ONLINE-ONLY SUPPLEMENTAL MATERIAL

Supplemental Materials and Methods

Supplemental Table 1: Details of participants who withdrew or were withdrawn from the study.

Supplemental Figure 1: Box and whisker plot of the slope estimates of individual serum losartan (A), EXP3174 (B), and EXP3179 (C) concentrations versus study visit. Panel D shows example chromatograms of losartan and its metabolites with different oral losartan doses.

Supplemental Table 2: Average knee strength by group and visit.

Supplemental Table 3: Correlations between energy metabolites and serum losartan, EXP3174, and EXP3179 and its metabolites.

Supplemental Figure 2: Heatmap showing all glycolysis, TCA cycle, and amino acid metabolism metabolites detected in patient serum samples.

Supplemental Figure 3: Effects of losartan doses on γ-aminobutyric acid biosynthetic pathway (GABA) metabolites.

Supplemental Materials and Methods

Participant Recruitment

Participants were included if they were age 70 and older and classified as prefrail based on the physical frailty phenotype (PFP)[1]. Participants were recruited from both a Johns Hopkins University (JHU) Institutional Review Board (IRB)-approved registry of older adults willing to participate in research studies and the community through newspaper advertisements. The study was approved by the JHU IRB with approval #NA_00078435 and registered at ClinicalTrials.gov #NCT01989793. All potential participants underwent initial phone screening, and those who qualified were provided with a consent form to review before their in-person screening visit. Potential participants were excluded if they had unstable cardiovascular disease, the current use of ARBs or angiotensin-converting enzyme (ACE) inhibitors, a prior allergic reaction to or hyperkalemia while taking any ARB, chronic renal failure with a glomerular filtration rate of < 30 mL/min/1.73 m^2, the daily use of nonsteroidal anti-inflammatory agents, the use of steroids, lower-extremity disability that would prevent muscle strength testing, echocardiogram-diagnosed cardiac failure as evidenced by a left ventricular ejection fraction less than 50%, cognitive impairment based on a Mini-Mental State Examination score < 24, or a blood pressure level consistently less than 110/70 mmHg.

Study Design

Study Visits

The study visits took place in the Institute for Clinical and Translational Research (ICTR) clinical research unit (CRU) on the Johns Hopkins Bayview Medical Center campus. There were four in-person CRU visits every eight weeks and six phone calls between visits to monitor safety and compliance. Participants who gave their informed consent attended a screening visit, during which their prefrail status was confirmed based on grip strength and walking speed assessments and self-reported physical activity/fatigue/weight loss. A study physician obtained a medical history and performed a physical assessment. Participants underwent an echocardiogram, baseline phenotypic measurements of lower-extremity peak torque, 6-minute walk test, and blood collection for molecular measurements. Once they were deemed eligible to enroll, participants were randomized to either the control group or the treatment group with a computerized randomization program. Subjects were started on 25 mg tablets (or the placebo) and were instructed to take the pills every morning. Every eight weeks, those in the losartan treatment arm were given an increased dose of losartan, from 25 mg to 50 mg to 100 mg. Matching placebo pills were also given out at these intervals to those in the control group. Validation of compliance was achieved by asking participants about their pill (placebo or losartan) intake. Patients were evaluable if they received 100% of the planned treatment dose. For those in the losartan group, noncompliance was confirmed by a lack of detectable serum losartan levels. Participants were monitored for potential side effects or symptoms listed in the exclusion criteria, such as hypotension, falls with fracture, hyperkalemia, and elevated creatinine.

Physical Measures

The primary outcome, isokinetic strength, was measured as the bilateral knee concentric strength using a Biodex System 3 dynamometer set at an angular velocity of 30 deg/sec through a joint arc from 90 degrees to 30 degrees (0 degrees = full extension) as previously described(24). Five trials were performed, separated by 60-second intervals. Peak torque was defined as the highest muscular force output during repetition and was expressed in Newtons (ft-lbs), adjusted for body weight, and defined as the average of the three middle trials. Right and left knee averages were further averaged to have one measurement per patient per visit.

Frailty, which was the secondary outcome, was measured using the PFP criteria of unintentional weight loss, weakness, poor endurance, slowness, and low physical activity (1;25). Weight loss was considered any unintentional weight loss >5% of the participant's body weight over the last year or a body mass index (BMI) less than 18.5 kg/m^2. Weakness was defined as a grip strength at or below the sex- and body mass index-adjusted cutoff points established in the Cardiovascular Health Study (CHS) (1). Poor endurance was measured by self-reported exhaustion questions derived from the Women's Health and Aging Studies (WHAS) (25). Slowness was based on the time to walk 4 meters and was defined by the CHS and WHAS cutoff points adjusted for sex and standing height (1;25). Low physical activity level was calculated from a weighted score of kilocalories expended per week based on a self-report on an abbreviated Minnesota Leisure Time Activities Questionnaire (MLTAQ) using the established cutoff points for each sex (10;25). Those meeting none of the five criteria were categorized as robust, those with one or two criteria were categorized as prefrail, and those with three or more criteria were considered frail. In this study, only older individuals who were prefrail at baseline were included.

Phlebotomy and Blood Processing

Blood collection was also performed in the morning on the day of each visit before the losartan dose was increased or the placebo was continued. Blood was drawn from each participant into serum separator tubes, which were inverted five times, allowed to clot for 30 minutes, and centrifuged at 1,100 xg for 15 minutes in a fixed-angle rotor. Serum aliquots were then transferred to cryovials and stored at -80°C until the lab assays were performed. The number of freeze-thaw cycles was limited to ≤ 3.

Laboratory Analyses

Blood Chemistry and Serum Cytokines

Comprehensive metabolic panels were analyzed by the JHU Bayview Hospital Clinical Laboratory. Serum cytokines [interleukin-6 (IL6), tumor necrosis factor α (TNFα), tumor necrosis factor α receptor 1 (TNFαR1), and interleukin-1β (IL1β)] were assayed using a quantitative sandwich ELISA (Mesoscale Diagnostics, Rockville, MD, USA) following the manufacturer's protocol. The performance characteristics of the cytokine immunoassay included a lower limit of detection (LLOD) of 0.09 pg/ml and a lower limit of quantitation (LLOQ) of 0.30 pg/ml. These measures were performed in the JHU Institute for Clinical and Translational Research (ICTR) Core Laboratory.

Losartan, EXP3174, and EXP3179

Samples from the losartan treatment group were analyzed for losartan, EXP3174, and EXP3179 by ultra-performance liquid chromatography (LC/MS/MS). The analytes were extracted from the serum by protein precipitation using acetonitrile containing an internal standard (thioridazine). Chromatographic separation was achieved with an Agilent Zorbax-XDB C18 analytical (no guard) column (4.6 × 50 mm, 3.5 µm) with a water/acetonitrile/formic acid mobile phase (30:70:0.1, v:v) using isocratic flow at 0.7 mL/minute for a total of 3 minutes. The column effluent was monitored using a Sciex 5500 QTrap mass spectrometer with electrospray ionization operating in positive mode. The mass spectrometer was programmed to monitor the following MRM transitions: 437.1>207.2 for EXP3174; 421.1>207.1 for EXP 3179; 423.1>207.2 for losartan; and 371.0>126.0 for thioridazine. The calibration curves were computed using the area ratio peak of the analysis to the internal standard based on a quadratic equation with a $1/x^2$ weighting function over the range of 5 – 2,000 ng/ml for losartan, 25 – 2,000 ng/mL for EXP3174, and 0.1 – 200 ng/mL for EXP3179.

Metabolomics

Metabolites were extracted, and their concentrations in the losartan treatment group were obtained using the AbsoluteIDQ kit p180 (Biocrates Life Science AG, Austria) following the manufacturer's protocol for the API5500 LC/MS/MS System (AB SCIEX, USA) running with Analyst 1.5.2 software and equipped with an electrospray ionization source, a Shimadzu CBM-20A command module, LC-20AB pump, Shimadzu SIL-20AC-HT autosampler and a CTO-10Ac column oven heater as previously described (26). Briefly, 10 µl of plasma-EDTA samples were pipetted onto the center of the spots in each well of a 96-well Biocrates kit. The samples were dried with a Microvap 118 from Organomation Associate nitrogen evaporator (Berlin, MA) at room temperature (RT) for 30 min. Fifty microliters of 5% PITC reagent was added and incubated for 20 min, and the plate was dried under nitrogen for 1 hour. Then, 300 µl of 5 mM ammonium acetate in methanol was added to each well and incubated at RT on a shaker (450 rpm) for 30 min. The plate was then centrifuged at 100 xg for 2 min, resulting in approximately 350 µl of sample extract in the capture plate. Fifty microliters of each sample was transferred to an empty 96 deep-well plate. The extracts were diluted for LC by adding 450 µl of 40% methanol (in HPLC-grade water) to each well. Then, the extracts were diluted for FIA by adding 490 µl of FIA running solvent (Biocrates solvent diluted with HPLC grade methanol). The LCMS plate was run first, with 10 µl injected onto the Eclipse XDB C18, 3.5 µm, 3.0x100 mm with a Phenomenex C18 Security Guard Cartridge, 3.0 mm ID. The mobile phase consisted of solvent A (water containing 0.2% formic acid) and solvent B (acetonitrile containing 0.2% formic acid), with the following gradient: 0-0.5 min: 0% B, 5.5 min: 95% B; 6.5 min: 95% B; 7.0 min: 0% B; and 9.5 min: 0% B. Evaluation of the samples was carried out using MetIDQ software. The FIA plate was run with 20 µl injection directly into the MS at a flow of 30 µl/min with water/acetonitrile (1:1) containing 0.2% formic acid as the mobile phase, with the following flow rate program: 0-1.6 min: 30 µl/min; 2.4 min: 200 µl/min; 2.8 min: 200 µl/min and 3.00 min: 30 µl/min. Concentrations were calculated using Analyst/MetIDQ software. PITC, ammonium acetate, water, methanol, and acetonitrile (LC-MS grade) were purchased from Sigma Aldrich. Of the 186 preconfigured metabolites and ratios in the kit, 121 metabolites were quantifiable in our serum samples.

TCA cycle and glycolysis metabolites were extracted from the serum samples using 80% (vol/vol) mass spectrometry grade methanol. The supernatant underwent Speed-vac to remove the methanol. Lyophilization was then performed to remove the water. Then metabolites were then re-suspended in 50% (vol/vol) acetonitrile diluted with mass spectrometry grade water. Data acquisition from the samples was performed using a Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer and a Vanquish UPLC system. The Vanquish UPLC autosampler withdrew 2 uL of each sample maintained at 4° C. The reverse-phase chromatography used had the mobile aqueous phase of 0.1% formic acid in MS grade water and the mobile organic phase of 0.1% formic acid in acetonitrile. The column used was a Discovery® HSF5 reverse phase HPLC column (Sigma), which was kept at 35° C together with a column guard. The total data acquisition time was 11 minutes. Acquired data were analyzed using Thermo Scientific Compound Discoverer® and TraceFinder® software. The intensities of each metabolite were obtained by integrating chromatographic peaks. Fold change was calculated by dividing the intensities by the average intensity of the baseline group for each metabolite.

Statistical Analysis

Participant characteristics, baseline chemistry lab values, physical measurements, and inflammatory lab values were summarized stratified by study arm using means and standard deviations (SD) or medians and interquartile ranges (IQR) for continuous variables and frequencies for categorical variables. Thirty-seven patients were recruited for the study. 25 subjects completed all study visits and included 10 on treatment and 15 who received placebo.

Study Arm Comparisons

Population-average marginal generalized linear models were fit to assess differences between the treatment arms for the primary (isokinetic strength) and secondary (frailty) outcomes as well as walking speed, the 6-minute walk test (m), and dominant-hand grip strength (kg). The change from baseline at each visit was used as the dependent variable. The independent variables were baseline outcome level, study arm, visit and visit by arm interaction to evaluate whether the change in outcome by arm differed by visit. If the interaction term was not statistically significant at the 0.05 level, the final model included only the study arm, baseline outcome, and visit. Additional adjustments were performed for age and sex. The models were estimated using generalized estimating equations (GEEs) with an exchangeable working correlation structure.

Frailty status was dichotomized as frail, represented in these analyses by a score of 3 or more, or not frail/prefrail, which were defined as a score of 0 or 1-2, respectively. To compare frailty status by arm, the GEE model described above was extended to include binomial family and logit links to estimate the odds ratios for frailty in the losartan and placebo groups, with adjustment for visit.

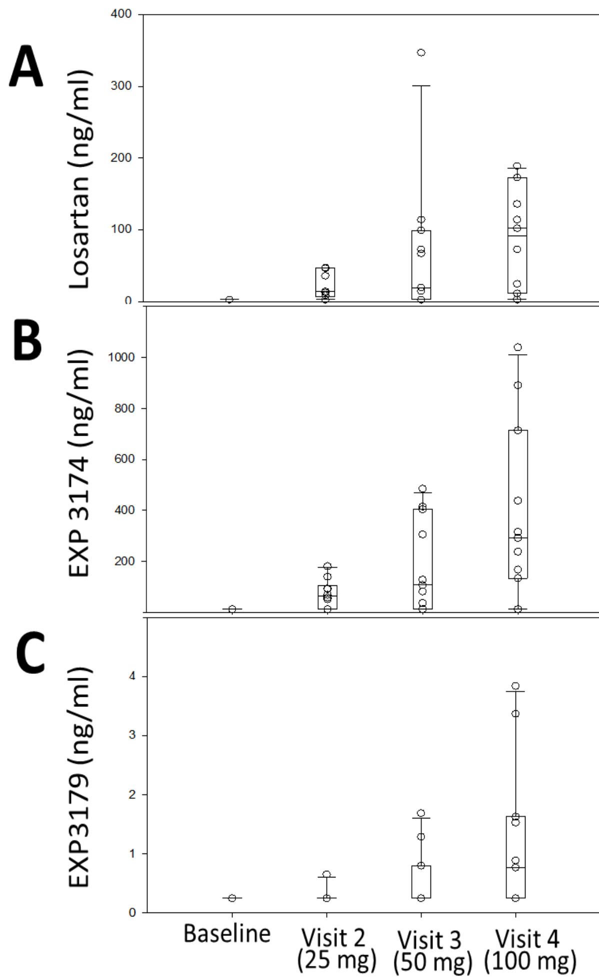
Losartan Group Analyses

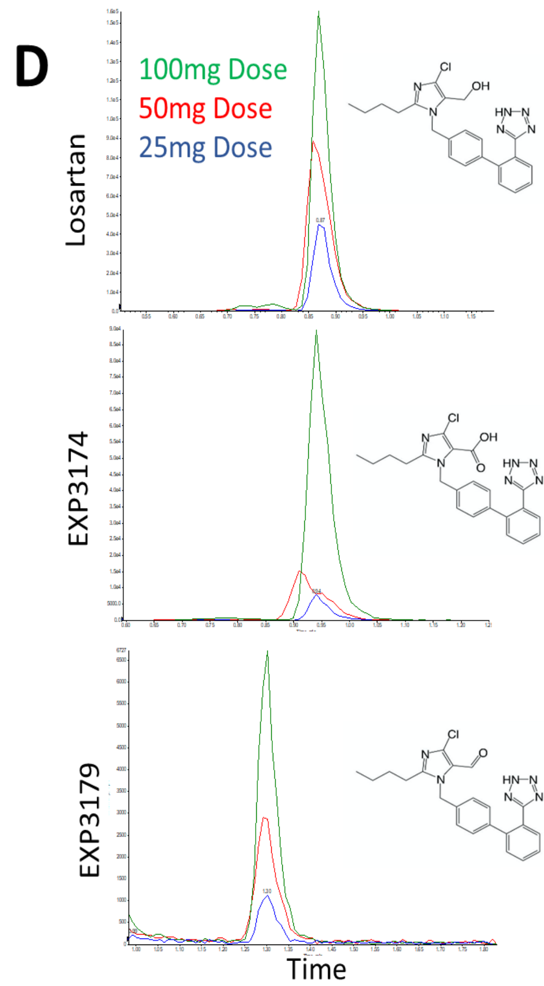
We assessed the changes in the outcomes according to losartan dose in the treatment arm and the correlations of losartan and its metabolite concentrations with the outcomes. These analyses should be considered exploratory; therefore, no multiplicity adjustments were used.

To assess the changes in the outcomes with an increase in the losartan dose, population-average marginal generalized linear models with logarithmic links and robust variance were used with visit as the predictor to estimate the percent changes in outcomes. In the results section, we report the changes observed at the end of the study (visit 4: 100 mg). These models for the active arm were refit to include losartan or its serum metabolite concentration levels and visit to assess the associations of physical measures, serum cytokines, oxidative stress markers, targeted metabolomics outcomes with the concentrations of losartan and its metabolites while controlling for the dose/visit. The level of statistical significance was at the 0.05 level. The analysis was performed using SAS (*Statistical Analysis* Software *9.4*, SAS Institute Inc, Cary, *North Carolina*, USA) and STATA 15 statistical software (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC.).

Placebo Control Group	**Losartan Treatment Group**
Participant Withdrew	Participant Withdrew
1 – Serious adverse event (myocardial infarction) not related to the study	2 – Adverse events (fatigue)
1 – Needed to restart ACEI	2 – Missed appointments/stopped communicating
1 – Lacked transportation	1 – Lacked transportation
1 – Daily medication for 6 months was too burdensome	1 – Daily medication for 6 months was too burdensome
	Participant Withdrawn by Investigators
	1 – Increased creatinine with hyperkalemia
	1 – Blood pressure consistently <110/70

Supplemental Table 1: Details of participants who withdrew or were withdrawn from the study.





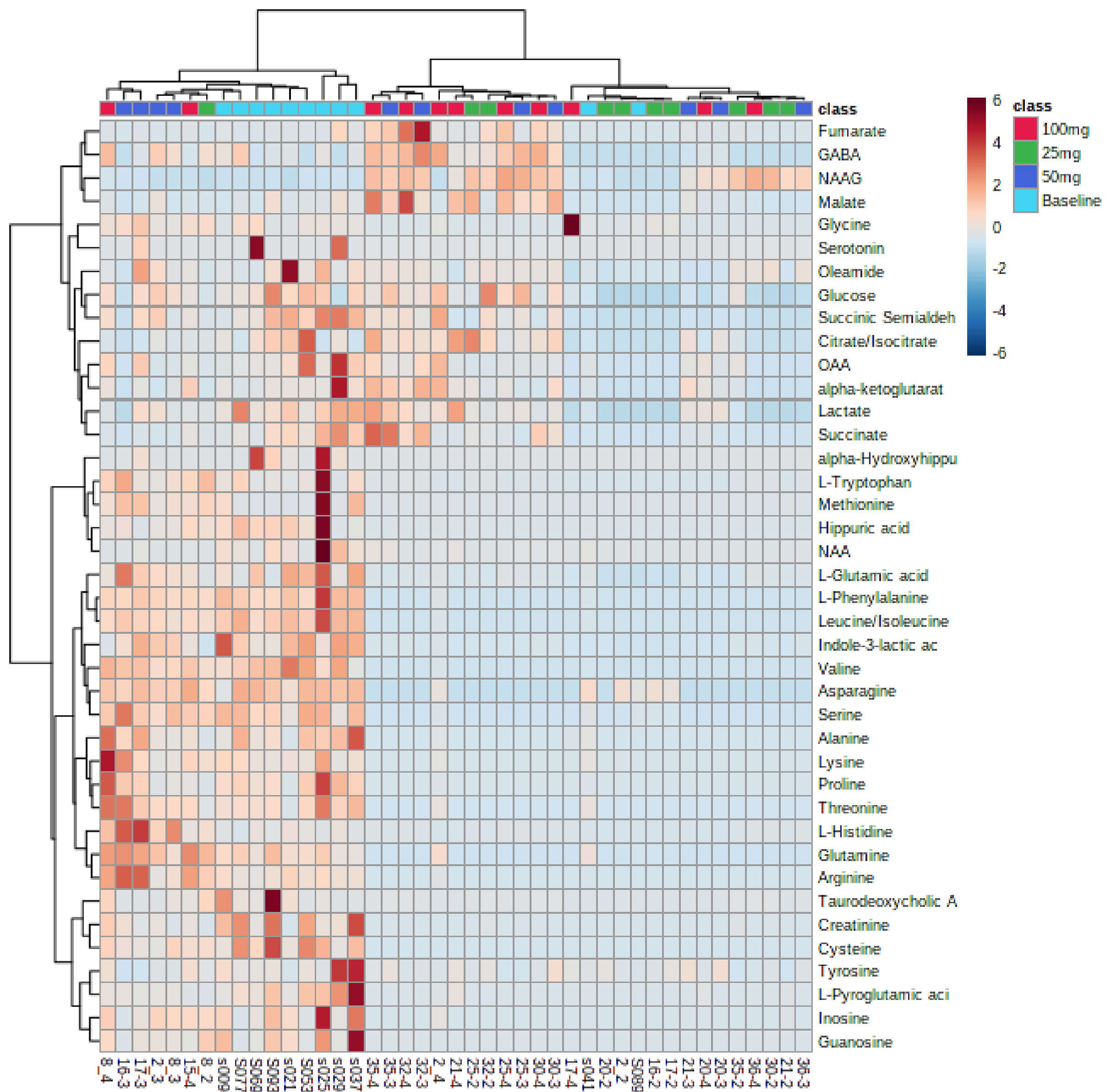
Supplemental Figure 1: Box and whisker plot of the slope estimates of individual serum losartan (A), EXP3174 (B), and EXP3179 (C) concentrations versus study visit. The box and whiskers represent the 10th, 25th, 50th, 75th, and 90th percentiles of the data. Samples that were below the limits of quantitation were plotted as one-half of that value [losartan 2.5 ng/mL (A), 12.5 ng/mL EXP3174 (B), and 0.05 ng/mL EXP3179 (C)]. Panel D shows example chromatograms of losartan and its metabolites with different oral losartan doses.

	Study Visit			
	1	**2**	**3**	**4**
Study Arm (N)	Mean (SD)			
Losartan (10)*	74.6 (34.7)	79.3 (32.0)	69.1 (32.7)	67.3 (30.9)
Placebo (15)**	66.8 (25.3)	62.6 (25.3)	62.7 (23.5)	63.6 (26.1)

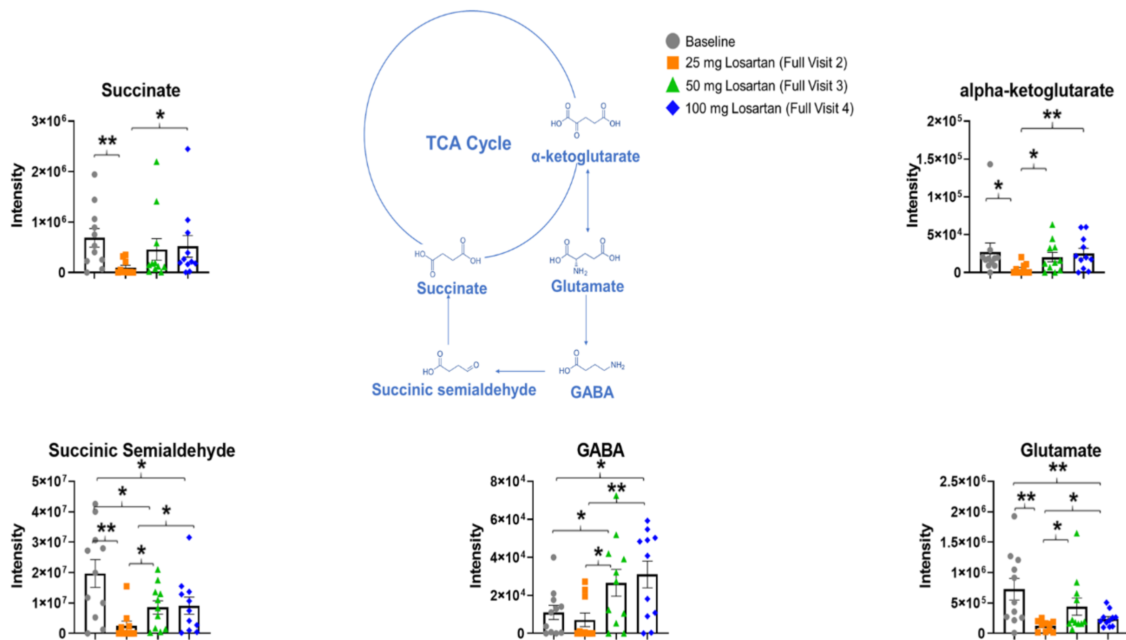
Supplemental Table 2: Average knee strength by group and visit. *two patients missed visit 2 in losartan group, **one patient missed visit 4 in placebo group

Energy Metabolites	Losartan	P-value	EXP3174	P-value	EXP3179	P-value
N-Acetyl Aspartate	-	-	↓	**0.05**	-	-
Asparagine	↓	**0.05**	↓	**0.05**	↓	**0.05**
Cysteine	↓	**0.05**	↓	**0.05**	↓	**0.05**
Leucine/Isoleucine	↓	**0.001**	↓	**0.001**	↓	**0.001**
Serine	↓	**0.05**	↓	**0.05**	↓	**0.05**
Valine	↓	**0.01**	↓	**0.05**	↓	**0.01**
Hippuric acid	↓	**0.05**	-	-	-	-
Indole-3-Lactic acid	↓	**0.01**	↓	**0.05**	↓	**0.01**
L-Phenylalanine	↓	**0.01**	↓	**0.01**	↓	**0.01**
L-Pyroglutamic acid	↓	**0.05**	-	-	-	-
L-Glutamic acid	-	-	-	-	↓	**0.05**
N-Acetyl-Aspartyl-Glutamate	↑	**0.001**	↑	**0.001**	↑	**0.001**
Malate	-	-	-	-	↑	**0.05**

Supplemental Table 3: Correlations between energy metabolites and serum losartan, EXP3174, and EXP3179 and its metabolites. Upward arrows indicate positive correlations, and downward arrows indicate negative correlations.



Supplemental Figure 2: Heatmap showing all glycolysis, TCA cycle, and amino acid metabolism metabolites detected in patient serum samples.



Supplemental Figure 3: Effects of losartan doses on γ-aminobutyric acid biosynthetic pathway (GABA) metabolites. Comparisons of the distributions of metabolites by losartan dose in the treatment group were conducted using Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test. *$p<0.05$, **$p<0.01$, ***$p<0.005$