nevertheless, the definition of ECDs can be more intricate, and advanced scoring systems incorporating additional variables have been suggested to enhance predictive accuracy and identify subgroups of ECDs linked to diverse long-term outcomes (98).

Kidneys acquired from donors after cardiac death (DCD) draw attention due to short-term data suggesting an increased incidence of primary nonfunction and delayed graft function due to severe ischemia-reperfusion injury. However, long-term data from various countries reveal similar outcomes for DCD kidneys when compared to kidneys from standard criteria donors (SCD) (98).

Integrated scoring approaches that encompass demographic information, serum creatinine levels, and histological data from preimplantation biopsies can enhance the accuracy of long-term prognosis predictions. For instance, certain histological scoring systems have demonstrated enhanced long-term outcomes for kidneys from donors aged over 60 when evaluated histologically before implantation. Composite scores derived from donor serum creatinine levels, donor hypertension, and the percentage of

sclerotic glomeruli have also been employed to anticipate unfavorable short-term and long-term outcomes (103,104).

In conclusion, precisely categorizing the donor and evaluating the "quality" of the transplanted kidney through suitable scoring methods are crucial for establishing trustworthy long-term predictions in kidney transplantation.

2.7.8 Diabetes mellitus

Diabetes mellitus (DM) is a prevalent cause of kidney damage globally, affecting a significant portion of the population. It is estimated that DM accounts for approximately 30% to 40% of all cases of kidney damage worldwide. This indicates the substantial burden of DM on kidney health and the importance of addressing its impact on kidney disease management and treatment (75). DM is a persistent metabolic disorder identified by elevated blood sugar levels, potentially resulting in complications that impact various organs, including the kidneys. The global prevalence of DM has been steadily increasing, driven by factors such as sedentary lifestyles, unhealthy diets, and rising obesity rates. As a consequence, the incidence of DM-related kidney damage is also on the rise, posing significant challenges for healthcare systems worldwide (105).

The impact of DM on kidney transplantation is significant. Studies have shown that kidney transplant recipients with pre-existing diabetes or who develop diabetes after transplantation have poorer outcomes compared to non-diabetic recipients. Mortality rates tend to be higher in diabetic recipients, particularly among those under 40 years old (106). This may be attributed to the association of DM with other comorbidities, such as hypertension and obesity, which further contribute to the overall risk profile.

Efforts to address DM-related kidney damage involve comprehensive management strategies. These may include early detection and diagnosis of DM, optimal glycemic control through lifestyle modifications and medication, blood

pressure management, and regular monitoring of kidney function. Additionally, healthcare providers emphasize the importance of preventive measures, such as regular screenings and education on healthy lifestyle choices, to reduce the risk of DM-related complications, including kidney damage (107–109).

2.7.9 Gender

Emerging evidence suggests that gender disparities exist in various aspects of kidney disease, including the burden of the disease, access to renal replacement therapies (RRT), and subsequent health outcomes. These disparities highlight the importance of considering gender as a factor in understanding and addressing kidney disease (110).

One aspect of gender inequality in kidney disease is the burden of the disease itself. Studies have shown that certain kidney conditions, such as CKD, ESRD, and certain types of glomerulonephritis, may be more prevalent in either males or females. For example, some studies suggest that males have a higher incidence of CKD and ESRD, while certain glomerular diseases, like lupus nephritis, are more common in females. These variations in disease prevalence contribute to disparities in the overall burden of kidney disease (111,112).

Access to renal replacement therapies, such as dialysis or kidney transplantation, is another area where gender disparities can be observed. Studies have shown that there may be differences in access to and utilization of RRT based on gender. For example, some research indicates that males may have higher rates of transplantation compared to females, possibly due to factors such as biological suitability, referral patterns, or differences in healthcare-seeking behavior. These disparities in access to transplantation can impact treatment outcomes and long-term survival rates (110). Furthermore, the development and progression of kidney disease may differ between genders. Some studies suggest that males may experience a more rapid decline in kidney function, leading to earlier initiation of RRT (111). On the other hand, females, particularly during reproductive years, may face unique challenges related to kidney

diseases, such as pregnancy-associated kidney complications or hormonal influences on disease progression (113). Understanding these gender-specific factors can help tailor management strategies and improve outcomes.

Numerous studies, encompassing analyses from extensive registries, have presented contradictory results concerning the influence of recipient gender on survival following kidney transplantation. Despite some authors finding no notable disparity in survival rates between men and women, divergent outcomes from other studies have fueled a debate regarding the effect of recipient gender on graft survival (111,114,115).

2.7.10 Acute rejection episodes

Episodes of acute rejection can exert an influence on the long-term survival outcomes in kidney transplantation. Acute rejection denotes the immune system's reaction to the transplanted kidney, resulting in inflammation and potential harm to the organ. It is identified as a substantial risk factor for graft failure and a decrease in long-term survival. Various studies have demonstrated that the presence of acute rejection episodes correlates with a heightened risk of graft failure and a reduction in overall survival rates. The severity and frequency of these episodes can vary among individuals, underscoring the importance of prompt diagnosis and appropriate treatment to minimize damage to the transplanted kidney (116,117).

The primary goal of kidney transplantation is to secure prolonged graft survival and enhance patient outcomes. Achieving this objective involves continuous endeavors to refine immunosuppressive protocols, improve donor-recipient matching, and elevate post-transplant care. These initiatives are geared towards minimizing the occurrence of acute rejection episodes and optimizing the enduring survival of the transplanted kidney. Immunosuppressive medications are commonly administered to prevent and manage acute rejection episodes by suppressing the immune response and mitigating the risk of rejection. Rigorous monitoring of transplant recipients and

consistent follow-up visits play a pivotal role in promptly detecting any signs of rejection and intervening as necessary (118).

2.4 Introduction of Metabolomics

Enzymes in the digestive system play a crucial role in extracting the energy and essential components required to create and sustain the biological organization necessary for cell survival. This energy and these building blocks are obtained from the chemical bonds present in proteins, lipids, and polysaccharides, which constitute a major portion of the food we consume. Before our cells can utilize these molecules, they undergo digestion facilitated by enzymes, enabling the release and utilization of their chemical bond energy and the acquisition of necessary nutrients (119). In the intestines, carbohydrates, proteins, and fats, which are the primary macronutrients found in food, undergo digestion and are transformed into their fundamental components. Carbohydrates undergo breakdown into sugars, proteins are metabolized into amino acids, and fats undergo breakdown into fatty acids and glycerol (119,120). The body absorbs these compounds, which serve as energy sources, into the bloodstream. The bloodstream transports them to the cells. Metabolism encompasses a vast array of chemical processes occurring simultaneously in all living cells of the body. These metabolic reactions are regulated by the body and contribute to the overall functioning and vitality of the organism (121).

Metabolites, which are small organic compounds, play a crucial role in driving fundamental cellular functions. They are involved in processes such as storage, energy production, signal transduction, and apoptosis. Metabolites can act as both products and substrates in various biological pathways. Typically, they have a mass range of 50 - 1500 daltons (Da) (122,123). The collection of small molecules and their interactions within a biological system is referred to as the metabolome. These diminutive molecules act as the final outcomes of cellular regulatory processes, and their concentrations can be seen as the ultimate reaction of biological systems to genetic variations and environmental influences. In comparison to other omics technologies like proteomics, genomics, and transcriptomics, the abundance of small molecules

within cells, biofluids, tissues, or organisms provides the most comprehensive representation of the molecular phenotype (122,124–127).

The term "omics" is used to describe a field of study within the biological sciences that focuses on specific areas ending with "-omics." Examples of these disciplines include genomics, transcriptomics, proteomics, and metabolomics (128). Biological processes are intricate outcomes resulting from the intricate interplay between genes, RNA, proteins, and metabolites. This intricate network of interactions is referred to as the interactome. Environmental factors exert a substantial influence on the interactome, thereby impacting disease processes. To effectively develop novel treatments and monitor diseases, it is crucial to comprehend the role of the interactome in the pathogenesis of various disorders (123). As depicted in Figure 1, transcriptomics, proteomics, and genomics offer insights into the genotype, providing valuable information. However, these approaches have limitations in capturing the entirety of cellular events beyond providing partial information about the phenotype (123,124). Genomics is dedicated to investigating the complete collection of genes within an individual or another organism. In contrast, transcriptomics explores variations in RNA and mRNA, while proteomics delves into the comprehensive analysis of large-scale proteome expressions (129,130).

Metabolomics, the most recent addition to the omics sciences, focuses on the quantitative and qualitative assessment of metabolites. Metabolites are significant intermediates and end products of metabolism. Metabolomics combines high-throughput analytical techniques with bioinformatics to enable comprehensive analysis of metabolites (131–133). Metabolomics possesses a significant advantage in that it not only provides comprehensive data on the exact cellular processes but also captures the impact of environmental factors. Unlike the genome, the metabolome offers a snapshot of an individual's pathophysiological state at a specific moment, thereby reflecting the dynamic interplay between genetic factors and the environment (134–136). Additionally, metabolomics offers valuable insights that aid in advancing our understanding of metabolic mechanisms, metabolic behaviors, and the identification of metabolic biomarkers within a specific biological condition. This

information serves to accelerate research and enhance our knowledge of metabolic processes.

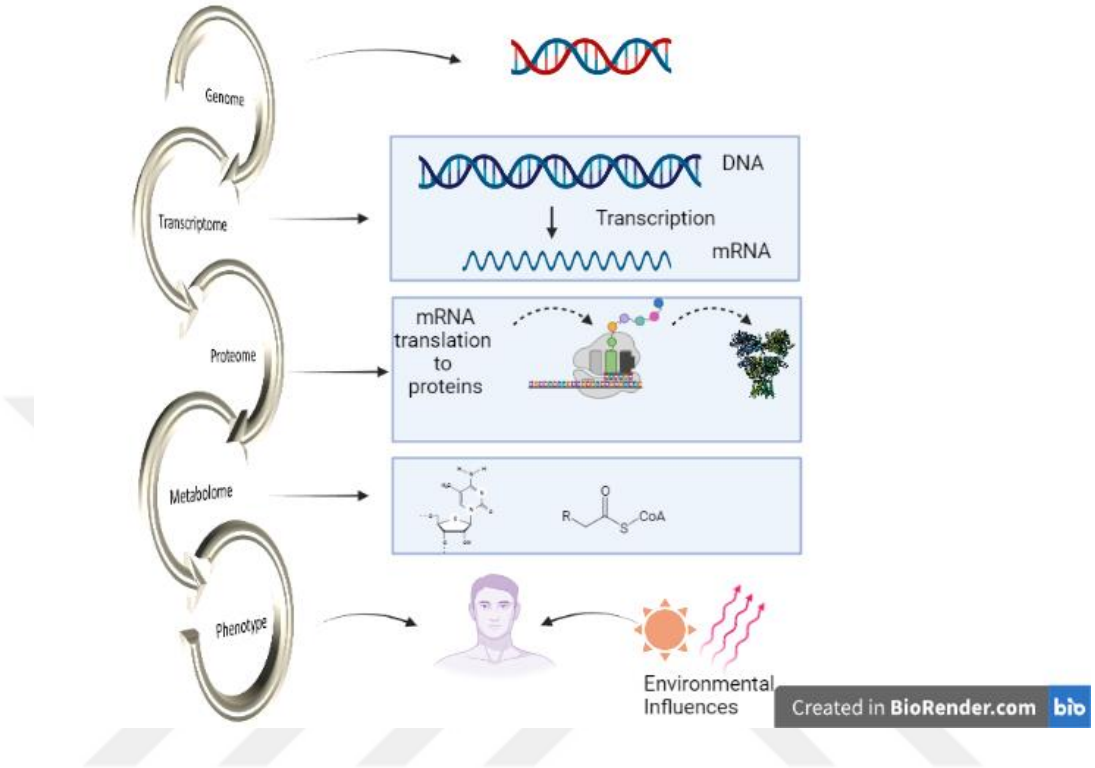

Figure1. Overview of different omics-sciences such as genomics, transcriptomics, and proteomics (129).

2.5 Application of Metabolomics

As shown in Figure 2, similar to other omics technologies such as genomics, transcriptomics, and proteomics, metabolomics finds wide application in a variety of fields. These include kidney and urology research (137–143), cancer studies, (144), neuroscience research (144,145), immunology research (146), agricultural practices (147), nutrition & cosmetics (148,149), etc.

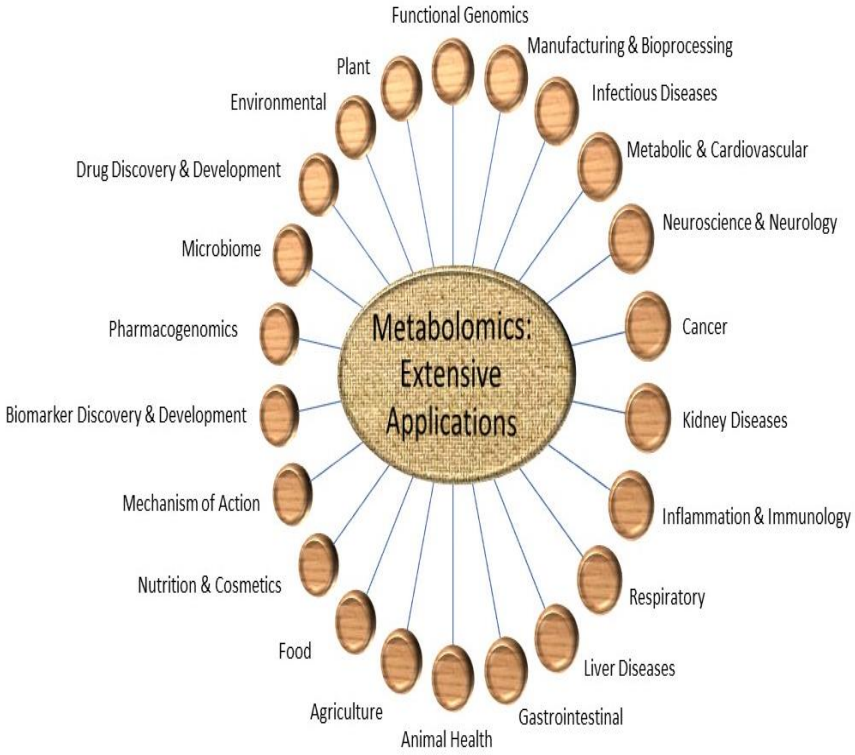

Figure 2. Extensive applications for metabolomics

2.6 Metabolomics Approach

There are different approaches available for analyzing and quantifying metabolites from various samples, depending on the purpose of the study. Generally, these approaches can be categorized into three main groups: untargeted and targeted methods. Below, we provide a brief description of these approaches.

2.6.1 Untargeted analysis

The non-targeted metabolomics approach plays a crucial role in monitoring biological variations by uncovering unknown compounds present in biological samples like urine, serum, tissue, saliva, and more. It serves as a discovery process and is widely utilized to investigate and identify changes in these samples (150,151). Ideally, having a singular analytical technique that can concurrently measure hundreds to thousands of metabolites is ideal. However, no single method can identify all metabolites in a biological system. To enhance the detection of metabolites,

metabolomics experiments commonly utilize a combination of analytical platforms such as Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS), and Nuclear Magnetic Resonance (NMR) methods. Advanced chemometric methods like multivariate analysis are essential for comprehending the extensive data generated. These techniques can effectively reduce extensive datasets into more manageable subsets (152). Despite the recent advancements in technology, untargeted metabolomics approaches have certain drawbacks. These include the time-consuming processing of large volumes of raw data, challenges in identification, classification, and limited coverage by analytical platforms. Consequently, the untargeted approach poses significant difficulties in the detection and identification of metabolites. As depicted in Figure 3, it offers advantages such as being comprehensive and unbiased, yielding a high amount of information and enabling the discovery of unexpected compounds in samples. However, it also has its disadvantages, including semi-quantitative results, a potential for a high number of false positives and false negatives, with many metabolites remaining undetected, and difficulties in interpreting the data (153).

2.6.2 Targeted metabolomics

Targeted approaches, unlike untargeted approaches, are designed experiments that focus on identifying and quantifying a specific set of known metabolites. These methods aim to measure the targeted metabolites with high precision and accuracy, allowing for precise identification and quantification of the desired compounds (154,155).

The quantification of metabolites in targeted approaches can be achieved through the use of internal or external standards. This practice is instrumental in the development of effective methods, as it enhances the sensitivity and selectivity of the targeted approach compared to non-targeted methods. By incorporating standards, the accuracy and reliability of metabolite quantification are improved, leading to more robust and precise results (156). Furthermore, in the targeted approach, sample preparation techniques can be optimized based on the specific metabolites of interest

and downstream analysis requirements. This optimization simplifies the data analysis process and aids in the interpretation of the biological significance of the results. By tailoring the sample preparation protocols, researchers can enhance the efficiency and accuracy of the targeted analysis, leading to more meaningful and reliable conclusions (157).

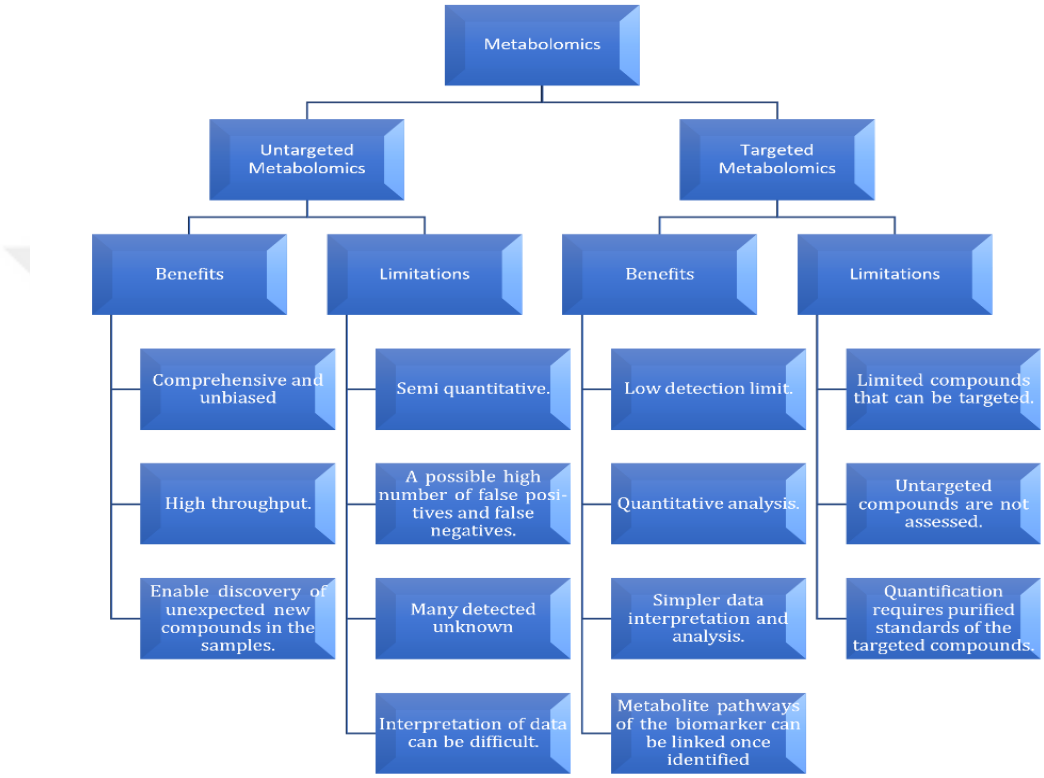

Figure 3. Comparison of benefits and limitations of untargeted and targeted metabolomics, modified from Selamat et at (153).

2.7 Workflow of Mass Spectrometry-Based Untargeted Metabolomics

The untargeted metabolomics approach encompasses several steps that necessitate meticulous planning and design. While there are publicly available protocols that focus on specific classes of metabolites, no single protocol can be universally applied to all applications. To ensure efficiency and reliability, the experimental protocols for each study are thoughtfully designed and optimized based on the hypothesis, sample type, metabolites of interest, and the analytical platform being used. This tailored approach accounts for the specific requirements of the study and maximizes the accuracy and effectiveness of the results. The workflow of metabolomics can significantly differ

depending on the specific question being addressed. Figure 4 provides a summary of the common procedures employed in a metabolomics study. In the case of non-targeted metabolomics, the workflow typically begins with sample preparation, involving the extraction of metabolites from the biological sample using an appropriate solvent for subsequent analytical analysis. This is followed by sample collection, data acquisition, data processing, statistical analysis, metabolite identification, interpretation of metabolic pathways, and finally, biological validation. Each step in this workflow contributes to the comprehensive understanding of the metabolome and its relevance to the biological question at hand (158). Every step of this workflow plays a critical role in the identification of reliable biomarkers in metabolomics studies (159).

2.7.1 Sample collection

Sample preparation holds immense significance in metabolomics studies, as it has a direct impact on the composition of the analyzed extract. This, in turn, can influence the resulting metabolome. Thus, sample preparation stands out as one of the most crucial steps in ensuring accurate and representative analysis in metabolomics studies (160–162). The sample preparation step is often regarded as a critical bottleneck in metabolomic analysis. This is because errors or inaccuracies during sample preparation can have a detrimental impact on the overall quality and reliability of the study. Therefore, special attention and care must be given to this step to minimize potential errors and ensure the integrity of the results (162). The search for biomarkers in biological samples encompasses various steps, which may vary depending on the sample type and the chosen metabolomics approach, whether it is targeted or untargeted (163). Extracting metabolites from blood, urine, or tissue for a comprehensive study is a challenging endeavor. Due to the diverse chemical and physical properties of metabolites, as well as their wide dynamic range of concentrations, it is not always feasible to extract all metabolites from a sample using a single solvent. Therefore, multiple extraction procedures employing different solvent systems may be necessary to ensure comprehensive metabolite extraction. This approach accounts for the varied properties and concentrations of metabolites, allowing for a more thorough analysis (162).

The selection of an appropriate sample preparation strategy significantly impacts the success of an experiment by influencing both the observed metabolite profile and the quality of the data. Sample preparation techniques in metabolomics studies can vary depending on the organism or cell structure being studied. There is no universally applicable sample preparation technique that suits all biological samples in metabolomics. Several factors should be considered when choosing sample preparation techniques for metabolomic studies. These factors include ensuring non-selectivity, simplicity, and rapidity of the technique, high reproducibility, effective metabolism quenching, and metabolite extraction methods that involve a minimal number of steps. By considering these factors, researchers can optimize the sample preparation process and improve the accuracy and reliability of their metabolomic analyses (164,165).

Sample selection plays a crucial role in metabolomics, as the suitability of the chosen samples greatly influences the resulting outcomes. Traditionally, plasma, serum, and urine have been extensively utilized for prognostic or diagnostic purposes in various diseases. Among these, urine stands out as a highly advantageous sample choice. It offers several benefits, including ease of collection, simple storage requirements, abundance, and non-invasiveness. Moreover, urine provides a direct reflection of the individual's overall condition, enabling the monitoring of biological responses to therapy and facilitating a more comprehensive evaluation of health risks (165).

To facilitate an effective metabolomic analysis, it is essential to prioritize rapid sampling due to the rapid turnover of metabolites in living organisms. However, it is important to recognize that after sampling, metabolites can undergo changes. Therefore, it is critical to swiftly collect samples and take immediate measures to halt any ongoing biochemical activity. Prompt sample collection helps in preserving the metabolite composition and prevents further alterations. This can be achieved by techniques such as rapid freezing or the addition of specific chemical inhibitors. By ensuring rapid sampling and promptly implementing measures to cease biochemical activity, the integrity of the metabolomic analysis can be upheld, enabling accurate

characterization of the metabolome of interest (166,167). Ensuring experimental reproducibility necessitates the prompt cessation of any chemical or enzymatic reactions that might occur immediately after sampling, as they have the potential to alter the organism's original metabolite profile. Quenching is a well-established method for swiftly deactivating all biochemical and enzymatic activities within organisms. Given that cellular metabolism is a dynamic process, alterations can impact metabolic turnover. Hence, employing proper quenching techniques is crucial to mitigate this concern. Various quenching methods are available, including pH changes using perchloric acid or rapid temperature reduction achieved by immersing the sample in liquid nitrogen. Additionally, the utilization of methanol, either at a high temperature (>80°C) or low temperature (<40°C), induces a sudden temperature shock, effectively interrupting biochemical reactions. Utilizing suitable quenching methods guarantees the preservation of the metabolite profile, facilitating precise metabolomic analyses (167–170).

2.7.2 Data acquisition

In this section, analytical platforms used for the extraction and analysis of metabolites are examined in detail. Due to the extensive diversity within the metabolome, there is no single protocol for metabolite extraction. However, combining a series of extraction protocols is important to increase metabolome coverage. Furthermore, analyzing all metabolites with a single analytical method is currently not feasible. To expand the scope of the metabolome, it is necessary to utilize multi-platform approaches. Among these platforms, multi-platform approaches incorporating techniques such as NMR and mass spectrometry (UPLC-QTOF-MS) stand out. These platforms enable comprehensive analysis of the metabolome and broaden the scope of metabolite identification.

2.7.2.1 Analytical platforms

Given the extensive diversity of molecules within the metabolome, there is no singular protocol for metabolite extraction. However, a combination of extraction

protocols can enhance metabolome coverage. Moreover, analyzing the entire range of metabolites with a single analytical method is currently not feasible. To increase metabolome coverage, it is necessary to employ multi-platform approaches utilizing techniques such as NMR and mass spectrometry (UPLC-QTOF-MS). These platforms are highly suitable and widely utilized in metabolomics research, enabling comprehensive analysis of the metabolome and expanding the scope of metabolite identification.

2.7.2.1.1 UPLC-QTOF-MS

Compared to techniques like gas chromatography (GC) or NMR (particularly 1H NMR), the widespread utilization of liquid chromatography (LC) for non-targeted metabolic profiling in metabolomic applications is relatively new (171). Published applications utilizing LC-MS in metabolomics likely began to emerge in the early twenty-first century (172,173). LC-MS-based analysis of biological samples has gained popularity due to several advantages it offers. Firstly, LC-MS allows for analysis without the need for derivatization, which simplifies the workflow and saves time. Secondly, it provides high sensitivity, enabling the detection of low-abundance metabolites in complex biological matrices. Additionally, LC-MS offers good spectral information content, allowing for the characterization of analytes based on their mass and fragmentation patterns. Another advantage of LC-MS is its ease of use, making it accessible to researchers in various laboratories. In comparison, techniques like high-field NMR spectroscopy may require specialized expertise and instrumentation, making them less readily available. Furthermore, the cost of LC-MS instrumentation has become more affordable over time, making it a cost-effective choice for many research laboratories. Considering these advantages, LC-MS has become a popular and widely adopted technique for metabolomic analysis in biological samples. Its versatility, sensitivity, spectral information content, accessibility, and cost-effectiveness make it an attractive option for researchers exploring complex metabolite profiles in various biological systems (174–176).

In the early stages, LC systems utilized conventional high-performance liquid chromatography (HPLC) with separations carried out using 3 to 5-mm particles. However, there has been a significant shift towards the use of smaller particles, typically below 2 mm, and the adoption of higher pressures in ultra-high-pressure liquid chromatography (UHPLC) techniques compared to conventional HPLC. The move towards smaller particle sizes allows for improved separation efficiency and faster analysis times. Smaller particles provide a larger surface area for interaction with analytes, resulting in enhanced resolution and peak capacity. This advancement in particle technology has enabled researchers to achieve higher chromatographic performance and better separation of complex mixtures. Additionally, UHPLC employs higher pressures to drive the mobile phase through the column at faster rates. The increased pressure results in reduced analysis times while maintaining or even enhancing chromatographic performance. UHPLC systems often utilize specialized columns, instruments, and detectors capable of withstanding the higher pressures generated during the analysis. The transition from conventional HPLC to UHPLC has revolutionized LC-based metabolomics analysis by offering improved separation efficiency, faster analysis times, and enhanced sensitivity. These advancements have significantly contributed to the evolution of LC-MS-based metabolomics research, enabling more comprehensive and efficient profiling of metabolites in biological samples (177–179).

In metabolomics, both normal-phase and reversed-phase (RP) columns find applications, but RP columns such as C18 and C8 are predominantly used. These RP columns have become the go-to choice for metabolomic separations due to their versatility and compatibility with a wide range of metabolites. RP separations are particularly effective for hydrophobic or nonpolar metabolites. However, it is important to note that NP separations can provide complementary information and insights into the metabolome. This has been demonstrated in various studies, including metabolic profiling of urine. For instance, the use of hydrophilic interaction liquid chromatography (HILIC) coupled with MS and reverse-phase high-performance liquid chromatography-mass spectrometry (RP-HPLC-MS) has revealed distinct and complementary views of the metabolome. HILIC separates polar and hydrophilic

metabolites based on their interaction with a hydrophilic stationary phase. This technique allows for the analysis of hydrophilic and polar metabolites that may not be effectively retained or resolved on RP columns. By combining the information obtained from both HILIC-MS and RP-HPLC-MS, researchers can gain a more comprehensive understanding of the metabolome. Therefore, while RP columns like C18 and C8 remain the most commonly used in metabolomics, NP separations, such as HILIC, offer valuable complementary information and can be employed to gain a more comprehensive view of the metabolome in conjunction with RP separations. The choice of the column type depends on the specific research objectives, the nature of the metabolites of interest, and the desired analytical insights (167,173,180,181).

In RP-based separations, commonly used organic solvents include water, acetonitrile, methanol (182–185), tetrahydrofuran (THF) (186–191), and 2-propanol (187,192). These solvents are used to create the mobile phase, which elutes the analytes from the RP column (193). The mobile phase composition is fine-tuned to attain the desired separation of metabolites according to their hydrophobicity. Water serves as the polar component, while organic solvents like acetonitrile, methanol, THF, and 2-propanol provide the necessary organic phase for effective separation. On the other hand, HILIC separations utilize a similar mobile phase composition to RP-LC. The mobile phase in HILIC typically consists of an aqueous component (e.g., water) and an organic solvent (e.g., acetonitrile, methanol). This composition allows for the retention and separation of polar and hydrophilic metabolites in the hydrophilic stationary phase (194).

They are proficient in dissolving a wide array of metabolites, encompassing both polar and non-polar compounds. Acetonitrile and methanol are often used in combination with water as mobile phase solvents in LC-MS separations. Their miscibility with water allows for the creation of gradient elution systems, enabling efficient chromatographic separation of metabolites with varying polarities (175,195).

These organic solvents offer several advantages in metabolomics. They possess desirable physicochemical properties, including low volatility, stability, and

compatibility with mass spectrometry. Moreover, they can effectively extract metabolites from biological samples, ensuring high recovery and reproducibility (196,197)

It is important to note that the choice and composition of organic solvents depend on the specific analytical requirements, the nature of the metabolites being analyzed, and the chromatographic system employed. Different solvent systems can be optimized for different metabolite classes, allowing for comprehensive metabolomic coverage and efficient separation in LC-MS analyses (198).

2.7.2.1.2 Nuclear Magnetic Resonance

Nuclear magnetic resonance spectroscopy is extensively utilized in metabolomics investigations as a highly efficient analytical tool. It has the unique ability to identify and quantify various types of metabolites with equal efficiency. In this regard, NMR is considered a universal detector, unlike other detection techniques that rely on analyte separation (199). NMR spectroscopy utilizes the inherent magnetic properties of atomic nuclei within metabolites. By subjecting a sample to a strong magnetic field and applying radiofrequency pulses, NMR can generate valuable information about the chemical composition and concentration of metabolites (200).

One of the significant advantages of NMR is that it does not require analyte separation prior to detection. This feature sets it apart from other techniques such as chromatography. NMR directly analyzes the complex mixture of metabolites present in a sample, providing comprehensive insights into the entire metabolome. NMR can effectively identify and quantify a wide range of metabolites, including small molecules, organic acids, amino acids, sugars, lipids, and more. It offers excellent reproducibility and robustness, ensuring reliable quantification of metabolites across different samples. Furthermore, NMR spectroscopy is non-destructive, allowing for the preservation of valuable samples for additional analyses. It is a versatile tool that provides rich spectral information, enabling the characterization of metabolites based on their unique chemical shifts, coupling patterns, and other spectral features. While

NMR may have certain limitations in terms of sensitivity compared to mass spectrometry (MS)-based techniques, it remains an essential and widely applied tool in metabolomics research. Its ability to analyze and quantify metabolites without the need for separation makes it a valuable asset for understanding the complex metabolic profiles of biological samples (201–203).

NMR spectroscopy provides valuable information about metabolites in complex mixtures. Resonance peaks in the NMR spectrum are associated with spectral intensities and frequencies, which reflect chemical changes. This enables the determination and evaluation of compound concentrations without the need for extensive sample preparation. One of the key advantages of NMR is its ability to detect and differentiate various chemical species in a single experiment. This capability allows for the identification of different metabolites present in a mixture, even when they have diverse chemical properties. As a result, NMR offers a comprehensive analysis of complex samples in metabolomics studies. Another benefit of NMR spectroscopy is its relative ease of use. It is a versatile technique that can resolve almost any combination of compounds. By optimizing parameters and experimental conditions, NMR can effectively distinguish and characterize different metabolites, making it a powerful tool in metabolomics research. Overall, NMR spectroscopy provides insights into metabolite concentrations and facilitates the analysis of complex mixtures without extensive sample preparation. Its ability to detect diverse chemical species in a single experiment, along with its flexibility and ease of use, make it a valuable and widely employed technique in metabolomics studies (153).

2.7.3 Data analysis

Metabolomic datasets commonly encompass hundreds to thousands of variables (204–208). This abundance of variables introduces statistical challenges and can impact data quality due to several factors, including variations in instrumental conditions, sample heterogeneity, and reduced signal detection (instrumental errors). These factors can lead to issues such as misidentification of metabolites and poor quantitation. To guarantee the dependability and precision of the data, it is crucial to

enforce rigorous quality control measures and carry out preprocessing steps before engaging in data analysis (209).

The specific approach to data preprocessing in metabolomics depends on the nature of the experiment and the data obtained. One of the primary challenges in data analysis is the high volume of data points generated in metabolomic experiments, which can pose difficulties for effective data modeling. Consequently, reliable preprocessing of this data is essential to ensure accurate and meaningful analysis. The cornerstone of successful data preprocessing lies in implementing appropriate quality control measures during the experimental design and execution. This includes incorporating rigorous protocols to minimize sources of variation, such as controlling instrumental parameters, optimizing sample handling procedures, and implementing replicate measurements. By ensuring these quality controls are in place, researchers can enhance the reliability and reproducibility of the data (209).

Various commercial software tools are available for the preprocessing of metabolomics data generated from mass spectrometry (MS) experiments. These tools offer a range of functionalities to perform essential data processing steps, ensuring the quality and reliability of the data. XCMS (210), MZmine (211), MS-Dial (212), MetAlign (213) and commercial software MarkerView (214), Compound Discoverer (215), MarkerLynx (216), and Progenesis QI (217), etc. Data preprocessing for MS data involves a series of steps to enhance the quality and extract meaningful information from the raw data. These steps typically include baseline correction, noise filtering, normalization, peak detection, peak alignment, and spectral deconvolution (218,219).

The first step in data preprocessing for mass spectrometry (MS) data is peak picking/detection and deconvolution. This crucial step aims to identify and assign features (mass-to-charge ratios/ retention time (m/z-RT pairs)) to each measured ion in a sample. The peak-picking algorithm operates on the extracted ion chromatograms, taking into consideration noise filtering and baseline correction to accurately capture and deconvolute peaks. Noise filtering is an essential component of peak picking, as it

helps to distinguish true signals from background noise. Various noise filters can be applied in the preprocessing of MS data, including the Savitzky-Golay filter, Gaussian filter, and wavelet-based filters. These filters are designed to reduce the impact of noise on peak detection, improving the sensitivity and specificity of the algorithm. Baseline correction is another important step in peak picking. It corrects any systematic variations or baseline shifts in the data that could interfere with peak detection. By adjusting the baseline, the algorithm can better identify and assign peaks, improving the accuracy of feature detection. The combination of noise filtering and baseline correction in peak-picking algorithms enhances the detection of true peaks in the MS data. It helps to minimize false positives and improve the overall quality of the detected features. This step sets the foundation for subsequent data analysis, such as peak alignment, quantification, and identification. Researchers can choose from different noise filtering techniques and baseline correction methods based on their specific needs and the characteristics of their data. The selection of appropriate filters and parameters is crucial to achieving optimal peak-picking results and improving the subsequent analysis and interpretation of the metabolomics MS data (219,220).

Correcting the baseline in data preprocessing helps eliminate the influence of background density variation on peak detection. Deconvolution, on the other hand, is a critical step that aims to separate overlapping peaks and improve peak quantification. By deconvolving overlapping peaks, it becomes easier to accurately measure and quantify individual components. One approach to deconvolution is the use of an isotope pattern to cluster isotopic peaks that correspond to the same compound. This clustering simplifies the resulting data matrix, making it easier to interpret and analyze. However, it's important to note that deconvolution introduces the potential for errors and additional variability in the process. The accuracy of the deconvolution algorithm and the quality of the data will greatly impact the reliability of the results. Deconvolution algorithms attempt to resolve the underlying signals from overlapping peaks by estimating their individual contributions. While these algorithms can be effective, they are not without limitations. Complex peak shapes, overlapping signals from closely eluting compounds, and variations in peak intensities can pose challenges and lead to errors in deconvolution. It is crucial to carefully validate and assess the

quality of the deconvolution results. This can be done through comparison with known standards, replicate analyses, and thorough evaluation of the data. Quality control measures should be implemented throughout the process to monitor and minimize potential errors and variability introduced by deconvolution. Overall, deconvolution is a powerful tool to improve peak quantification and resolve overlapping peaks in metabolomics data. However, its implementation requires careful consideration and validation to ensure accurate and reliable results (218,219).

During chromatographic separation, it is common for retention time (RT) shifts to occur due to various factors such as changes in the mobile phase, column aging, variations in temperature and pressure, and sample matrix effects. As a result, the elution of a metabolite may exhibit slight variations in RT across different samples. This poses a significant challenge, especially when analyzing a large number of samples in a long experimental run. To address this challenge, peak alignment algorithms are employed in the preprocessing stage of MS analysis. The primary objective of these algorithms is to align peaks detected in different data samples, allowing them to be classified based on common peaks and assigned to corresponding features in the data table (221).

Data normalization is a crucial step in metabolomics data analysis to mitigate systematic bias, technical variations, and discrepancies resulting from the input of large amounts of data. The objective of data normalization is to account for these variations and ensure accurate comparisons and reliable interpretation of metabolomic profiles. In the context of data normalization, each row of the data table, representing a specific metabolite or feature, is divided by a normalization factor. This factor is calculated to account for factors that can introduce variation, such as differences in sample concentration or other experimental variables. For instance, when analyzing metabolite concentrations in urine samples, the amount of water used as a solvent may vary between samples. As a result, the measured metabolite concentrations could primarily reflect dilution effects rather than actual changes in metabolic responses. To address this variation and obtain meaningful comparisons, a normalization factor is calculated for each row of the data table. The normalization factor is determined based

on suitable reference points or internal standards present in the data. These reference points could be stable endogenous compounds or exogenous standards added to the samples. By dividing each row of data by the normalization factor, the influence of factors such as dilution or varying experimental conditions is minimized, allowing for more accurate comparisons of metabolite levels across samples. Common methods for data normalization in metabolomics include total ion count normalization, median normalization, probabilistic quotient normalization, and normalization based on stable endogenous compounds. The choice of the normalization method depends on the specific characteristics of the dataset and the experimental design. Data normalization helps uncover true biological variations by reducing the impact of technical variations and systematic biases. It ensures that metabolite concentrations are represented relative to a common baseline, enabling valid comparisons and identification of meaningful patterns and differences in metabolic responses. In summary, data normalization in metabolomics is a vital step to correct for systematic biases and technical variations. By dividing each row of the data table by a normalization factor, it accounts for differences in sample concentration or other experimental factors, enabling accurate comparisons and reliable interpretation of metabolomic profiles (221).

To assign biological or scientific significance to the spectral peaks obtained through data preprocessing, compound identification is a crucial step in metabolomics analysis. Compound identification involves comparing the experimental spectra with reference spectra in established databases to determine the identity of the metabolites. Several reference databases are commonly used for compound identification in metabolomics studies. These databases contain information about known metabolites, including their spectral data, chemical properties, and associated biological pathways. Some popular databases used for compound identification in metabolomics include Human Metabolome Database (HMDB), MassBank, METLIN, LIPID MAPS Structure Database (LMSD), Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.7.3.1 Human metabolome database

HMDB is a widely utilized and openly available electronic repository designed specifically for clinical chemistry, metabolomics and biomarker discovery. Providing extensive and intricate details on small molecule metabolites in the human body, the database is structured to integrate or interlink three categories of data: molecular biology/biochemistry data, chemical data and clinical data. It serves as a valuable resource for researchers and clinicians interested in understanding the chemical composition, biological functions, and clinical relevance of metabolites. HMDB encompasses an extensive repository of 220,945 entries for metabolites, covering both lipid-soluble compounds and water-soluble. Each entry includes extensive information such as chemical structures, names, physicochemical properties, metabolic pathways, enzyme reactions, disease associations, and tissue/cellular locations. Researchers can leverage HMDB for various applications, including metabolite identification, pathway analysis, biomarker discovery, and the exploration of metabolic alterations in health and disease. The database provides a comprehensive platform for integrating and exploring metabolomics data, facilitating cross-referencing of metabolite information with clinical and biological contexts (222).

2.7.3.2 MassBank

MassBank is a public mass spectral database that provides information on the mass spectra of various chemical compounds. It serves as a valuable resource for the identification and characterization of metabolites, drugs, environmental pollutants, and other organic compounds. MassBank contains curated and annotated mass spectra obtained from different analytical techniques, such as mass spectrometry (MS) and tandem mass spectrometry (MS/MS). The database includes spectra for a wide range of compounds, including small molecules, natural products, drugs, and metabolites. Researchers can search MassBank using different criteria, such as compound names, molecular formulas, or mass spectral peaks. The database provides detailed information on the spectral peaks, including their m/z, intensity values, and fragmentation patterns. One of the key features of MassBank is its spectral comparison

functionality. Users can compare their own experimental mass spectra with those available in the database to aid in compound identification. This can be particularly useful in metabolomics studies, where researchers need to identify and annotate the detected metabolites. MassBank is continually updated with new entries and spectra, and it is freely accessible to the scientific community. The database serves as a valuable tool for researchers in fields such as metabolomics, toxicology, environmental science, and drug discovery, enabling them to identify and characterize compounds based on their mass spectral signatures (223).

2.7.3.3 METLIN

METLIN, available at http://metlin.scripps.edu, is a comprehensive and extensively annotated database of molecular standards. It serves as a valuable resource in the field of metabolomics, providing researchers with a diverse collection of metabolite information. The database contains more than 850,000 molecular standards, offering a wide range of chemical structures and compound classes. Each entry in METLIN is meticulously curated and includes detailed annotations, such as chemical formulas, systematic names, molecular weights, and structural information. This comprehensive annotation enables researchers to accurately identify and characterize metabolites of interest. METLIN offers a user-friendly interface that allows researchers to search for metabolites based on various criteria, including compound name, molecular formula, mass, and spectral features. The database also provides spectral data, such as mass spectra and tandem mass spectrometry (MS/MS) spectra, allowing users to compare experimental spectra with reference data for identification purposes. In addition to its extensive collection of metabolite standards, METLIN incorporates a variety of additional features to enhance metabolomics research. These include the ability to search for metabolites based on specific biological sources or pathways, as well as tools for metabolite structure prediction and fragmentation analysis. METLIN is regularly updated and maintained by a team of experts, ensuring the inclusion of new metabolites and the accuracy of the database. It serves as a valuable resource for metabolomics researchers, facilitating the identification and annotation of metabolites in complex biological samples, advancing

our understanding of metabolic pathways, and aiding in the discovery of potential biomarkers and therapeutic targets (224).

2.7.3.4 LIPID MAPS

The LIPID MAPS Structure Database (LMSD) is a comprehensive resource that focuses on biologically relevant lipids. It serves as a repository of lipid structures and associated annotations, facilitating research in lipidomics and lipid-related areas. The LMSD contains a vast collection of lipid structures, providing detailed information on their chemical compositions, classes, subtypes, and structural features. It encompasses a wide range of lipid categories, including sphingolipids, glycerolipids, glycerophospholipids, prenol lipids, sterol lipids, saccharolipids, and polyketides. As of August 2022, the LMSD encompasses more than 47,000 lipid entries. These entries include not only well-known lipids but also rare or specialized lipid species. The database is continuously updated and curated to ensure accurate and up-to-date information. Researchers and scientists in the fields of lipidomics, biochemistry, and cell biology can utilize the LMSD for lipid identification, characterization, and classification. The database provides an extensive collection of lipid structures along with associated metadata, including lipid names, systematic names, chemical formulas, mass spectra, and experimental data. The LIPID MAPS Structure Database serves as a valuable resource for studying the diverse roles and functions of lipids in biological systems. It enables researchers to explore lipid structures, investigate lipid-related pathways and networks, and further our understanding of lipid metabolism, signaling, and their implications in various biological processes and diseases (225).

2.7.3.5 Kyoto encyclopedia of genes and genomes

KEGG is a comprehensive and widely used database that integrates biological information related to genes, proteins, pathways, and diseases. It provides valuable resources for understanding the functional and molecular aspects of cellular systems. KEGG encompasses a diverse range of data, including genomic, chemical, and network information. It offers extensive coverage of various organisms, including

humans, animals, plants, and microorganisms. The database contains information on genes and proteins, their functions, metabolic pathways, signaling pathways, and disease-related pathways. One of the key features of KEGG is its pathway maps, which illustrate the complex interactions and relationships between genes, proteins, and small molecules in various biological pathways. These pathway maps help researchers visualize and interpret the functional implications of genomic and molecular data. In addition to pathway information, KEGG provides annotations for genes and proteins, including their sequences, functions, and associated diseases. It also offers resources for drug discovery and development, such as information on drug targets, drug metabolism, and drug-related pathways. KEGG is continually updated and expanded, incorporating new knowledge and discoveries in the field of genomics and molecular biology. It serves as a valuable tool for researchers in various disciplines, including bioinformatics, systems biology, drug discovery, and disease research, enabling them to explore and analyze complex biological systems and uncover novel insights into biological processes and disease mechanisms (219). Additionally, spectral library matching tools such as mzCloud can be utilized for compound identification. These tools compare the experimental spectra with a library of reference spectra, allowing for spectral matching and identification of metabolites based on similarity scores. Compound identification using reference databases and spectral matching provides valuable insights into the identity and biological relevance of the detected metabolites. It enables the annotation of spectral peaks with specific metabolites and aids in the interpretation of metabolomics data in the context of metabolic pathways, disease associations, and biological processes.

2.7.3.6 Univariate and multivariate analysis techniques

The main goal of employing univariate and multivariate analysis techniques is to extract meaningful information from data and derive biologically relevant insights related to the specific research question. In statistical metabolomics data analysis, chemometric approaches are commonly utilized, with principal component analysis (PCA) (226) and partial least squares regression (227) being extensively applied methods. These techniques aid in the identification and interpretation of features

obtained from non-targeted metabolomics experiments, allowing for the prioritization of metabolites with potential biological significance.

Multivariate approaches play a crucial role in simplifying the complexity of high-dimensional metabolomic datasets, making them more manageable and facilitating their interpretation (209). PCA is an unsupervised method commonly employed in metabolomics data analysis. It does not require prior knowledge and begins by exploring the relationships among the original variables. Through variance maximization, PCA transforms these variables into independent composite indicators known as principal components. By graphing two to three principal components, PCA facilitates the visual identification of distinctions in metabolic patterns and clustering outcomes among various groups. Loading plots generated from PCA aid in the identification of initial variables that contribute to intergroup classification, serving as potential biomarkers (158,209,228). PCA serves as a crucial multivariate tool frequently employed as an initial analysis and quality control step for metabolomic data, allowing observation of intergroup classification patterns and identification of data outliers in metabolomics datasets. PCA serves as a vital multivariate tool used for preliminary analysis and quality control of metabolomic data. It allows for the detection of intergroup classification patterns and the identification of data outliers in metabolomics datasets. Additionally, PCA can assess whether quality control samples cluster together, providing insights into the overall assay quality. For instance, in a study conducted by Kim et al., PCA was employed to examine urinary metabolomic profiling data for the noninvasive diagnosis of acute T cell-mediated rejection following kidney transplantation. The tightly clustered quality control samples observed on the PCA score plot confirmed the reliability of the metabolomics data and the stability of the instrument's performance (23).

Another commonly employed classification technique in the analysis of metabolomics data is the score plot of Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). It integrates regression models with dimensionality reduction and discriminant analysis of regression outcomes, using distinct discriminant thresholds. The score plot of OPLS-DA is frequently used to illustrate the

classification impact of the model. A greater separation between the two groups on the graph is indicative of a more pronounced classification effect (209). It is crucial to highlight that when applying OPLS-DA, cross-validation is essential to assess the model's performance, and the results of OPLS-DA should be interpreted alongside the cross-validation outcomes. This practice helps avoid overfitting issues that can arise when working with metabolomic data (229).

The univariate analysis serves as an initial exploration of data features that could play a crucial role in determining specific conditions. This type of analysis focuses on a single variable (such as metabolite density) as the input for statistical analysis. In essence, univariate testing involves comparing the statistical values (usually mean or median) of samples between two or multiple study groups, such as disease and healthy control groups. There are various univariate statistical tests available for comparing means or medians, and the choice depends on factors such as dataset heteroscedasticity, normality, and independence. Commonly used univariate statistical tools for metabolomic data include paired or unpaired t-tests, analysis of variance (ANOVA), the Kruskal-Wallis test and Wilcoxon rank sum test, among others. The selection of a specific test depends on the experimental design and the distribution of the data (209,230).

Overall, the combination of univariate and multivariate analysis techniques in metabolomics data analysis allows for comprehensive exploration, interpretation, and extraction of biologically relevant information from complex metabolomic datasets.

3 MATERIALS AND METHODS

3.1 Study Participants

Table 2 presents the clinical and demographic features of the participants. The research involved sixty-one individuals who underwent kidney transplantation and were exclusively administered tacrolimus. The post-transplant follow-up period for all transplant recipients was one year, during which their hospital stay was assessed in the first week, and subsequent evaluations were conducted at the third, sixth months, and one year. Urine samples were collected from these kidney transplant recipients within a 24-hour period post-transplantation. In our study, there were no instances of rejection observed during the one-year follow-up period. Moreover, throughout the corresponding follow-up period, no occurrences of delayed graft function were observed among the patients. The individuals' glomerular filtration rates were calculated using the equations from the Chronic Kidney Disease-Epidemiology Collaboration. It is important to note that all surgeries were performed using organs from living donors. The study received ethical approval from the Acibadem Mehmet Ali Aydinlar University Human Scientific and Ethical Review Committee under approval ID 2022-02/30. Written informed consent was obtained from all participants before their inclusion in the study.

Table 2. Demographic and transplant characteristics of patients, data are presented as mean ± SD, median (25th, 75th percentiles), or counts (n) for various parameters, including cold ischemia, Human Leukocyte Antigens (HLA), Estimated Glomerular Filtration Rate (eGFR), c-reactive protein (hs-CRP), body mass index (BMI), panel-reactive antibody (PRA), and gender (F: Female, M: Male).

		1W			3M			6M		
		Group 1 (N=36)	**Group 2 (N=25)**	**P-Value**	**Group 1 (N=29)**	**Group 2 (N=24)**	**P-Value**	**Group 1 (N=32)**	**Group 2 (N=23)**	**P-Value**
Recipient Age in years		44.4 (40.0- 56.8)	38.7 (30.2-48.4)	9.58E-03	40.0 (31.0-62.0)	33.0 (19.0-48.0)	1.68E-03	40.9 (40.0-62.0)	44.1 (28.9-48.4)	NS
Donor Age in years		47.0 (36.0-57.0)	52.0 (45.0-58.0)	1.00E-02	46.0 (33.0-59.0)	56.0 (49.0-58.0)	2.20E-02	44.0 (33.0-57.0)	49.5 (42.5-56.8)	NS
Diabetes (n)	NO	27	18	NS						
	YES	9	7							
Warm ischemia (s)		179 (160-200)	171 (153-184)	1.99E-02	180 (165-210)	184 (169-233)	NS	178 (162-205)	169 (135-196)	NS
Cold ischemia (min)		63.5 (58.0-72.0)	66.0 (54.0-78.0)	NS	64.0 (57.0-68.0)	66.0 (61.0-78.0)	3.40E-02	65.00 (56.0-68.0)	74.00 (61.0-78.0)	2.05E-02
Number of the HLA-mismatch		4 (3-5)	4 (3-5)	NS	4 (3-5)	3 (3-4)	NS	4 (3-5)	4 (3-5.75)	NS
Time on Dialysis Pre-Transplant (month)		4.0 (0.0-12.0)	2.0 (0.0-10.0)	NS	3.00 (0.0-11.0)	7.0 (0.0-12.0)	NS	2.5 (0.0-11.0)	0.0 (0.0-7.0)	NS
Recipient Gender	F/M	17/19	7/18	1.40E-05	12/17	9/15	3.90E-02	14/18	10/13	2.68E-03
Donor Gender	F/M	15/21	17/8	NS						

Table 2. Demographic and transplant characteristics of patients, data are presented as mean ± SD, median (25th, 75th percentiles), or counts (n) for various parameters, including cold ischemia, Human Leukocyte Antigens (HLA), Estimated Glomerular Filtration Rate (eGFR), c-reactive protein (hs-CRP), body mass index (BMI), panel-reactive antibody (PRA), and gender (F: Female, M: Male) (Continued from the previous page)

		1W			3M			6M		
		Group 1 (N=36)	**Group 2 (N=25)**	**P-Value**	**Group 1 (N=29)**	**Group 2 (N=24)**	**P-Value**	**Group 1 (N=32)**	**Group 2 (N=23)**	**P-Value**
PRA (n)	PRA Class I									
	%0-5	34	24	NS						
	%5-25	2	1							
	PRA Class II									
	%0-5	33	24	NS						
	%5-25	3	1							
BMI (kg/m²) (mean±SD)		24.3 ± 4.4	26.5 ± 5.2	NS						
Creatinine Serum (mg/dL)		0.99 (0.81-1.17)	1.27 (1.06-1.67)	0.00	1.11 (0.9-1.33)	1.54 (1.22-1.83)	4.00E-06	1.13 (0.92-1.32)	1.52 (1.39-1.81)	3.16E-08
eGFR (mL/dk/1.73 m2)		71.7 (50.7-96.8)	78.4 (64.7-101.3)	0.04	74.5 (63.0-86.0)	64.0 (46.0-82.0)	1.30E-02	71.3 (62.4-87.2)	53.8 (47.8-60.4)	2.00E-06
Urea Nitrogen (BUN) (mg/dL)		22 (17-29)	25 (21-30)	0.00	21 (16-26)	26 (23-31)	1.00E-03	16 (12-20)	21 (18-35)	5.90E-04

Table 2. Demographic and transplant characteristics of patients, data are presented as mean ± SD, median (25th, 75th percentiles), or counts (n) for various parameters, including cold ischemia, Human Leukocyte Antigens (HLA), Estimated Glomerular Filtration Rate (eGFR), c-reactive protein (hs-CRP), body mass index (BMI), panel-reactive antibody (PRA), and gender (F: Female, M: Male) (Continued from the previous page)

		1W			3M			6M		
		Group 1 (N=36)	**Group 2 (N=25)**	**P-Value**	**Group 1 (N=29)**	**Group 2 (N=24)**	**P-Value**	**Group 1 (N=32)**	**Group 2 (N=23)**	**P-Value**
C-reactive protein (hs-CRP) (mg/dL)		1.05 (0.37-2.62)	0.58 (0.23-1.42)	0.00	0.14 (0.00-0.30)	0.07 (0.00-0.19)	4.90E-02	0.25 (0.07-0.75)	0.13 (0.01-0.49)	NS

3.2 Sample Preparation

Urine samples obtained for this study were promptly aliquoted and stored at -80 °C until further preparation. To analyze the urine metabolite profile, 500 µl of the urine samples underwent centrifugation at 10,000 g for 10 minutes at 4 °C to remove any particles. The resulting supernatants were then mixed with 1500 µl of chilled methanol and incubated overnight at -80 °C to eliminate proteins. Subsequently, they were centrifuged again at 10,000 g for 10 minutes at 4 °C, and the resulting supernatants were transferred to new Eppendorf tubes. The supernatants were further evaporated under a vacuum. To prepare a QC sample containing comprehensive metabolic information, 30 µL of each aliquoted urine sample was combined in a test tube. This QC sample was used to assess the quality requirements during UPLC-MS analysis data acquisition. As shown in Figure 4, the QC sample was injected into the system 15 times before the commencement of sample analysis to condition the column. Additionally, the QC sample was reinjected once at the beginning of the run, every 10 sample injections, and at the end of the sample injection process. This was done to evaluate the repeatability of the process as well as the precision and stability of the instrument. Following the completion of the sample analysis, a dilution series of the QC sample (1:2, 1:4, 1:8) in a dilution urine mixture was injected into the UPLC/ESI/QToF-MS system. This was followed by an injection of a blank sample for further analysis.

3.3 Urine Metabolite Profiling Using UPLC/ESI/QToF-MS

In the untargeted metabolomics analysis, a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 µm) from Waters Corp, USA, was employed. This column was coupled with a Waters AcquityTM UPLC system and a Xevo G2 XS QTof mass spectrometer (Waters MS Technologies, UK), operating in positive ionization (ESI+) polarity mode with an electrospray ionization (ESI) source. With the autosampler temperature held at 4 °C and the column compartment temperature set at 40 °C, the mobile phase composition consisted of 0.1% formic acid (FA) in water as mobile phase A and 0.1% FA in acetonitrile as mobile phase B. The elution profile was designed,

initiating with 99-85% A from 0.0 to 3.0 min at a flow rate of 0.5 mL/min, followed by 85-15% A from 3.0 to 6.0 min at a flow rate of 0.5 mL/min. Subsequently, a transition to 50-5% A occurred from 6.0 to 9.0 min at a flow rate of 0.5 mL/min, maintaining 5% A from 9.0 to 10.01 min at a flow rate of 0.5 mL/min. Finally, the elution was completed with 99% A from 10.01 to 14.0 min at a flow rate of 0.5 mL/min. Employing a 10 µL injection volume, both MS and MSE data scans were acquired at intervals of 0.2 s, with an interscan delay of 0.1 s.

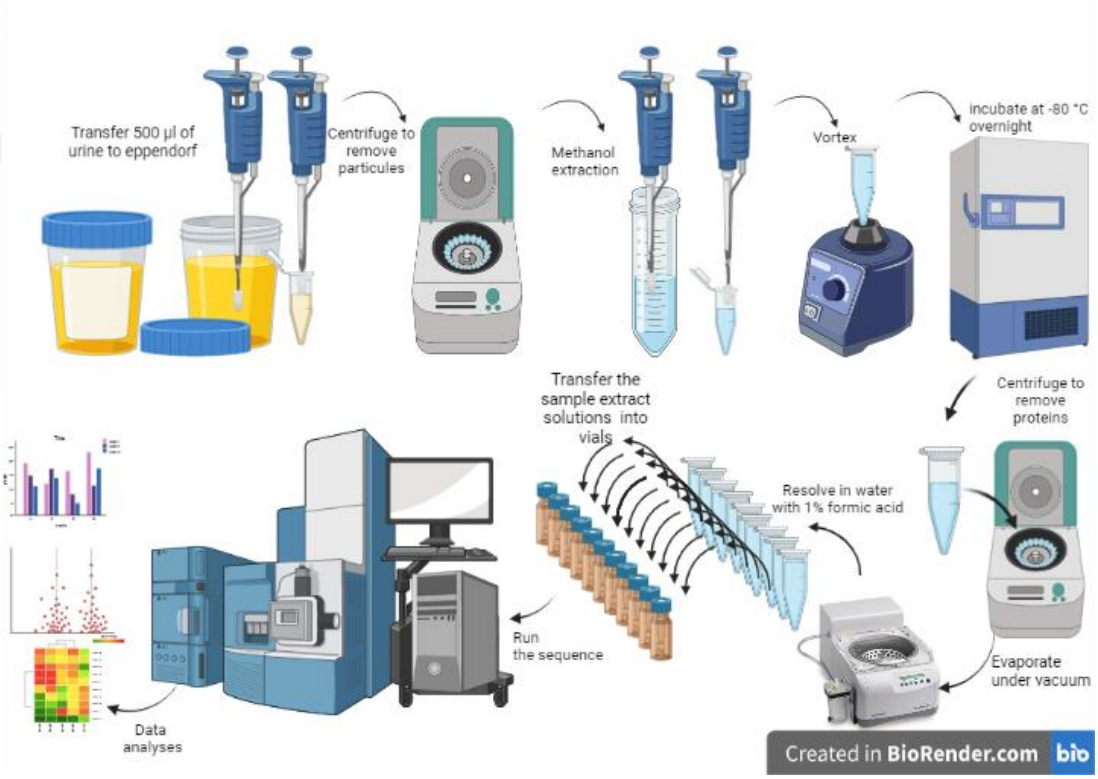

Figure 4. depicts the procedural workflow employed in this study. Following sample preparation, each sample underwent processing based on the designated run order. The concluding phase involved data processing and statistical analysis to pinpoint potential biomarker candidates.

During MSE data acquisition, collision energy underwent a gradual increase from 15 to 40V. For lock mass correction, leucine enkephalin (200 pg/µL, 0.1% FA: acetonitrile 1:1, v/v) was used. Lock spray was applied to ensure reproducibility and accuracy throughout the analysis, with lock mass data collected every 0.3 s and an interscan delay of 0.2 s. The MS scan range was set from 50 to 1200 m/z. The cone voltage and capillary voltage were adjusted to 40 and 3 kV, respectively. Desolvation

temperature, source temperature, and desolvation gas flow rate were set to 450 °C, 120 °C, and 800 L/h, respectively. Waters MassLynx v4.1 software controlled the entire acquisition process.

3.4 Data Processing and Statistical Analysis

The raw data set acquired from the UPLC/ESI/QToF-MS instrument was subjected to nonlinear alignment and normalization by commercial Progenesis QI 2.0 software to compare different runs and correct nonlinear RT shifts between related peaks in runs. The initial step involved transferring the raw data set to Progenesis QI software. The software then generated ion density maps by mapping the data with the m/z and RT of each MS signal. These ion maps were aligned in the RT direction and subjected to peak collection. The metabolomic data underwent standardization through Total Ion Current (TIC) normalization. Subsequently, an aggregated data set containing all the information from the sample files was created.

During the data preprocessing phase, we conducted a rigorous filtering process to ensure the selection of reliable features for subsequent multivariate analysis. Initially, we removed features that contributed more than 5% to the intensity from the Blank to eliminate noise or artifacts. To address missing values, we implemented the "80% rule," which involved removing variables that were missing in 80% or more of the samples, enhancing the quality and reliability of the data (231,232). Additionally, we excluded features with a QC coefficient of variation above 30% as well as unreliable features that demonstrated poor linearity in the dilution QC samples . This comprehensive approach further improved the data quality and suitability. The m/z and retention time datasets, along with their corresponding ion intensities, were then imported into SIMCA and MetaboAnalyst 5.0 for both univariate and multivariate data analysis (233). Establishing a threshold for statistical significance at a false discovery rate (FDR)-adjusted p-value of 0.05, the data underwent Pareto scaling, which involves mean-centering each variable and dividing it by the square root of its standard deviation. To further refine the differentiation between Group 1 and Group 2 at different time intervals (1st week, 3rd month, and 6th month) following kidney

transplantation, we employed Supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) modeling. OPLS-DA is a statistical technique that aims to maximize the discrimination between groups while considering the underlying correlation structure within the data. By applying OPLS-DA, the model sought to identify the metabolomic features that contribute the most to the separation between the two groups at different time points, thereby providing insights into potential biomarkers or metabolic changes associated with kidney transplantation. V-plots generated from the OPLS-DA model were utilized to enhance the identification of metabolic profile differences between the groups. These plots allowed for the visualization of the relative importance of differential variables and aided in obtaining a list of peaks or features that contribute significantly to the separation. The OPLS-DA model, which facilitates the comparison between groups, was constructed using the SIMCA software. Variables meeting the criteria of a VIP value exceeding 1.0, a fold change beyond 1.1 or below 0.91, quality control (QC) <30, and an adjusted p-value under 0.05 were regarded as potential biomarker candidates. These selection criteria ensured that the identified variables exhibited strong discriminatory power and statistically significant differences between the compared groups.

Putative annotations were conducted by referencing the MassBank and Human Metabolome Database (HMDB) (http://www.hmdb.ca). For the identification process, specific criteria were employed, including a mass error of less than 12 ppm, isotope similarity of greater than 70% between in silico fragmentation results, and the presence of fragments in high-energy spectra with at least one fragment exhibiting a mass error of less than 20 ppm (234). These criteria ensured the accuracy and reliability of the metabolite identifications.

In the course of this study, an in-depth examination of clinical and demographic variables was conducted across distinct groups, utilizing statistical methodologies. Continuous variables underwent scrutiny through the Mann-Whitney U test, a nonparametric assessment, and the t-test was employed for parametric analysis where applicable. The analysis of categorical variables was carried out using the Chi-square test. The statistical analysis was executed with the assistance of SPSS 22.0 software

(IBM Corp., Armonk, NY, USA). The presentation of Patient Demographic and Transplant Characteristics data were presented in various formats, such as mean ± SD, median (25th, 75th percentiles), or counts (n). The significance level was set at $P < 0.05$, establishing statistical significance in this comprehensive investigation.

3.4.1 Logistical regression modeling and receiver operating characteristic curves analysis

Utilizing IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, N.Y., USA), a stepwise backward conditional Binary Logistic Regression Analysis was undertaken to evaluate the discriminative capacity of metabolomic entities in distinguishing between Group 2 and Group 1. Employing the pertinent metabolites identified as significant biomarker candidates through ESI-positive analyses, the analysis integrated various parameters. This encompassed the inclusion of log-transformed values, log-transformed ratios, and serum creatinine ratios as explanatory variables. The comprehensive approach aimed to elucidate the intricate relationships and potential predictive value of these metabolites in the context of the study's objectives and the differentiation between the specified groups. The purpose of this analysis was to determine the contribution of these variables in predicting the membership of individuals in either Group 2 or Group 1. By employing a backward conditional approach, the analysis systematically eliminated non-significant variables from the model until the most predictive set of variables remained. In order to thoroughly evaluate the impact of potential clinical variables, acknowledged as significant kidney transplant risk factors, on the model's overall fit, an additional logistic regression model was developed. This supplementary model not only integrated the clinical explanatory variables such as diabetes, gender, WIT, HLA mismatch, CIT, and donor age but also included the identified significant biomarker candidates. The incorporation extended to encompass their log-transformed ratios, log-transformed values, and serum creatinine ratios as additional explanatory variables. This comprehensive approach sought to account for the multifaceted interplay between clinical factors and metabolomic entities, providing a nuanced understanding of their collective influence on the model's predictive capacity. The model performances were

evaluated and presented as ROC curves (Receiver Operating Characteristic curves). The areas under the ROC curves were used to determine the significance of the biomarkers. The ROC curves were plotted using MedCalc software, specifically version 12.7.0.0 (MedCalc Sofware, Ostend, Belgium). These analyses aimed to assess the predictive value and clinical significance of the identified biomarkers, taking into account both metabolomic entities and clinical variables, in the context of kidney transplantation.

4 RESULTS

4.1 Technical Reproducibility

The scrutiny of quality control samples, derived from the study samples, is a meticulous process that involves vigilant monitoring of critical analytical parameters, such as retention time, detector response, and mass accuracy. This comprehensive oversight is essential to ensure the reliability and accuracy of results obtained from analytical processes, thereby enhancing the overall dependability of study outcomes. Throughout our investigation, data analysis revealed a closely clustered pattern in the Quality Control (QC) samples, depicted in Figure 5-A and B.

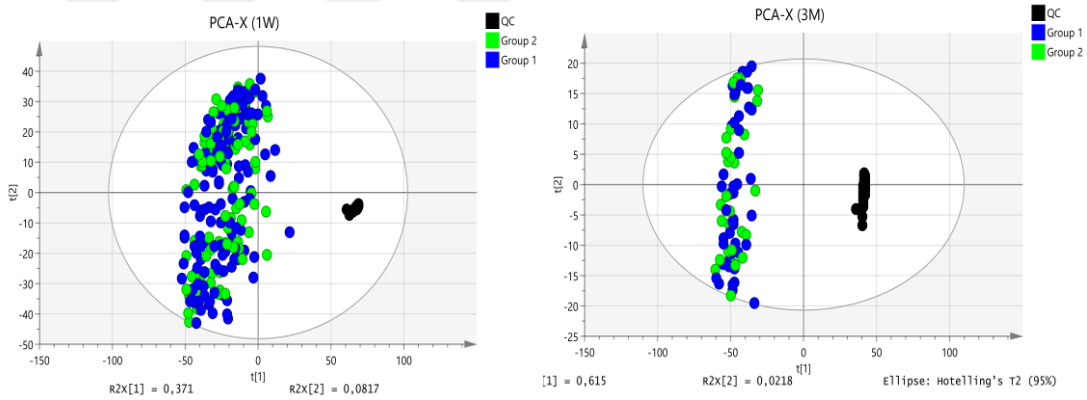

Figure 5. PCA-generated score plots visually depicting samples after kidney transplantation, with green circles representing individuals from Group 1, blue circles from Group 2, and black circles signifying the quality control (QC) group. Subfigures 5A and 5B present PCA score plots for Group 1, Group 2, and QC, capturing variations in the data during the first week and the third month. Within these figures, "QC" designates the quality control sample, "1W" denotes the first week, and "3M" signifies the third month.

This observation validates the dependability of the metabolomics data, affirming the efficiency and consistency of the employed method for human urine metabolite profiling. The compact clustering of QC samples indicates the precision of the analytical measurements, instilling confidence in the overall quality of the metabolomics analysis. This cohesive clustering pattern enhances the robustness of the study's findings and reinforces the reliability of the metabolomic profiling approach utilized.

4.2 Multivariate and Univariate Statistical Analysis

A fundamental aspect of this study was the stratification of kidney transplant recipients into two groups based on their serum creatinine levels. Group 1 comprised patients with serum creatinine levels below 1.5, while Group 2 consisted of individuals surpassing this threshold (8,14). Conducting metabolomic analyses on urine samples collected from all recipients at three different time points (1st week, 3rd month, and 6th month) post-transplantation constituted a pivotal component of this study. The primary objective was to discern potential differences in urinary metabolite profiles associated with kidney function and transplant outcomes through a comparative analysis between Group 1 and Group 2.

Utilizing the OPLS-DA model served as a strategic approach to identify potential biomarkers distinguishing between groups. The OPLS-DA score plots, systematically analyzed for the 1st week, 3rd month, and 6th month time points, unveiled conspicuous variations in metabolic profiles between Group 1 and Group 2. These graphical representations depicted explicit differences, signifying substantial variations in urinary metabolites at different post-transplantation intervals. The impressive performance of the model during the 1st week, as indicated by R2Y of 0.98 and Q2 of 0.54, not only attests to its robust fitting but also underscores its reliability. Correspondingly, the 3rd-month model, with R2Y of 0.95 and Q2 of 0.50, further solidifies the evidence of consistent and dependable separation between the groups (Figure 6-A, B). This heightened level of accuracy in both time points enhances our confidence in the model's ability to discern meaningful distinctions in urinary metabolite profiles. These findings imply that the identified metabolites possess the potential to function as biomarker candidates, facilitating the differentiation between groups post-kidney transplantation. Notably, the absence of significant variations in features during the 6th month after transplantation is observed. Furthermore, the OPLS-DA model developed for the 6th month suggests overfitting, prompting concerns about its accuracy in capturing the underlying patterns of the dataset.

The comprehensive validation process of the predictive capacity and significance of variables within the OPLS-DA models included an in-depth analysis using VIP plots, as illustrated in Figure 6-C and D.

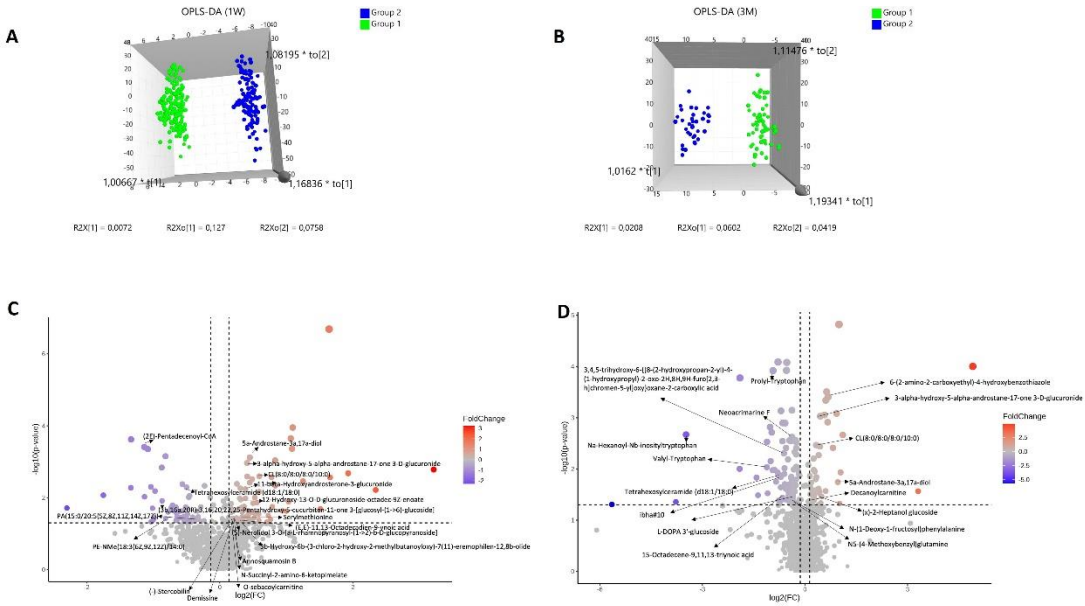

Figure 6. presents exhaustive OPLS-DA-generated score plots and volcano plots, offering a visual representation of samples from Group 1 (depicted by green circles) and Group 2 (depicted by blue circles) post-kidney transplantation. In Figure 6 (A, B), detailed volcano plots illustrate the unique metabolic profiles of Group 1 and Group 2 during the first week (A) and the third month (B) following transplantation. Further insights are provided in Figure 6 (C, D), delving into the OPLS-DA model results for Group 1/Group 2 in the first week (C) and Group 1/Group 2 in the third month post-kidney transplantation in ESI-mode (D). "1W" and "3M" respectively denote the first week and the third month.

These plots provided visual confirmation of metabolites exhibiting substantial abundance differences between groups, further validating the observed separation. To strengthen the reliability of the models, extensive statistical assessments were carried out, incorporating both CV-ANOVA and permutation tests. The CV-ANOVA results, with p-values of 1.61E-32 (1st week) and 3.46E-11 (3rd month), unequivocally demonstrated the robust predictive capability of the OPLS-DA models, reinforcing the confidence in their ability to discern meaningful distinctions in urinary metabolite profiles. In the rigorous permutation test, encompassing 200 permutations and meticulously evaluating R2 and Q2 values between authentic models and permuted models, the 1st-week comparison showcased the superiority of true models with R2

and Q2 values (0.952 and -0.336). Similarly, during the 3rd-month comparison, true models demonstrated better performance than permutations, R2 and Q2 values of 0.905 and -0.291, respectively. This not only underscores the robustness of authentic models in elucidating significant distinctions between groups but also emphasizes their exceptional capability to reliably capture the intricate patterns present in the dataset, ensuring the credibility of the observed outcomes.

4.3 1st Week and 3rd Month Statistics

The identification of a distinct subgroup within the HREG category relies significantly on the role played by serum creatinine levels. In an evaluation of discriminatory capabilities, ROC analysis was systematically employed to assess how serum creatinine distinguishes Group 2 from Group 1 within the HREG context. Specifically focusing on the 1st week post-transplantation, the Area Under the Curve (AUC) for serum creatinine yielded a calculated value of 0.73 (95% confidence interval: 0.674 to 0.785, p-value: 0.0001, model 1). This outcome underscores a moderate to good discriminatory power, pinpointing the effectiveness of serum creatinine in this particular temporal context within the HREG category. Through comprehensive multivariable statistical analyses, discernible variations emerged between Group 1 and Group 2 concerning serum creatinine levels at distinct time points. Specifically, during the 1st week, a noteworthy discrepancy in 19 metabolites was observed, while in the 3rd month, 17 metabolites exhibited significant distinctions. These disparities were identified based on specific criteria, including (adjusted p-value < 0.05, quality control (QC) < 30, a fold change (FC) > 1.1 or a FC < 0.91, VIP) > 1, as outlined in Table 3 and 4. The recognition of these metabolites accentuates their potential as biomarkers, offering a promising avenue for distinguishing between the two groups predicated on serum creatinine levels. The metabolomic analysis has unveiled differentially expressed metabolites, exhibiting substantial distinctions between Group 1 and Group 2, across a diverse array of classes. This comprehensive categorization spans fatty acyls, steroids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, amino acids, prenol lipids, and sophorolipids, as outlined in Tables 3 and 4. In order to evaluate the discriminative

potential of the identified metabolites, individual ROC curve analyses were conducted. During the initial week following kidney transplantation, the acquired AUC values displayed a range from 0.39 to 0.71, while in the third month, they varied from 0.32 to 0.76. These AUC values serve as quantitative indicators of the metabolites' efficacy in distinguishing between Group 1 and Group 2. It is pertinent to highlight that the observed variability in AUC values underscores the diverse discriminative capabilities of individual metabolites, with some manifesting weak to moderate discriminatory power. This nuanced assessment provides a detailed understanding of the varying contributions of different metabolites in the context of serum creatinine levels post-transplantation.

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1.

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
4.88_972.3000 m/z	0.46	-1,13	3.89E-04	3.41	HMDB0062229	Fatty Acyls	Lipids and lipid-like molecules	M+H-H2O	C36H62N7O17P3S	-10.38	95.62	(2E)-Pentadecenoyl-CoA		972.3000	4.88
6.61_257.2258 m/z	1.37	0,45	7.63E-04	3.12	HMDB0000458	Steroids and steroid derivatives	Lipids and lipid-like molecules	M+H-2H2O	C19H32O2	-2.14	91.91	5a-Androstane-3a.17a-diol		257.2258	6.61
6.38_449.2524 m/z	1.33	0,41	1.09E-03	2.96	HMDB0010365	Steroids and steroid derivatives	Lipids and lipid-like molecules	M+H-H2O	C25H38O8	-2.2	90.66	3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide		449.2524	6.38

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
6.30_932.5080n	1.51	0,59	2.54E-03	2.60	HMDB0116798	Glycerophospholipids	Lipids and lipid-like molecules	M+H-2H2O. M+Na. M+NH4. M+H. M+H-H2O. M+H+Na	C43H82O17P2	5.62	93.52	CL(8:0/8:0/8:0/10:0)	932.508	955.4972	6.3
8.63_628.3943m/z	0.71	-0,50	5.11E-03	2.29	HMDB0004961	Sphingolipids	Lipids and lipid-like molecules	M+2H	C62H114N2O23	-5.65	84.17	Tetrahexosylceramide (d18:1/18:0)		628.3943	8.63

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
5.63_482.2492n	1.31	0,39	5.77E-03	2.24	HMDB0010351	Steroids and steroid derivatives	Lipids and lipid-like molecules	M+H-H2O. M+CH3OH+H	C25H38O9	-4.87	85.8	11-beta-Hydroxyandrosterone-3-glucuronide	482.2492	465.2459	5.63
6.51_490.2773n	1.24	0,31	1.13E-02	1.95	HMDB0060118	Saccharolipids	Lipids and lipid-like molecules	M+H-H2O. M+NH4. M+K. M+H+Na	C24H42O10	-1	86.34	12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate	490.2773	473.2740	6.51

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
8.85_863.4992 m/z	0.55	-0,87	1.22E-02	1.91	HMDB0035345	Steroids and steroid derivatives	Lipids and lipid-like molecules	M+CH3OH+H	C42H70O16	-0.79	81.19	(3b.16a.20R)-3.16.20.22.25-Pentahydroxy-5-cucurbiten-11-one 3-[glucosyl-(1->6)-glucoside]		863.4992	8.85

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1**.** (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
6.14_553.2925 m/z	1.14	0,19	2.49E-02	1.60	HMDB0040844	Fatty Acyls	Lipids and lipid-like molecules	M+Na	C27H46O10	-10.94	96.47	(S)-Nerolidol 3-O-[a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside]		553.2925	6.14
6.21_269.1186 m/z	1.44	0,53	2.69E-02	1.57	HMDB0029045	Amino acids. peptides. and analogues	Organic acids and derivatives	M+CH3OH+H	C8H16N2O4S	8.62	90.29	Serylmethionine		269.1186	6.21

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
3.87_212.0793m/z	1.33	0,41	3.39E-02	1.47	HMDB0041278	Prenol lipids	Lipids and lipid-like molecules	M+H+Na	C20H29ClO6	-7.76	69.57	3b-Hydroxy-6b-(3-chloro-2-hydroxy-2-methylbutanoyloxy)-7(11)-eremophilen-12.8b-olide		212.0793	3.87
8.54_680.4339n	0.8	-0,32	3.40E-02	1.47	HMDB0114823	Sophorolipids	Lipids and lipid-like molecules	M+NH4. M+K. M+Na. M+H. M+H+Na	C34H64O13	-1.17	87.89	PA(15:0/20:5(5Z.8Z.11Z.14Z.17Z))	680.4339	703.4231	8.54

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
5.74_276.2073n	1.43	0,52	3.57E-02	1.45	HMDB0034382	Fatty Acyls (Long-chain fatty acids)	Lipids and lipid-like molecules	M+H-2H2O. M+H	C18H28O2	-5.99	88.71	(E.E)-11.13-Octadecadien-9-ynoic acid	276.2073	277.2146	5.74
5.31_308.2347n	1.19	0,25	3.62E-02	1.44	HMDB0031379	Prenol lipids	Lipids and lipid-like molecules	M+H-2H2O. M+K	C19H32O3	-1.46	91.6	Annosquamosin B	308.2347	273.2208	5.31
6.42_331.1142 m/z	1.18	0,24	3.70E-02	1.43	HMDB0012266	Amino acids. peptides. and analogues	Organic acids and derivatives	M+ACN+H	C11H15NO8	2	97.21	N-Succinyl-2-amino-6-ketopimelate		331.1142	6.42

Table 3. Features displaying notable distinctions between Group 1 and Group 2 during the first week, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1 (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw.pval	-LOG10(P)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	Neutral mass (Da)	m/z	Retention time (min)
4.54_328.2103m/z	1.14	0,19	3.71E-02	1.43	LMFA07070100	Fatty acyl carnitines	Lipids and lipid-like molecules	M+NH4	C17H31NO6	-4.85	75.22	O-sebacoylcarnitine		328.2103	4.54
8.60_732.5199m/z	0.57	-0,82	3.78E-02	1.42	HMDB0113193	Glycerophospholipids	Lipids and lipid-like molecules	M+CH3OH+H	C38H70NO8P	3.52	70.6	PE-NMe(18:3(6Z.9Z.12Z)/14:0)		732.5199	8.6
4.65_1017.5529n	1.22	0,29	3.87E-02	1.41	HMDB0034205	Steroids and steroid derivatives	Lipids and lipid-like molecules	M+2H.M+H+Na	C50H83NO20	1.99	86.01	Demissine	1017.553	520.7747	4.65
5.23_594.3393n	1.17	0,23	3.95E-02	1.40	HMDB0240259	Tetrapyrroles and derivatives	Organoheterocyclic compounds	M+H.M+Na.M+K	C33H46N4O6	-4.01	98.59	(-)-Stercobilin	594.3393	595.3466	5.23

Table 4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1.

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
3.40_301.1418n	0,52	-0,93	1,20E-04	3,92	HMDB0029028	Amino acids, peptides, and analogues	Organic acids and derivatives	M+NH4, M+CH3OH+H	C16H19N3O3	-2,71	97,1	Prolyl-Tryptophan	334,1753	3,4
4.76_239.0481m/z	1,60	0,68	3,95E-04	3,4	HMDB0062793	Amino acids, peptides, and analogues	Organic acids and derivatives	M+H	C10H10N2O3S	-1,57	92,3	6-(2-amino-2-carboxyethyl)-4-hydroxybenzothiazole	239,0481	4,76

Table 4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
6.38_449.2524m/z	1,49	0,57	8,81E-04	3,05	HMDB0010365	Steroids and steroid derivatives	Lipids and lipid-like molecules	M+H-H2O	C25H38O8	-2,2	90,7	3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide	449,2524	6,38
3.38_497.2481m/z	0,09	-3,48	2,13E-03	2,67	HMDB0037036	Prenol lipids	Lipids and lipid-like molecules	M+CH3OH+H	C23H32N2O8	-2,76	92,9	Na-Hexanoyl-Nb-inosityltryptophan	497,2481	3,38
4.66_549.1895m/z	0,77	-0,38	2,37E-03	2,63	HMDB0031112	Glycerolipids	Lipids and lipid-like molecules	M+NH4	C29H25NO9	5,25	93,4	Neoacrimarine F	549,1895	4,66

Table 4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
6.30_932.5080n	1,25	0,33	3,42E-03	2,47	HMDB0116798	Glycerophospholipids	Lipids and lipid-like molecules	M+H-2H2O, M+Na, M+NH4, M+H, M+H-H2O, M+H+Na	C43H82O17P2	5,62	93,5	CL(8:0/8:0/8:0/10:0)	955,4972	6,3

Table 4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
4.23_479.1579m/z	0,65	-0,62	4,95E-03	2,31	HMDB0130138	Glycerophospholipids	Lipids and lipid-like molecules	M+H-H2O	C23H28O12	6,31	90,2	3,4,5-trihydroxy-6-{[8-(2-hydroxypropan-2-yl)-4-(1-hydroxypropyl)-2-oxo-2H,8H,9H-furo[2,3-h]chromen-5-yl]oxy}oxane-2-carboxylic acid	479,1579	4,23

Table 4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
3.38_336.1907m/z	0,46	-1115 1,00	9,23E-03	2,03	HMDB0029138	Amino acids, peptides, and analogues	Organic acids and derivatives	M+CH3OH+H	C16H21N3O3	-3,6	95,6	Valyl-Tryptophan	336,1907	3,38
5.43_401.1518m/z	0,90	-0,16	1,34E-02	1,87	HMDB0029452	Amino acids, peptides, and analogues	Organic acids and derivatives	M+ACN+H	C15H21NO9	-10,14	81,9	L-DOPA 3'-glucoside	401,1518	5,43
8.63_628.3943m/z	0,59	-0,76	1,38E-02	1,86	HMDB0004961	Sphingolipids	Lipids and lipid-like molecules	M+2H	C62H114N2O23	-5,65	84,2	Tetrahexosylceramide (d18:1/18:0)	628,3943	8,63
5.99_316.2472m/z	1,10	0,14	1,38E-02	1,86	HMDB0000651	Fatty Acyls (Acyl carnitines)	Lipids and lipid-like molecules	M+H	C17H33NO4	-3,43	97,8	Decanoylcarnitine	316,2472	5,99

Table 4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
2.67_481.2531m/z	0,61	-0,71	1,45E-02	1,84	LMFA13040115	Fatty Acyls(Fatty acyl glycosides)	Lipids and lipid-like molecules	M+NH4	C24H33NO8	-2,94	90,6	ibha#10	481,2531	2,67
6.61_257.2258m/z	1,95	0,96	1,80E-02	1,74	HMDB0000458	Steroids and steroid derivatives (Androstane steroids)	Lipids and lipid-like molecules	M+H-2H2O	C19H32O2	-2,14	91,9	5a-Androstane-3a,17a-diol	257,2258	6,61
1.66_327.1313n	0,76	-0,40	2,14E-02	1,67	HMDB0037846	Amino acids, peptides, and analogues	Organic acids and derivatives	M+H-H2O, M+H	C15H21NO7	-1,42	96,1	N-(1-Deoxy-1-fructosyl)phenylalanine	310,1281	1,66

Table4. Features displaying notable distinctions between Group 1 and Group 2 during the third month, identified through stringent criteria, including an adjusted p-value below 0.05, quality control (QC) below 30, a fold change (FC) exceeding 1.1 or falling below 0.91, and Variable Influence on Projection (VIP) surpassing 1. (Continued from the previous page)

Rt_m/z	FC	log2(FC)	raw,pval	-log10(p)	Compound ID	CLASS	SUPER CLASS	Adducts	Formula	Mass Error (ppm)	Isotope Similarity	Description	m/z	Retention time (min)
5.63_243.1582m/z	1,40	0,48	3,44E-02	1,46	HMDB0035028	Fatty Acyls(Fatty acyl glycosides)	Lipids and lipid-like molecules	M+H-2H2O	C13H26O6	-3,12	93,4	(x)-2-Heptanol glucoside	243,1582	5,63
6.41_253.1578m/z	0,63345	-0,66	3,61E-02	1,44	HMDB0032673	Fatty Acyls (Long-chain fatty acids)	Lipids and lipid-like molecules	M+H-H2O	C18H22O2	-3,41	94,2	15-Octadecene-9,11,13-triynoic acid	253,1578	6,41
4.26_249.1258m/z	0,89048	-0,17	3,95E-02	1,4	HMDB0033598	Amino acids, peptides, and analogues	Organic acids and derivatives	M+H-H2O	C13H18N2O4	9,31	95,3	N5-(4-Methoxybenzyl)glutamine	249,1258	4,26

4.4 Combined Biomarkers Model

Utilizing a set of marker candidates rather than a single marker can enhance the discriminative capability and offer a more holistic insight into the biological system under examination. Stepwise backward logistic regression is a technique employed to choose the most relevant variables for incorporation into the regression model. By including both the metabolomic variables and clinical variables in the regression analysis, the aim is to create models that incorporate both types of information and potentially improve the accuracy of prediction or classification. This approach allows for the identification of the most relevant variables, considering their individual contributions as well as their interactionsn accordance with the outcomes presented in Table 5 and Table 6, our Stepwise Backward logistic regression analysis resulted in the development of five distinct models. Among these, three models incorporated clinical variables, while the remaining two excluded clinical variables. These models were systematically evaluated during both the 1st week and the 3rd month post-kidney transplantation. The construction of these models involved the integration of individual biomarker candidates, considering their log-transformed values, log-transformed ratios, and serum creatinine ratios as crucial components. In a targeted approach, the models devoid of clinical variables exclusively focused on metabolomic variables, incorporating their transformation ratios, transformations, and serum creatinine ratios. The primary aim of these models was to evaluate the discriminative capacity of the selected biomarkers, assessing their predictive capabilities in accurately classifying the respective groups. Conversely, the models incorporating clinical variables expanded their scope to encompass not only metabolomic variables but also supplementary clinical factors, including diabetes, gender, WIT, HLA mismatch, CIT, and donor age. The overarching objective of incorporating clinical variables into these models was to systematically assess the combined influence of both metabolomic and clinical factors in predicting the accurate classification of groups. The formulation encompassed a total of 10 models, constituting 5 models devised during the first-week post-transplantation and an additional set of 5 models crafted in the third month post-transplantation. These diverse models were purposefully constructed with varying combinations of variables, providing a nuanced and comprehensive analysis of

potential biomarkers and their interactions with clinical factors. This systematic approach facilitated an in-depth exploration of the intricate interplay between biomarkers and clinical variables within the specific time frames scrutinized in this study. This comprehensive analysis involved the development of five distinct models, each serving a unique purpose. In the foundational Model 1, the sole predictor variable was serum creatinine. Building upon this, Model 2 retained serum creatinine and introduced a range of pertinent clinical variables (diabetes, gender, WIT, HLA mismatch, CIT, and donor age). Model 3 took a different approach by incorporating the serum creatinine ratios of potential individual biomarker candidates as predictor variables. The subsequent Model 4 retained these ratios from Model 3 and augmented the model with the same additional clinical variables as seen in Model 2. The most expansive among them, Model 5, extended the scope of Model 4 by considering potential individual biomarker candidates, their log-transformed values, log-transformed ratios, and the inclusion of additional clinical variables, all evaluated during both the initial week and three months following kidney transplantation. This systematic progression allowed for an exploration of the impact of different variables on the predictive capacity of the models across distinct temporal frames.

In the assessment of logistic regression models during the initial week post-kidney transplantation, the Hosmer & Lemeshow (H-L) goodness-of-fit test yielded distinct results for each model: Model 1 demonstrated X^2 = 27.408 (p-value = 0.001), Model 2 exhibited X^2 = 27.545 (p-value = 0.001), Model 3 displayed X^2 = 12.874 (p-value = 0.116), Model 4 showed X^2 = 14.679 (p-value = 0.066), and Model 5 presented X^2 = 11.509 (p-value = 0.175). Simultaneously, the Nagelkerke (pseudo) R^2 values were as follows: Model 1 (0.099), Model 2 (0.328), Model 3 (0.173), Model 4 (0.414), and Model 5 (0.571). These outcomes collectively signify the fitness of the logistic regression models. The H-L test, gauging concordance between observed and predicted outcomes, indicated significant p-values of 0.001 for Model 1 and Model 2, suggesting a potential lack of fit. Conversely, Model 3, Model 4, and Model 5 demonstrated p-values of 0.116, 0.066, and 0.175, respectively, implying a better fit for these models. The Nagelkerke (pseudo) R^2 values, measuring the proportion of variance explained, highlighted Model 5 with the highest R^2 value of 0.571, followed

by Model 4 (0.414), Model 2 (0.328), Model 3 (0.173), and Model 1 (0.099). This intricate evaluation underscores the performance and explanatory power of each model in capturing the complexities of the data during the initial post-transplantation period.

Table 5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval.

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 1	Serum Creatinine	SC	2.450 (1.535-3.911)	0.896	0.239	1.72E-04	27.408	0.001	0.099
p	Serum Creatinine	SC	1.654 (1.024-2.671)	0.503	0.245	0.04	27.545	0.001	0.328
	Donor age	DA	1.051 (1.022-1.082)	0.05	0.015	0.001			
	Warm ischemia	WIT	0.981 (0.970-0.993)	-0.019	0.006	0.001			
	Diabetes	D	0.339 (0.163-0.708)	-1.081	0.375	0.004			
	HLA mismatch	HLA-MM	0.260 (0.140-0.484)	-1.347	0.317	2.18E-05			

Table 5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 2	Gender	G	0.242 (0.120-0.487)	-1.419	0.357	7.12E-05	27.545	0.001	0.328
Model 3	3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/Serum Creatinine	HMDB0010365/SC	1.027 (1.008-1.046)	0.027	9.00E-03	4.00E-03	12.874	0.116	0.173
	O-sebacoylcarnitine/Serum Creatinine	LMFA07070100/SC	1.025 (1.005-1.045)	0.025	0.010	0.012			
	PE-NMe(18:3(6Z,9Z,12Z)/14:0)/ Serum Creatinine	HMDB0113193/SC	0.663 (0.469-0.938)	-0.411	.177	0.020			

Table 5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 4	Donor age	DA	1.068 (1.036-1.100)	0.066	0.015	1.60E-05	14.679	0.066	0.414
	O-sebacoylcarnitine/Serum Creatinine	LMFA07070100/SC	1.025 (1.002-1.048)	0.024	.011	3.46E-02			
	3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/ Serum Creatinine	HMDB0010365/SC	1.023 (1.002-1.045)	0.023	.011	.031			
	Warm ischemia	WIT	0.978 (0.966-0.990)	-0.022	.006	4.85E-04			

Table 5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 4	PE-NMe(18:3(6Z,9Z,12Z)/14:0)/ Serum Creatinine	HMDB0113193/SC	0.623 (0.42--0.907)	-0.473	0.191	1.34E-02	14.679	0.066	0.414
	Diabetes	D	0.425 (0.198-0.910)	-0.856	0.389	2.76E-02			
	HLA mismatch	HLA-MM	0.275 (0.143-0.527)	-1.292	0.332	9.90E-05			
	Gender	G	0.244 (0.119-0.501)	-1.41	0.367	1.21E-04			

Table 5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 5	Log10(3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate)	HMDB0010365/HMDB0060118	0.43 (0.26-071)	-0.849	0.259	1.08E-03	11.509	0.175	0.571
	Log10((2E)-Pentadecenoyl-CoA)	HMDB0062229	0.34 (0.18-0.65)	-1.085	0.335	1.22E-03			
	Log10(3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/PE-NMe(18:3(6Z.9Z.12Z)/14:0))	HMDB0010365/HMDB0113193	3.71 (1.15-11.90)	1.310	0.595	2.77E-02			

Table5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL-Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 5	Log10 (N-Succinyl-2-amino-6-ketopimelate/3b-Hydroxy-6b-(3-chloro-2-hydroxy-2-methylbutanoyloxy)-7(11)-eremophilen-12.8b-olide)	HMDB0012266/HMDB0041278	1.85E-4 (6.00E-06-6.00E-03	-8.596	1.781	1.39E-06	11.509	0.175	0.571
	Log10((3b.16a.20R)-3.16.20.22.25-Pentahydroxy-5-cucurbiten-11-one 3-[glucosyl-(1->6)-glucoside]/PE-NMe(18:3(6Z.9Z.12Z)/14:0))	HMDB0035345/HMDB0113193	5.80E-02 (5.80E-03-0.71)	0.026	1.283	0.026			

Table5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL-Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 5	Log10 (N-Succinyl-2-amino-6-ketopimelate/CL (8:0/8:0/8:0/10:0))	HMDB0012266/HMDB0116798	5.26E-02 (6.51E-03-4.26E-01)	-2.944	1.067	5.78E-03	11.509	0.175	0.571
	HLA mismatch	HLA-MM	0.129 (0.054-0.308)	-2.049	0.445	4.14E-06			
	Diabetes	D	0.188 (0.075-0.471)	-1.671	0.468	3.58E-04			
	Warm ischemia	WIT	0.961 (0.945-0.0.978)	-0.040	0.009	4.77E-06			
	Gender	G	0.412 (0.180-0.943)	-0.887	0.423	0.036			

Table5. Binary Logistic regression analysis using stepwise backward conditional method in the 1st week after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 5	Donor age	DA	1.081 (1.045-1.119)	0.078	0.018	9.00E-06	11.509	0.175	0.571

In the evaluation of logistic regression models during the third month following kidney transplantation, the Hosmer & Lemeshow (H-L) goodness-of-fit test yielded distinct results for each model: Model 1 displayed X^2 = 4.635 (p-value = 0.796), Model 2 presented X^2 = 1.474 (p-value = 0.993), Model 3 exhibited X^2 = 2.754 (p-value = 0.949), Model 4 showed X^2 = 9.040 (p-value = 0.339), and Model 5 demonstrated X^2 = 6.914 (p-value = 0.546). Simultaneously, the Nagelkerke (pseudo) R^2 values were as follows: Model 1 (0.347), Model 2 (0.538), Model 3 (0.650), Model 4 (0.576), and Model 5 (0.610). Remarkably, all models displayed relatively high p-values, indicating a good fit for the data in the third month after transplantation. Further analysis of the Nagelkerke (pseudo) R^2 values emphasized that Model 3 exhibited the highest R^2 value of 0.650, followed by Model 5 (0.610), Model 4 (0.576), Model 2 (0.538), and Model 1 (0.347) (Table 6). This examination underscores the robustness of the models in effectively capturing the complexities of the data during the third month post-transplantation.

Table 6. Binary Logistic regression analysis using stepwise backward conditional method in 3rd month. after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval.

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 1	Serum Creatinine	SC	31.016 (6.220-154.658)	3,434	.820	2.80E-05	4.635	0.796	0.347
Model 2	Serum Creatinine	SC	25.192 (3.544-179.080)	3,227	1,001	1.26E-03	1.474	0.993	0.538
	Gender	G	21.747 (2.326-203.313)	3,079	1.140	6.93E-03			
	HLA mismatch	HLA-MM	0.219 (0.064-0.751)	-1,518	.628	1.60E-02			
	Diabetes	D	0.066 (0.07-0.631)	-2,715	1.151	1.80E-02			
Model 3	CL (8:0/8:0/8:0/10:0)/Serum Creatinine	HMDB0116798/SC	5.272 (2.089-13.300)	1,662	0.472	4.30E-04	2.754	0.949	0.65
	L-DOPA 3'-glucoside/Serum Creatinine	HMDB0029452/SC	0.284 (0.127-0.635)	-1,260	0.411	2.19E-03			

Table 6. Binary Logistic regression analysis using stepwise backward conditional method in 3rd month. after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 3	N-(1-Deoxy-1-fructosyl) phenylalanine/Serum Creatinine	HMDB0037846/SC	0.208 (0.074-0.585)	-1,569	.527	2.90E-03	2.754	0.949	0.65
	Na-Hexanoyl-Nb-inosityltryptophan/Serum Creatinine	HMDB0037036/SC	1.89E-04 (7.31E-08 - 0.488)	-8,574	4,009	1.89E-04			
	Tetrahexosylceramide (d18:1/18:0)/Serum Creatinine	HMDB0004961/SC	0.149 (0.034-0.653)	-1,902	0.753	1.16E-02			
Model 4	CL (8:0/8:0/8:0/10:0)/Serum Creatinine	HMDB0116798/SC	2.636 (1.269-5.475)	0.969	0.373	9.00E-03	9.04	0.339	0.576
	Donor age	DA	1.081 (1.018-1.147)	0.078	0.030	0.011			

Table 6. Binary Logistic regression analysis using stepwise backward conditional method in 3rd month. after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 4	Tetrahexosylceramide (d18:1/18:0)/Serum Creatinine	HMDB0004961/SC	0.139 (0.030-0.640)	-1,971	0.778	0.011	9.04	0.339	0.576
	Diabetes	D	0.061 (0.006-0.617)	-2,805	1,185	1.80E-02			
	Na-Hexanoyl-Nb-inosityltryptophan /Serum Creatinine	HMDB0037036/SC	2.22E-04 (3.48E-07 - 0.142)	-8,412	3,295	0.011			
Model 5	Log10(CL (8:0/8:0/8:0/10:0)	HMDB0116798	3.401 (1.421-8.139)	1,224	0.445	5.97E-03	6.914	0.546	0.61
	Donor age	DA	1.095 (1.026-1.169)	0.091	0.033	6.20E-03			
	Log10(L-DOPA 3'-glucoside)	HMDB0029452	0.311 (0.156-0.618)	-1,169	0.351	8.69E-04			

Table 6. Binary Logistic regression analysis using stepwise backward conditional method in 3rd month. after KTx. List of variables for each model that has a p-value lower than 0.05, Lower 95% CL: Lower 95% Confidence Interval, Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

							Hosmer & Lemeshow (H-L) goodness-of-fit test		**Nagelkerke (pseudo) R^2 values**
Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**B**	**SE**	**P-value**	**X^2**	**P-value**	**R^2**
Model 5	Log10(N-(1-Deoxy-1-fructosyl) phenylalanine)	HMDB0037846	0.194 (0.071-0.527)	-1,641	0.511	1.00E-03	6.914	0.546	0.61
	Log10(Tetrahexosylceramide (d18:1/18:0))	HMDB0004961	0.097 (0.018-0.516)	-2,329	0.851	6.19E-03			

The AUC values derived from the ROC analysis offer insights into the discriminative performance of various models. Summarily, the AUC ranges aid in evaluating the effectiveness of prognostic models as follows: AUC ranging from 0.9 to 1 indicates a good prognostic model, AUC ranging from 0.7 to 0.9 signifies a moderate prognostic model, and AUC ranging from 0.5 to 0.7 suggests a poor prognostic model. During both the initial week and the third month following kidney transplantation, ROC analysis was systematically employed to evaluate the discriminative efficacy of Group 2 in contrast to Group 1 across multiple logistic regression models, including Model 1, Model 2, Model 3, Model 4, and Model 5. As delineated in Table 2, the AUC values assigned to each model during the 1st week post-kidney transplantation elucidate their respective capacities in differentiating Group 2 from other groups. Notably, Model 5 emerges with the highest AUC value (0.900), underscoring its commendable discriminative ability. In comparison, Model 2 and Model 4 display moderate discriminative capabilities, boasting AUC values of 0.805 and 0.834, respectively. Meanwhile, Model 3 and Model 1 exhibit slightly lower discriminative abilities, each garnering an AUC value of 0.721 and 0.732, respectively. A comprehensive depiction of the ROC curve corresponding to the data presented in Table 7 is available in Figure 7, providing a visual representation of the discriminative performance of each model during the 1st week post-transplantation.

Table 7.The AUC, sensitivity, and specificity, along with their corresponding 95% confidence intervals (CI), for each predictive set of urine metabolites in the initial week post-KTx. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval.

Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**AUC. 95% CL**	**Sensitivity. 95% CL**	**Specificity. 95% CL**
Model 1	Serum Creatinine	SC	2.450 (1.535-3.911)	0.732 (0.674-0.785)	70.6 (60.7-79.2)	68.8 (60.9-75.9)
Model 2	Serum Creatinine	SC	1.654 (1.024-2.671)	0.805 (0.751-0.851)	77.5 (68.1-85.1)	76.4 (69.0 -82.8)
	Donor age	DA	1.051 (1.022-1.082)			
	Warm ischemia time	WIT	0.981 (0.970-0.993)			
	Diabetes	D	0.339 (0.163-0.708)			
	HLA mismatch	HLA-MM	0.260 (0.140-0.484)			
	Gender	G	0.242 (0.120-0.487)			
Model 3	3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/Serum Creatinine	HMDB0010365/SC	1.027 (1.008-1.046)	0.721 (0.662-0.774)	65.1 (55.0-74.2)	72.2 (64.5-79.0)
	O-sebacoylcarnitine/Serum Creatinine	LMFA07070100/SC	1.025 (1.005-1.045)			

Table **7**. The AUC, sensitivity, and specificity, along with their corresponding 95% confidence intervals (CI), for each predictive set of urine metabolites in the initial week post-KTx. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**AUC. 95% CL**	**Sensitivity. 95% CL**	**Specificity. 95% CL**
Model 3	PE-NMe(18:3(6Z.9Z.12Z)/14:0)/ Serum Creatinine	HMDB0113193/SC	0.663 (0.469-0.938)	0.721 (0.662-0.774)	65.1 (55.0-74.2)	72.2 (64.5-79.0)
Model 4	Donor age	DA	1.068 (1.036-1.100)	0.834 (0.783-0.877)	70.9 (61.1-79.4)	84.2 (77.5-89.5)
	O-sebacoylcarnitine/Serum Creatinine	LMFA07070100/SC	1.025 (1.002-1.048)			
	3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/ Serum Creatinine	HMDB0010365/SC	1.023 (1.002-1.0045)			
	Warm ischemia time	WIT	0.978 (0.966-0.990)			
	PE-NMe(18:3(6Z.9Z.12Z)/14:0)/ Serum Creatinine	HMDB0113193/SC	0.623 (0.42-0.907)			
	Diabetes	D	0.425 (0.198-0.910)			
	HLA mismatch	HLA-MM	0.275 (0.143-0.527)			
	Gender	G	0.244 (0.119-0.501)			

Table **7**. The AUC, sensitivity, and specificity, along with their corresponding 95% confidence intervals (CI), for each predictive set of urine metabolites in the initial week post-KTx. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL- Uppper 95% CL)**	**AUC. 95% CL**	**Sensitivity. 95% CL**	**Specificity. 95% CL**
Model 5	Log10(3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/12-Hydroxy-13-O-D-glucuronoside-octadec-9Z-enoate)	HMDB0010365/HMDB0060118	0.43 (0.26-071)	0.90 (0.857 0.934)	86.4 (78.2-92.4)	81.7 (74.7-87.3)
	Log10((2E)-Pentadecenoyl-CoA)	HMDB0062229	0.34 (0.18-0.65)			
	Log10(3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide/PE-NMe(18:3(6Z.9Z.12Z)/14:0))	HMDB0010365/HMDB0113193	3.71 (1.15-11.90)			
	Log10 (N-Succinyl-2-amino-6-ketopimelate/3b-Hydroxy-6b-(3-chloro-2-hydroxy-2-methylbutanoyloxy)-7(11)-eremophilen-12.8b-olide)	HMDB0012266/HMDB0041278	1.85E-4 (6E-06-6E-03)			

Table **7**. The AUC, sensitivity, and specificity, along with their corresponding 95% confidence intervals (CI), for each predictive set of urine metabolites in the initial week post-KTx. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

Predictive models	**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL-Uppper 95% CL)**	**AUC. 95% CL**	**Sensitivity. 95% CL**	**Specificity. 95% CL**
Model 5	Log10((3b.16a.20R)-3.16.20.22.25-Pentahydroxy-5-cucurbiten-11-one 3-[glucosyl-(1->6)-glucoside]/PE-NMe(18:3(6Z.9Z.12Z)/14:0))	HMDB0035345/HMDB0113193	5.8E-02 (5.8E-03-0.71)	0.90 (0.857 0.934)	86.4 (78.2-92.4)	81.7 (74.7-87.3)
	Log10 (N-Succinyl-2-amino-6-ketopimelate/CL (8:0/8:0/8:0/10:0))	HMDB0012266/HMDB0116798	5.3E-02 (6.5E-03-4.3E-01)			
	HLA mismatch	HLA-MM				
	Diabetes	D				
	Warm ischemia time	WIT				
	Gender	G				
	Donor age	DA				

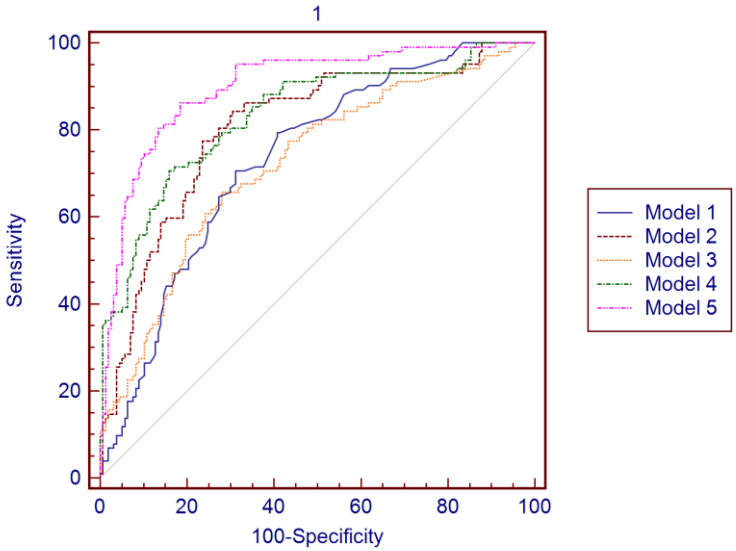

Figure 7. ROC curves, offering a comprehensive overview of the discriminative capacities inherent in logistic regression models (Model 1 to Model 5) during the first week post-kidney transplantation.

Based on the AUC values outlined in Table 3, Model 3 emerges as the most efficacious model in the third month post-kidney transplantation, boasting an AUC of 0.924 (95% Confidence interval: 0.851 to 0.968, p-value = 0.0001). Notably, this specific model surpasses all others in terms of AUC, underscoring its robust discriminatory capacity in effectively distinguishing between Group 2 and Group 1 during the third month following kidney transplantation. For a visual representation of the ROC curve corresponding to the data presented in Table 8, please refer to Figure 8.

Table 8. The AUC, sensitivity, and specificity, along with 95% confidence intervals (CI), for each predictive panel of urine metabolites at the third month. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval.

Predictive models		**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL-Uppper 95% CL)**	**AUC. 95% CI**	**Sensitivity. 95% CI**	**Specificity. 95% CI**
Model 1		Serum Creatinine	SC	31.016 (6.220-154.658)	0.788 (0.691-0.865)	55.9 (37.9 - 72.8)	88.3 (77.4 - 95.2)
Model 2		Serum Creatinine	SC	25.192 (3.544-179.080)	0.886 (0.804-0.942)	82.4 (65.5 - 93.2)	80.0 (67.7 - 89.2)
		Gender	G	21.747 (2.326-203.313)			
		HLA mismatch	HLA-MM	0.219 (0.064-0.751)			
		Diabetes	D	0.066 (0.07-0.631)			
Model 3		CL (8:0/8:0/8:0/10:0)/Serum Creatinine	HMDB0116798/SC	5.272 (2.089-13.300)	0.924 (0.851-0.968)	85.7 (69.7 - 95.1)	86.7 (75.4 - 94.0)
		L-DOPA 3'-glucoside/Serum Creatinine	HMDB0029452/SC	0.284 (0.127-0.635)			
		N-(1-Deoxy-1-fructosyl) phenylalanine/Serum Creatinine	HMDB0037846/SC	0.208 (0.074-0.585)			
		Na-Hexanoyl-Nb-inosityltryptophan/Serum Creatinine	HMDB0037036/SC	1.89E-04 (7.31E-08 -0.488)			

Table 8. The AUC, sensitivity, and specificity, along with 95% confidence intervals (CI), for each predictive panel of urine metabolites at the third month. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval. (Continued from the previous page)

Predictive models		**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL-Uppper 95% CL)**	**AUC. 95% CI**	**Sensitivity. 95% CI**	**Specificity. 95% CI**
Model 3		Tetrahexosylceramide (d18:1/18:0)/Serum Creatinine	HMDB0004961/SC	0.149 (0.034-0.653)	0.924 (0.851-0.968)	85.7 (69.7 - 95.1)	86.7 (75.4 - 94.0)
Model 4		CL (8:0/8:0/8:0/10:0)/Serum Creatinine	HMDB0116798/SC	2.636 (1.269-5.475)	0.897 (0.818-0.950)	91.4 (76.9 - 98.1)	76.7 (64.0 - 86.6)
		Donor age	DA	1.081 (1.018-1.147)			
		Tetrahexosylceramide (d18:1/18:0)/Serum Creatinine	HMDB0004961/SC	0.139 (0.030-0.640)			
		Diabetes	D	0.061 (0.006-0.617)			
		Na-Hexanoyl-Nb-inosityltryptophan /Serum Creatinine	HMDB0037036/SC	2.22E-04 (3.48E-07 -0.142)			
Model 5		Log10(CL (8:0/8:0/8:0/10:0)	HMDB0116798	3.401 (1.421-8.139)	0.910 (0.833-0.959)	80.0 (63.1 - 91.5)	91.7(81.6 - 97.2)
		Donor age	DA	1.095 (1.026-1.169)			

Table 8. The AUC, sensitivity, and specificity, along with 95% confidence intervals (CI), for each predictive panel of urine metabolites at the third month. List of variables for each model that has a p-value lower than 0.05. AUC: Area Under the Curve. Lower 95% CL: Lower 95% Confidence Interval. Upper 95% CL: Upper 95% Confidence Interval.

Predictive models		**Variables**	**Compound ID**	**Odd ratios (Lower 95% CL-Uppper 95% CL)**	**AUC. 95% CI**	**Sensitivity. 95% CI**	**Specificity. 95% CI**
Model 5		Log10(L-DOPA 3'-glucoside)	HMDB0029452	0.311 (0.156-0.618)	0.910 (0.833-0.959)	80.0 (63.1 - 91.5)	91.7(81.6 - 97.2)
		Log10(N-(1-Deoxy-1-fructosyl) phenylalanine)	HMDB0037846	0.194 (0.071-0.527)			
		Log10(Tetrahexosylceramide (d18:1/18:0))	HMDB0004961	0.097 (0.018-0.516)			

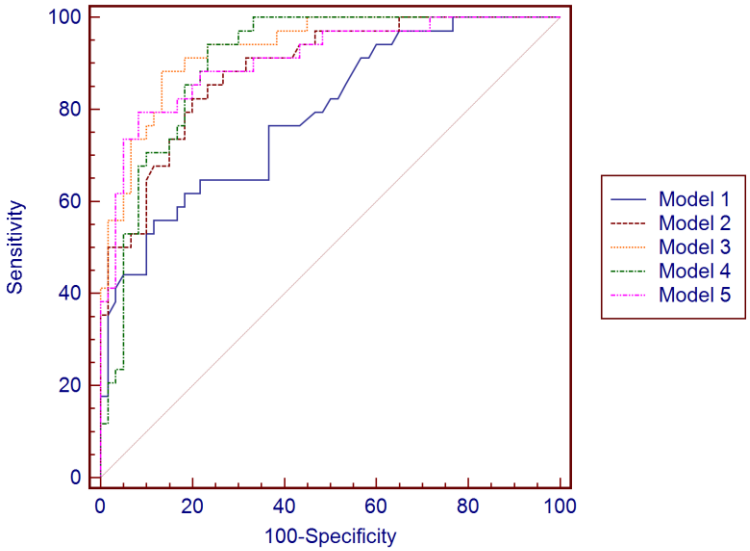

Figure 8. ROC curves, offering a comprehensive overview of the discriminative capacities inherent in logistic regression models (Model 1 to Model 5) during third month post-kidney transplantation.

Within Model 5, scrutinizing the first week post-kidney transplantation, the most impactful variable contributing to the model's efficacy was the ratio of HMDB0010365 to HMDB0113193, revealing a noteworthy odds ratio of 3.71 (95% confidence interval: 1.15-11.90) with a p-value of 0.027. Conversely, in Model 3, during the third month after transplantation, the variable exerting the most substantial influence on the model was the ratio of HMDB011679 to serum creatinine (SC), exhibiting a remarkable odds ratio of 5.272 (95% confidence interval: 2.089-13.300) and a p-value of less than 0.0001. These results underscore the statistical significance of the identified biomarker ratios in relation to the studied outcome, such as graft survival or kidney function, during the specified time frames. Remarkably, CIT was excluded from all models due to its lack of statistical significance, suggesting that it did not exhibit a notable association with the investigated outcome in any of the models at either time point.

5 DISCUSSION

Kidney transplantation is widely recognized as the optimal renal replacement therapy, offering the best combination of cost-effectiveness, successful treatment outcomes, and improved quality of life for patients. Extensive research consistently emphasizes the critical importance of kidney function within the initial year post-transplantation, considering it a pivotal determinant influencing graft survival. Prior retrospective studies have specifically highlighted the role of serum creatinine levels at both 6 and 12 months, as well as the dynamics between these time points, as crucial factors linked to graft survival. These findings suggest a robust correlation between kidney function and LTGS, thereby underscoring the enduring significance of monitoring and assessing renal function during the early post-transplantation period. [14]. The identified findings underscore the paramount importance of diligent monitoring and effective management of kidney function throughout the initial year post-transplantation, emphasizing the imperative role it plays in securing optimal outcomes for transplant recipients. This recognition emphasizes the critical need for comprehensive strategies aimed at preserving and enhancing renal function during this crucial period, ultimately contributing to the long-term success and well-being of transplant recipients. Throughout this study, an extensive surveillance was undertaken on individuals who underwent kidney transplantation (KTx) over the course of one year. Following this duration, the classification of patients was contingent upon the assessment of serum creatinine levels recorded at the 12th month post-transplantation. The categorization of patients was bifurcated into two distinct groups: Group 1, encompassing individuals with serum creatinine levels below 1.5, and Group 2, comprising those with serum creatinine levels surpassing 1.5. This division allowed for the comparison and analysis of outcomes and prognostic markers between the two groups. Presently, the monitoring of kidney transplants primarily relies on non-specific biochemical tests, such as serum creatinine, and invasive pathological examinations. However, these methods may have limitations in accurately predicting LTO in HREGL patient groups. Therefore, there is a critical need for a non-invasive approach that can provide accurate predictions of LTO with high selectivity and specificity in these patient populations. Such a method may greatly contribute to improving patient

care and decision-making in post-transplant management. Moreover, it is crucial to identify the key urinary metabolites during the early stages of graft adaptation. In this study, our objective was to explore whether models integrating potential biomarker candidates with clinical variables could function as an effective screening tool for predicting LTO in patient groups within HREGL at the earliest available time. This study stands as the inaugural endeavor within the existing literature, seeking to prognosticate LTO in patient cohorts within the HREGL category through the application of non-targeted liquid chromatography-mass spectrometry-mass spectrometry urine metabolomic analysis. In this investigation, urine samples were obtained from individuals who underwent kidney transplantation at three distinct time intervals: the initial week, third month, and sixth month post-transplantation. The metabolites within these samples were assessed for differences among distinct groups at each time interval. In the initial week post-transplantation, 19 features surfaced as the most dependable predictors distinguishing between groups, while in the third month after transplantation, 17 features were identified for the same purpose. The identified characteristics, spanning diverse metabolite classes including fatty acyls, glycerophospholipids, steroids, sphingolipids, saccharolipids, amino acids, prenol lipids, glycerolipids, and sophorolipids, provided a comprehensive insight into the metabolomic landscape. Notably, the absence of significant differences in metabolite profiles during the sixth month post-transplantation suggests a potential phase of recovery for patients during this specific time period. During the recovery phase following kidney transplant surgery, the initial metabolic alterations undergo substantial transformations, driven by the interplay of stress and the adaptation to the newly transplanted organ. This dynamic stage of recuperation is characterized by pronounced and significant metabolic disruptions, reflecting the intricate physiological adjustments occurring within the recipient's system. However, During the process of stabilization and improvement in overall health observed in patients, the initial metabolic changes encountered during the recovery phase exhibit a tendency to normalize. The body's concerted efforts to reestablish a state of homeostasis, characterized by a balanced physiological condition, become evident through the successful adaptation and stabilization of kidney function by the 6th month. This achievement suggests a potential progression towards a metabolic baseline, resulting

in a diminished number of significantly different metabolites.

The utilization of a panel of marker candidates, as opposed to relying on a single marker, within a biological system has the potential to augment the overall discriminatory capability. Each marker in the panel may contribute its own discriminating ability, and by combining multiple markers, the panel can capture different aspects of the biological system and provide a more comprehensive view of the condition or disease being studied. Utilizing a marker panel, consisting of a variety of markers, not only holds the potential to significantly improve the accuracy, sensitivity, and specificity of predictive or diagnostic models but also contributes to a comprehensive understanding of biological processes. It allows for the consideration of multiple molecular pathways or biological processes that may be involved in the disease or condition, leading to a more robust and reliable assessment (235). In this study, Stepwise Backward logistic regression was employed to select the most significant variables for inclusion in the regression models. The objective was to create models that incorporate both metabolomic variables and clinical variables, aiming to improve the accuracy of prediction or classification. In this comprehensive investigation, a total of 10 models were formulated, encompassing 5 models designed to analyze the initial week post-transplantation and an additional 5 models tailored for the third month post-transplantation. The outcomes of the study indicate that Model 5 showcased the most favorable performance during the first week after kidney transplantation, boasting an impressive AUC of 0.900 (95% Confidence interval 0.857 to 0.934, p-value = 0.0001). Likewise, in the third month post-transplantation, Model 3 emerged as the most efficacious, demonstrating a noteworthy AUC of 0.924 (95% Confidence interval 0.851 to 0.968, p-value = 0.0001). These results underscore the nuanced dynamics of predictive models across distinct time points, emphasizing the importance of tailoring models to specific post-transplantation periods for optimal efficacy. The results obtained from our study provide valuable insights into the potential biomarkers and their predictive ability for outcomes in kidney transplantation, particularly when comparing the first week and third month with the 12th month. By examining the metabolite profiles and identifying significant changes in various metabolite classes, including lipids, steroid hormones,

glycerophospholipids, sphingolipids, amino acids, and fatty acids, we were able to gain a deeper understanding of the metabolic alterations occurring during the early stages of graft adaptation. The discernment of metabolites linked to the risk of early graft loss presents prospective biomarkers, offering the potential to contribute to the prediction of patient outcomes. These biomarkers may serve as indicators of pathological processes, metabolic dysregulation, and cellular damage, which are crucial factors in determining the success or failure of kidney transplantation. Furthermore, the ability of the identified metabolites to distinguish between different time points, such as the first week and third month compared to the 12th month, highlights their potential as dynamic biomarkers that reflect the evolving metabolic changes during the early stages of graft accommodation. By incorporating these biomarkers into predictive models and considering clinical variables, our study demonstrates the potential to improve the accuracy of outcome prediction and aid in patient management and intervention. These discoveries bear significant implications for the realm of kidney transplantation, furnishing valuable t for tools risk categorization, early identification of complications, and the formulation of targeted therapeutic strategies aimed at enhancing long-term graft survival.

Donor-related factors are crucial in determining the long-term success of kidney transplantation. Differentiating between kidneys from living and deceased donors is an important classification (96), with kidneys from living donors generally showing better outcomes due to shorter ischemia times and higher organ quality. Existing literature has acknowledged that donor age constitutes a pivotal factor influencing long-term survival following kidney transplantation (97).

In our investigation, we employed various models to assess the influence of diverse variables on the outcome of kidney transplantation during both the initial week and third month. By examining the AUC values derived from our analysis, The assessment of each model's discriminative capacity between Group 2 and Group 1 revealed that, during the initial week, Model 5 exhibited the highest AUC value of 0.900 among the evaluated models, indicating its proficiency in effectively distinguishing Group 2 from Group 1 based on the studied outcome. In the initial week

following kidney transplantation, the analysis conducted by Model 5 highlighted the prominent impact of the ratio between HMDB0010365 and HMDB0113193 on the studied outcome. This specific ratio exhibited a noteworthy odds ratio of 3.71 (95% confidence interval: 1.15-11.90) and a statistically significant p-value of 0.027, underscoring its significant association with the observed outcome. In Model 5 of our binary logistic regression analysis, concentrating on the initial week post-transplantation, we examined the influence of the HMDB0010365 to HMDB0113193 ratio on kidney function outcomes. The odds ratio of 3.71 signifies that with each one-unit rise in this ratio, the likelihood of encountering kidney dysfunction in Group 2 (individuals with deteriorating kidney function) is 3.71 times greater compared to Group 1 (individuals with improving kidney function). This suggests that elevated values of the HMDB0010365 to HMDB0113193 ratio are significantly linked to an increased probability of kidney dysfunction in the early post-transplantation period. The 95% confidence interval, ranging from 1.15 to 11.90, provides a range within which we are reasonably certain the true odds ratio lies. In this case, it suggests that the true odds ratio for the HMDB0010365 to HMDB0113193 ratio is likely to fall within this interval. Furthermore, the p-value of 0.027 indicates the statistical significance of this finding. In practical terms, this means that the relationship between the HMDB0010365 to HMDB0113193 ratio and early kidney dysfunction is unlikely to be due to random chance. Instead, it suggests a genuine association between these variables. In summary, a higher HMDB0010365 to HMDB0113193 ratio in the first week after transplantation is linked to an increased risk of kidney dysfunction, as evidenced by the elevated odds ratio and its statistical significance. This finding could have clinical implications for identifying patients at higher risk of early kidney dysfunction and tailoring their post-transplant care accordingly.

In the third month, Model 3 exhibited the most noteworthy efficacy among the evaluated models after kidney transplantation, boasting an AUC of 0.924 (95% confidence interval: 0.851 to 0.968) and a p-value of 0.0001. This signifies that Model 3 possesses robust discriminatory capabilities, efficiently discerning between Group 2 and Group 1 during this specific time frame. Its elevated AUC value underscores precise predictions and dependable differentiation between the two groups in the third

month following kidney transplantation. These results underscore the potential importance of these particular biomarker ratios as predictive factors in the distinct time periods following kidney transplantation. These findings demonstrate a statistically significant connection between these biomarker ratios and the studied outcome, such as graft survival or kidney function, within the specified time frames.

The interplay among ischemia-reperfusion injury, CIT, acute rejection, and long-term graft survival in kidney transplantation constitutes a subject of ongoing research, with conflicting outcomes reported in the literature. Certain investigations have indicated that an extended duration of cold ischemia is linked to diminished long-term graft survival, implying that prolonged periods of cold ischemia might adversely affect graft outcomes (82–84). Nonetheless, other investigations have not identified a substantial correlation between Cold Ischemia Time (CIT) and the prolonged survival of grafts (85). Within our comprehensive analysis, CIT failed to demonstrate a substantial correlation with the examined outcome, leading to its exclusion from all models as it did not achieve statistical significance. This suggests that, in our study, CIT did not play a substantial role in influencing the studied outcome at either time point.

The analysis revealed that a significant number of metabolites that differed between the groups were lipids. Glycerophospholipids and sphingolipids, essential components of the phospholipid bilayer, have been linked to the onset and advancement of glomerular diseases (236). These findings suggest that the alteration of lipid metabolism may have implications for the pathogenesis of kidney-related disorders. Phosphatidylcholine, the predominant glycerophospholipid produced through the Kennedy pathway in healthy and developing kidney tissue, plays a role in the constitution of renal cell membranes. It exhibits enhanced biosynthesis in response to growth signals, representing one of the early cellular responses in kidney cells. A study illustrated a correlation between phosphatidylcholine and T cell-mediated rejection in pediatric patients, suggesting its participation in the pathophysiology of kidney rejection (237). This study accentuated the significance of the metabolite HMDB0113193, intricately involved in phosphatidylcholine biosynthesis, and

highlighted aberrant cardiolipin levels as potential indicators for early prediction, with a specific focus on high-risk patient groups. Cardiolipin, a phospholipid found predominantly in the mitochondrial inner membrane, plays a vital role in electron transport, oxidative phosphorylation, maintenance of mitochondrial membrane integrity, formation of respiratory chain complexes, cristae structure maintenance, protein translocation into mitochondria, and apoptosis regulation (238,239). The restoration of renal function after kidney transplantation has been found to have an indirect impact on cardiolipin levels (239). The findings of this study imply a temporal decline in the expression of HMDB0116798, signifying cardiolipin, from the first week to three months post-transplantation. This observation suggests that the restoration of renal function may play a pivotal role in contributing to the dynamic alterations observed in cardiolipin levels during the initial phases of kidney transplantation.

Beyond glycerophospholipids, sphingolipids, acting as integral structural constituents of plasma membranes across all eukaryotic cells, actively participate in a diverse array of cellular processes. The multifaceted roles they play underscore their critical significance in sustaining cellular structure and coordinating fundamental cellular activities. They have been acknowledged for actively contributing to the pathogenesis of numerous human diseases. Sphingolipids have surfaced as bioactive signaling molecules overseeing diverse cellular physiological functions, encompassing critical processes like cell adhesion, cell proliferation, cell migration, inflammatory response, and apoptosis (240). Disruptions in sphingolipid metabolism may result in the intracellular buildup of sphingolipids, particularly within distinct glomerular cells such as podocytes. This buildup has been linked to the development and advancement of kidney diseases (241). Disrupted sphingolipid homeostasis can contribute to podocyte dysfunction, glomerular inflammation, and the disruption of glomerular filtration barrier integrity. In-depth comprehension of the intricate role played by sphingolipids in the pathogenesis of kidney diseases not only yields valuable insights but also presents opportunities for interventions and the identification of innovative therapeutic targets in the management of renal disorders. Our study unveiled a noteworthy association between HMDB0004961, a glycosphingolipid

within the sphingolipid subclass, and an elevated risk of early graft failure in kidney transplant recipients. This observation implies that disruptions or aberrations in glycosphingolipid levels may contribute to the pathogenesis of graft failure.

These earlier studies have provided substantial evidence supporting the association between elevated levels of testosterone and androgens and an augmented susceptibility to ischemia-reperfusion injury, shedding light on the intricate interplay between hormonal factors and the physiological response to this particular type of injury (242,243). In the later phases of kidney failure, moderate to severe sexual dysfunction frequently arises. Extensive evidence indicates that decreased levels of circulating sex steroidal hormones not only associate with clinical hypogonadism but also elevate the likelihood of premature cardiovascular diseases and graft loss. Apart from hypogonadism, these hormones significantly contribute to the development and advancement of kidney failure, impacting adverse outcomes that extend beyond hypogonadism alone (244–247). Recent data indicates that normal levels of sex hormones are typically reinstated around six months following a successful kidney transplantation. This reinstatement aligns with the resolution of uremia, a condition intricately associated with kidney failure. The temporal correlation between the restoration of hormonal balance and the alleviation of uremic conditions underscores the interplay between kidney function, hormonal regulation, and overall physiological homeostasis (247–250). Playing a pivotal role, steroid hormones oversee a multitude of physiological functions, encompassing reproduction, stress management, immune system regulation, and metabolic activities (251). Among these, androgens, comprising testosterone, androstenedione, and dehydroepiandrosterone sulfate, stand out as notable contributors circulating in the systemic circulation among various steroid hormones (252). Prior research has indicated an elevated risk of ischemia-reperfusion injury associated with testosterone and androgens (242,243). Concentrations of sex steroid hormones, comprising both androgens and estrogens, have been intricately linked to the progressive deterioration of renal function and heightened indications of kidney damage. This complex interplay between sex steroid hormones and renal health underscores the need for comprehensive investigations to elucidate the underlying mechanisms. Additionally, steroids have become a focal point

in research, not only due to their association with kidney damage but also regarding their potential implications in maintenance therapy for kidney transplants. Delving into the dual facets of sex steroid hormones, their impact on renal health, and the therapeutic potential of steroids in transplantation, contributes to a holistic understanding of their role in kidney-related conditions (86,253,254). In our investigation, steroid hormones were integrated as a lipid type in our model, emphasizing their heightened sensitivity and specificity in discriminating patients in Group 2. The study identified HMDB0000458, HMDB0010365, HMDB0010351, HMDB0035345, and HMDB0034205 as noteworthy steroid hormone metabolites with the capacity to differentiate between groups during the initial week after kidney transplantation. These research findings not only signify a noticeable rise in the expression levels of HMDB0010365 and HMDB0000458 metabolites but also suggest a dynamic temporal shift in their abundance, emphasizing the distinguishable increase in metabolic alterations occurring during the transition from the first week to the third month post-transplantation. The observed changes in these metabolites' levels over this specific time frame may hold valuable insights into the evolving metabolic landscape and potential implications for post-transplantation outcomes. Nonetheless, no substantial alterations were noted in the metabolite levels at the six-month time point. These findings imply that the early post-transplantation period induces changes in the expression of these specific metabolites, which may subsequently stabilize or revert to baseline levels by the sixth month. This points towards a significant impact on the biosynthesis of steroid hormones in patient groups at HREGL. The investigation suggests that steroid hormones could function as viable candidates for the early prediction of LTO in patient groups with HREGL. The incorporation of steroid hormones into the model underscores their significance as crucial metabolite structures in forecasting LTO within patient groups exhibiting HREGL. Moreover, our results indicate the potential candidacy of HMDB0060118, a saccharolipid within its designated class, for inclusion in a metabolic biomarker model. This implies that saccharolipids, in conjunction with other lipid types within the model, might contribute to the progression of early graft loss. The significance of these findings extends beyond the scrutiny of membrane-related lipids to include steroid hormones and saccharolipids, emphasizing their potential utility as biomarkers for predicting long-

term outcomes in patient groups facing the heightened risks associated with end-stage renal disease.

Our observations revealed significant changes in the expression patterns of fatty acids and amino acids, exerting a notable impact on the group predisposed to early graft loss. Specifically, variations in amino acids, including HMDB0012266, HMDB0029138, and HMDB0029452, were identified, alongside alterations in lipid profiles. Previous research has emphasized the correlation between disruptions in lysine synthesis and the onset of acute rejection in recipients of renal transplants, as well as the occurrence of post-transplant diabetes mellitus (255,256). The metabolite HMDB0012266, identified as an intermediate product in lysine synthesis (260), demonstrated higher levels in Group 2 relative to Group 1. The noted elevation in HMDB0012266 levels implies a potential correlation with an augmented risk of early graft loss, underscoring its candidacy as a predictive biomarker for anticipating such risks in kidney transplant recipients. Fatty acid metabolism disruptions, implicated in conditions such as CKD and kidney transplantation, emerge as contributing factors fostering not only cellular damage but also mitochondrial dysfunction (257–259). This intricate interplay within the metabolic landscape underscores the pivotal role of long-chain acyl-CoA, intricately involved in fatty acid metabolism, membrane modifications, and diverse physiological processes, as a significant player linked to these multifaceted metabolic disturbances (260). The outcomes of our investigation emphasize the significance of HMDB0062229 and HMDB0012266 as biomarkers associated with the risk of early graft loss within the first week following kidney transplantation. Classified under the classes of fatty acids and amino acids, these metabolites provide valuable insights into the dynamic metabolic shifts during the early stages of graft adaptation, presenting themselves as promising candidates for predictive markers that can aid in identifying patients susceptible to unfavorable outcomes.

Limitations are encountered in the study, particularly concerning the relatively small sample size. It is essential to employ a focused LC-MS approach within a more extensive longitudinal investigation to establish the predictive efficacy for long-term

patient outcomes at HREGL. It is imperative to extend this research to improve the clinical utility of these biomarkers in the management of kidney transplant patients, providing more dependable insights and ultimately enhancing patient outcomes. Another constraint in our study arises from the possible introduction of variability into the metabolomic data owing to the lack of standardized dietary conditions or fasting prerequisites during sample collection. This aspect may affect the accuracy and comprehensibility of our findings. Additionally, although our study sought to elucidate metabolomic profiles primarily from a human standpoint, it is essential to recognize the difficulties in precisely determining whether metabolites originate from human or microbial sources. Despite the sophistication of current methodologies, they may not entirely distinguish between these two origins. Our samples mostly derived from human subjects, and the study did not explicitly investigate microbial contributions. Further research is necessary to ascertain the source of these metabolites, whether human or microbial.

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6. CONCLUSION

In summary, our approach involved the development of ten models through a backward conditional method, wherein Model 5 showcased remarkable performance for Group 2 during the first week, boasting an AUC of 0.900. Similarly, Model 3 excelled at the third month with an AUC of 0.924. These findings underscore the considerable potential of our models in effectively predicting early LTO in individuals facing the heightened risk of premature graft loss. Our study may illuminate the metabolic alterations linked to early graft loss in kidney transplantation, providing valuable insights into the underlying mechanisms. These findings offer potential for guiding targeted interventions and therapeutic strategies designed to improve outcomes for kidney transplant recipients. The incorporation of these identified biomarkers into predictive models not only holds promise for augmenting risk stratification but also provides valuable insights that can guide targeted interventions aimed at optimizing graft survival, especially in patient populations characterized by heightened risk factors. However, it is essential to underscore that further research and validation in larger patient cohorts are necessary to confirm the utility of these biomarkers and investigate their practical implementation in clinical practice.

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9 CURRICULUM VITAE