METHODS and PROTOCOLS

Metabolomics Analysis

Plasma samples stored at -80 °C were thawed on ice, followed by adding 800 µL methanol:distilled water (9:1, v/v) and vortexed for 1 minute. The samples were then centrifuged at 15000 rpm for 10 minutes. From the supernatant, 250 µL aliquots were taken for both GC-MS and LC-qTOF-MS analyses and transferred into 1.5 mL Eppendorf tubes. Additionally, 100 µL from each sample was pooled to prepare quality control (QC) samples, and 250 µL aliquots from the QC pool were transferred into 1.5 mL Eppendorf tubes. All samples were dried in a vacuum centrifuge (Christ RVC 2-18 CD plus, Osterode am Harz, Germany). Urine samples stored at -80 °C were thawed on ice, followed by adding 100 µL urease (15 Units) and incubated at 37°C for 4 hours. Subsequently, 800 µL methanol/distilled water (9:1, v/v) was added and vortexed for 1 minute. Urine samples were prepared in the same manner as plasma samples and dried in a vacuum centrifuge along with QC samples.

GC-MS based metabolomics analysis

The dried samples were derivatized by adding 20 µL of methoxyamine hydrochloride solution, vortexed for 1 minute, and incubated at 30°C for 90 minutes. The samples were brought to room temperature and then 50 µL of N-methyl-N-trimethylsilyl trifluoroacetamide + trimethylchlorosilane (MSTFA + 1% TMCS) solution was added and vortexed for 1 minute, followed by incubation at 37°C for 30 minutes. The derivatized samples were analyzed under optimized conditions using a DB-5MS stationary phase column (30 m + 10 m duraguard x 0.25 mm i.d., 0.25 µm film thickness). The temperature program for the oven was configured as follows, with a total runtime of 37.5 minutes: initially maintained at 60 °C for 1 minute, followed by a ramp-up to 325 °C at a rate of 10 °C per minute, and then held at this temperature for 10 minutes. The injection volume was adjusted to 1 µL in splitless mode. Mass spectra were recorded for ions ranging from 50 to 650 m/z with an event time of 0.30 seconds. High-purity helium (>99.999%) was used as the carrier gas at a flow rate of 0.99 mL/min. The purge flow was set to 5 mL/min, and the injector temperature was maintained at 250 °C. The mass spectrometry (MS) parameters were specified as follows: the ion source temperature was set to 230 °C, the interface temperature was adjusted to 290 °C, and the solvent delay time was 5.91 minutes. Mass scanning was performed in the 50–650 m/z range with an event time of 0.30 seconds using GC–MS Solution (version 4.20). The derivatized samples were analyzed using a

GC-MS system (GC-MS QP-2010 Ultra, Shimadzu, Japan) equipped with a DB-5MS column (30 m + 10 m DuraGuard × 0.25 mm i.d., 0.25 µm film thickness) under the optimized conditions specified in **Table 1.**

Table 1. GC-MS conditions for metabolomics analysis

PARAMETER	VALUE
Oven Temperature Program	Temperature increase from 60 °C (held for 1 minute) to 325 °C at 10 °C/min (held for 10 min)
Analysis Time	37.5 minutes
Injection Volume	1 µL (splitless)
Carrier Gas	Helium at 0.99 mL/min
Msd Transfer Line Temperature	290 °C
Solvent Delay Time	5.91 minutes
Mass Range	50-650 Dalton

LC-qTOF-MS based metabolomics analysis

For LC-qTOF-MS based metabolomic profiling, dried samples were reconstituted in 200 µL of 0.1% formic acid (FA) in acetonitrile and water, vortexed for 1 minute, and centrifuged at 10,000 rpm for 10 minutes. A total of 175 µL of supernatant was transferred into vials and injected into the LC-qTOF-MS system (Agilent 6530, Agilent Technologies, USA). Chromatographic separation was performed using a C18 column (1.0 × 150 mm, 2.7 µm) under gradient elution conditions, as detailed in **Table 2**. The mobile phases consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The flow rate was maintained at 0.15 mL/min. The gradient initiated with 10% mobile phase B, maintained for 1 minute, then progressively increased to 90% by 1-14 minutes and was held between 14-15 minutes. This was followed by a decrease from 90% to 10% by 20 minutes and maintained at 10% until 25 minutes to allow for column re-equilibration. The column temperature was controlled at 60°C throughout the procedure. QC samples, prepared by pooling all individual samples, were analyzed with different collision energies (10, 20, and 40 eV) in targeted MS/MS mode to facilitate peak identification. The qTOF-MS parameters included the following settings: gas temperature at 300°C; nebulizer pressure at 35 psi; drying gas flow rate at 8 L/min; VCap voltage at 3500 V; fragmenter voltage at 175 V; and skimmer voltage at 65 V. Further instrument details are provided in **Table 3**.

Table 2. Gradient elution program

TİME (MİN)	% MOBİLE PHASE B
0	10
1	10
14	90
15	90
20	10
25	10

*% 0.1 FA İN ACETONİTRİLE

Table 3. LC-qTOF-MS instrument parameters.

Parameter	Positive Ionization	Negative Ionization
Mass range (amu)	50-650	50-650
Scan speed (spectrum/s)	2	2
Spray voltage (kV)	3500	3500
Skimmer voltage (V)	65	65
Gas temperature (°C)	300	300
Gas flow rate (L/min)	8	8
Nebulizer (psi)	35	35