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Title: Metabolic Shifts in Plasma Amino Acids and Related Metabolites in Response to SGLT2 Inhibition and Hyperglycemia in Type 1 Diabetes

Authors: Luxcia Kugathasan
Nagarjunachary Ragi
Subrata Debnath
Vikas S Sridhar
Soumya Maity
Tianqing Feng
Esmeralda Trevino
Bruce Perkins
David ZI Cherney
Kumar Sharma

Running Title: SGLT2 inhibition and hyperglycemia on plasma metabolites

Abstract: Objective: Regulated kidney function is dependent on maintaining efficient energy utilization. Our aim in this study was to determine the effects of acute, ambient hyperglycemia and sodium-glucose cotransporter-2 (SGLT2) inhibition on plasma amino acid metabolism in patients with type 1 diabetes (T1D). Methods: The ATIRMA trial, a single-arm study, evaluated the effects of 8 weeks of oral empagliflozin (25 mg/day) in 40 young adults with T1D. The study involved consecutive two-

day assessments of clamped euglycemia and hyperglycemia at both baseline and post-treatment. MetaboAnalyst 6.0 categorized 35 metabolites into significant pathways, which were statistically compared using principal component analysis. Findings: Acute hyperglycemia induced changes to ten metabolic pathways, including but not limited to, increases in cysteine and methionine metabolism (0.52{plus minus}0.12, p<0.0001), valine, leucine and isoleucine biosynthesis (0.31{plus minus}0.10, p=0.002); and nitrogen metabolism (0.11{plus minus}0.03, p=0.003). Introduction of empagliflozin was associated with a decrease in adenine, and increase in cysteine and methionine metabolism (0.31{plus minus}0.13, p=0.02) when maintained under euglycemia and a decrease in nitrogen metabolism under hyperglycemia (-0.07{plus minus}0.04, p=0.04). Conclusion: Our findings show that SGLT2 inhibition counteracts the hyperglycemia-induced changes in plasma amino acid metabolism, potentially improving energy efficiency and metabolic health, though more research is needed to confirm these metabolic effects.

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Metabolic Shifts in Plasma Amino Acids and Related Metabolites in Response to SGLT2 Inhibition and Hyperglycemia in Type 1 Diabetes

Luxcia Kugathasan BSc[1,2,3*], Nagarjunachary Ragi PhD[4,5*], Subrata Debnath MBBS PhD[4,5], Vikas S. Sridhar MD[6], Soumya Maity PhD[4,5], Tianqing Feng BSc[4,5], Esmeralda Treviño BSc[4,5], Bruce A. Perkins MD MPH[7,8], David Z.I. Cherney MD PhD[1,2,3†], Kumar Sharma MD[4,5†]

Affiliations:

[1]Department of Medicine, Division of Nephrology, University Health Network, Toronto, Ontario, Canada
[2]Department of Physiology, University of Toronto, Toronto, Ontario, Canada
[3]Cardiovascular Sciences Collaborative Specialization, University of Toronto, Toronto, Canada
[4]Center for Precision Medicine, University of Texas Health San Antonio, Texas, USA
[5]Division of Nephrology, Department of Medicine, University of Texas Health San Antonio, Texas, USA
[6]Division of Nephrology, University of British Columbia, Vancouver, British Columbia, Canada
[7]Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, ON, Canada
[8]Division of Endocrinology and Metabolism, Department of Medicine, University of Toronto, Toronto, Ontario, Canada.

*Co-first authors
[†]Co-senior authors

Corresponding author:

Luxcia Kugathasan, BSc
University Health Network
585 University Avenue, 1N128
Toronto, ON M5G 2N2, Canada
Telephone: +1 (416) 340 4800 ext. 8508
Email: luxcia.kugathasan@uhn.ca

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ABSTRACT

Objective: Regulated kidney function is dependent on maintaining efficient energy utilization. Our aim in this study was to determine the effects of acute, ambient hyperglycemia and sodium-glucose cotransporter-2 (SGLT2) inhibition on plasma amino acid metabolism in patients with type 1 diabetes (T1D).

Methods: The ATIRMA trial, a single-arm study, evaluated the effects of 8 weeks of oral empagliflozin (25 mg/day) in 40 young adults with T1D. The study involved consecutive two-day assessments of clamped euglycemia and hyperglycemia at both baseline and post-treatment. MetaboAnalyst 6.0 categorized 35 metabolites into significant pathways, which were statistically compared using principal component analysis.

Findings: Acute hyperglycemia induced changes to ten metabolic pathways, including but not limited to, increases in cysteine and methionine metabolism (0.52±0.12, $p<0.0001$), valine, leucine and isoleucine biosynthesis (0.31±0.10, $p=0.002$); and nitrogen metabolism (0.11±0.03, $p=0.003$). Introduction of empagliflozin was associated with a decrease in adenine, and increase in cysteine and methionine metabolism (0.31±0.13, $p=0.02$) when maintained under euglycemia and a decrease in nitrogen metabolism under hyperglycemia (–0.07±0.04, $p=0.04$).

Conclusion: Our findings show that SGLT2 inhibition counteracts the hyperglycemia-induced changes in plasma amino acid metabolism, potentially improving energy efficiency and metabolic health, though more research is needed to confirm these metabolic effects.

INTRODUCTION

The glycemic benefits of sodium-glucose cotransporter-2 (SGLT2) inhibitors in individuals with type 1 diabetes (T1D) has been consistently reported in the few randomized controlled trials conducted to date, namely the DEPICT (1-4), EASE (5) and inTandem (6-9) trials. However, growing safety concerns, particularly regarding the heightened risk of diabetic ketoacidosis (DKA) (10-12), have raised questions about their long-term use, leading to a reconsideration of their role in T1D glycemic management. SGLT2 inhibitors have proven cardiovascular and kidney benefits in patients with type 2 diabetes (T2D) (13-15). Considering the substantial residual risk for cardiovascular disease and kidney failure in individuals with T1D despite guideline-directed therapy (16, 17), the use of SGLT2 inhibitors may improve the overall benefit-risk balance in patients with T1D.

The development of cardiovascular and kidney events follows similar pathophysiological pathways in both T1D and T2D (18, 19). Emphasizing the common pathways targeted by SGLT2 inhibitors provides a strong rationale for their clinical investigation and potential use in T1D management. Impaired amino acid metabolism is a distinct metabolic hallmark of diabetes, likely developed as a consequence of insulin resistance, changes in protein turnover, and kidney dysfunction (20). This bioenergetic mismatch accelerates a pathological shift in mitochondrial substrate utilization, affecting multiple organ systems (21). It is hypothesized that a key metabolic effect of SGLT2 inhibition is the induction of a 'pseudo-fasting' state, where increased glucosuria drives a shift in metabolism from glucose utilization to the enhanced use of circulating amino acids, ketones and lipids for energy (22, 23). This metabolic shift suggests that SGLT2 inhibition modulates mitochondrial energetics by enhancing the utilization of amino acid substrates at a systemic level (24). Moreover, many non-amino acid metabolites (e.g., adenine),

which are intermediates or byproducts of pathways linked to amino acid catabolism (25, 26), accompany the changes in amino acid levels due to SGLT2 inhibition (27), particularly in processes related to energy production and cellular function (28). Whether this metabolic benefit of SGLT2 inhibition extends to patients with T1D is unknown.

Our current analysis evaluated the changes in individual and grouped plasma amino acid levels and related metabolites in a cohort of patients with T1D in response to 1) hyperglycemia, 2) SGLT2 inhibitor empagliflozin under clamped euglycemia and 3) empagliflozin under clamped hyperglycemia. We hypothesized that SGLT2 inhibition will attenuate any increase in plasma metabolite levels in response to hyperglycemia.

MATERIALS AND METHODS

Research Design and Study Participants

We performed a secondary exploratory analysis of the 8-week Adjunctive-To-Insulin and Renal Mechanistic (ATIRMA) trial (NCT01392560) (29) to examine changes in plasma metabolites associated with empagliflozin and acute hyperglycemia. The ATIRMA study was an open-label, single-arm, mechanistic trial with the primary objective to evaluate the physiological effects of 8 weeks of 25 mg empagliflozin on glomerular hyperfiltration in 40 young adults with T1D who were normotensive and normoalbuminuric (30-35). Of the 40 patients, 27 were classified as hyperfiltering (with an estimated glomerular filtration rate [eGFR] $\geq$ 135 mL/min/1.73 m^2) (36), while the remaining 13 were considered normofiltering (with an eGFR of 90-134 mL/min/1.73 m^2). Of the 40 participants, 20 were female and 20 were male. Patient characteristics for this cohort are published elsewhere (29).

The ATIRMA trial was structured into three phases: a 2-week placebo run-in period, an 8-week treatment phase with a daily dose of 25 mg of empagliflozin, and a 2-week follow-up period (as depicted in **Figure 1**). Boehringer Ingelheim Ltd. Canada was the supplier of empagliflozin drug. After the run-in and treatment phases, glycemic exposure was assessed over two consecutive days: Day 1 involved clamped euglycemia (plasma glucose of 4-6 mmol/L), and Day 2 involved clamped hyperglycemia (plasma glucose of 9-11 mmol/L). To mitigate the risk of hypoglycemia associated with empagliflozin, both prandial and basal insulin doses were initially reduced by 30%. This initial reduction was subsequently adjusted under the guidance of the investigator, resulting in an average decrease of 16.4% (29). The study was approved by the Research Ethics Board at the University Health Network in Toronto, Canada, and informed consent was obtained from all participants prior to the start of the trial.

Measurement of Plasma Amino Acids and Related Metabolites

Amino acids and related metabolites were measured from plasma samples by bulk liquid chromatography (LC) and mass spectroscopy (MS) analysis (**Supplemental Table S1**). The plasma (10 µL) samples were transferred into a fresh 96-well plate and extracted with the 80% methanol. The plate was vortexed for 1 min followed by centrifuge the samples for 10 minutes at 9000 RPM at 4^0C. Next, 5 µL of supernatant was transferred into 96-well plate for LC/MS analysis. Analysis was performed on Thermo Q Exactive HF-X Orbitrap mass spectrometer (Thermo, San Jose, CA) interfaced with heated electrospray ionization source (HESI) and coupled with Thermo Vanquish HPLC system. An aliquot of 2.5 µL of the sample was injected into the instrument using an autosampler. The chromatographic separation took place on an Agilent ZORBAX HILIC PLUS column with 3.5 µm particle size and with the dimensions 2.1

×100 mm with a phase composition of 10 mM ammonium formate, 0.05% formic acid in Millipore water (component A), and 0.05% formic acid in acetonitrile (component B) at 0.3 mL/ min flow rate (37). Data acquisition and the data processing was carried out by using Thermo Xcalibur Quant Browser software version 4.2.47.

Retention time of metabolites were tabulated in the **Supplemental Table S2** and for assignment of metabolite identities, commercially available pure standards (≥90–99% purity; primarily from Sigma-Aldrich and MedChemExpress) were analyzed under identical LC-MS conditions as the experimental samples. Standards included, but were not limited to, nicotinic acid, 5-methylthioadenosine, SAM, SAH, ADMA, SDMA, MMA, GABA, and a comprehensive amino acid mix (MSK-A2-US-1.2, CIL Inc.); full details including CAS numbers, vendors, and purities are provided in **Supplemental Table S3**. Metabolite identities were confirmed by matching the retention time (±0.05 min) and accurate mass (±5 ppm) to those of the standards. MS/MS fragmentation spectra were acquired for selected metabolites and compared to spectra from reference standards. Retention times and signal intensities of internal standards and quality control samples were monitored across runs to ensure consistency. Any drift in retention time or changes in peak shape triggered manual review of the assignment. Peak integration was checked and adjusted as needed to maintain accuracy in quantification. By combining accurate mass, retention time, and MS/MS fragmentation approach ensures robust metabolite identification and facilitates reproducibility of results.

Mapping Amino Acid Metabolic Networks

MetaboAnalyst 6.0 software (38) was employed to classify 19 plasma amino acids and 14 related metabolites into physiologically relevant metabolic pathways. Pathway analysis was

conducted according to standardized human metabolic pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. To evaluate the likelihood of random occurrences within specific metabolic pathways, we performed an overrepresentation analysis using hypergeometric distributions. A false discovery rate (FDR) threshold of ≤ 0.1 was established to select pathways that were significantly represented by the metabolites.

Thirty-five measured plasma metabolites were grouped into thirteen metabolic pathways by MetaboAnalyst 6.0: cysteine and methionine metabolites; arginine metabolites; valine, leucine, and isoleucine metabolites; alanine, aspartate, and glutamate metabolites; arginine and proline metabolites; glyoxylate and dicarboxylate metabolites; glycine, serine and threonine metabolites; one carbon pool by folate metabolites; glutathione metabolism; taurine and hypotaurine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; phenylalanine metabolism; and nitrogen metabolites (**Supplemental Table S4**).

To reduce dimensionality, principal component analysis (PCA) was used to project the metabolites from each pathway onto the first principal component, resulting in Eigenvalues that indicated the contribution of each metabolite group to their respective pathways. It is noteworthy that the phenylalanine metabolic pathway and the phenylalanine, tyrosine and tryptophan biosynthesis metabolic pathway contained identical amino acid compositions, leading to a combined PCA for both pathways.

Statistical Analyses

We conducted linear regression analyses for repeated measures to evaluate changes in individual plasma metabolite levels and grouped metabolites following PCA. The results were expressed as Z-scores derived from linear mixed-effects models. We specifically examined

differences across four conditions: baseline clamped euglycemia, baseline clamped hyperglycemia, post-treatment clamped euglycemia, and post-treatment clamped hyperglycemia. This was done using two-tailed hypothesis testing based on a least squares means model with an unstructured random-effects covariance matrix for the intercept and independent errors. Each visit was treated as a repeated measure for the respective subject.

To enhance the normal distribution of residuals and mitigate skewness, the data underwent log transformation before PCA. To analyze the interaction effects of empagliflozin treatment and acute hyperglycemia, we conducted an interaction analysis for both individual and grouped metabolites. Changes in individual plasma amino acids and grouped pathways with empagliflozin and acute hyperglycemia were reported as differences in least squares means estimates of log-transformed values. Two-tailed hypothesis testing was utilized at a significance level of 5% to compare the treatment effects on grouped metabolites. For the individual metabolites, we applied the Benjamini-Hochberg procedure to correct for multiple comparisons, assuming independence and defining significant results as an FDR of ≤ 0.05. The adjusted p-values from the Benjamini-Hochberg procedure are reported. This approach was chosen to reduce the likelihood of false positives in our exploratory analysis of individual metabolites. All analyses were carried out using SAS System Version 9.4 (SAS Institute, Cary, NC).

RESULTS

Baseline Characteristics

The baseline characteristics of the ATIRMA trial have been previously reported (29, 32). This secondary analysis included 27 individuals with hyperfiltration (estimated glomerular filtration rate [eGFR] $\geq$ 135 mL/min/1.73m^2) and 13 with normofiltration (eGFR 90-134

206 mL/min/1.73m^2), all with T1D. The mean age of participants was 24.3 ± 5.1 years, with a mean
207 diabetes duration of 17.1 ± 7.1 years (33). The cohort was equally distributed by sex and had a
208 mean body-mass index of 24.5 ± 3.2 kg/m^2. None were taking renin-angiotensin system
209 blockers, statins, or metformin.
210
211 **Plasma Amino Acid Metabolism in Response to Acute Hyperglycemia**
212 Under acute hyperglycemia, the level of six individual amino acids were significantly
213 altered compared to euglycemic levels (**Figure 2**). Specifically, hyperglycemia was associated
214 with decreases in methionine (–0.20, p=0.004), serine (–0.14, p=0.004), threonine (–0.15,
215 p=0.01), glutamine (–0.07, p=0.02), proline (–0.15, p=0.03), and alanine (–0.19, p=0.004)
216 (**Table 1**).
217 Of the grouped pathways identified using the 19 available plasma amino acids and 14
218 related metabolites, hyperglycemia significantly changed ten out of the twelve pathways. This
219 includes increases in cysteine and methionine metabolism; one carbon pool by folate; arginine
220 biosynthesis; valine, leucine and isoleucine biosynthesis; arginine and proline metabolism;
221 alanine, aspartate and glutamate metabolism; glyoxylate and dicarboxylate metabolism; glycine,
222 serine and threonine metabolism; and nitrogen metabolism, as well as decreases in taurine and
223 hypotaurine metabolism (**Figure 3; Table 2**).
224
225 **Plasma Amino Acid Metabolism in Response to SGLT2 inhibition under Euglycemia**
226 Of the 35 measured plasma metabolites, empagliflozin under clamped euglycemia
227 significantly changed the levels of six individual amino acids and adenine metabolite (**Figure 2**).
228 Specifically, empagliflozin was associated with increases in alanine (0.14, p=0.03), kynurenine

(0.07, *p*=0.03), and mono-methyl arginine (0.40, *p*=0.004) as well as decreases in L-alpha-aminobutyric acid (–0.26, *p*=0.004), citrulline (–0.15, *p*=0.03) and acetyl-lysine (–0.12, *p*=0.04) (**Table 3**). Empagliflozin was also associated with a decrease in adenine (–0.43, *p*=0.04). Under clamped euglycemia, empagliflozin was associated with significant decreases in taurine and hypotaurine metabolism and significant increases in cysteine and methionine metabolism (**Table 4; Figure 4**).

Plasma Amino Acid Metabolism in Response to SGLT2 inhibition under Hyperglycemia

Empagliflozin under clamped hyperglycemia significantly changed the levels of twelve individual amino acids (**Table 3**). Specifically, empagliflozin was associated with increases in phenylalanine (0.006, *p*=0.01), tryptophan (0.07, *p*=0.04), kynurenine (0.15, *p*=0.046), mono-methyl arginine (0.32, *p*=0.004) and alanine (0.16, *p*=0.009), whereas decreases in cysteine (–0.29, *p*=0.009), acetyl-lysine (–0.19, *p*=0.004), symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) (–0.29, *p*=0.04), citrulline (–0.15, *p*=0.01), taurine (–0.13, *p*=0.04), cystathionine (–0.28, *p*=0.046) and L-alpha-Aminobutyric acid (–0.18, *p*=0.009) was observed. Moreover, of the 35 individual metabolites, adenine was identified to have significant treatment by glycemia interaction where the effect of empagliflozin on adenine was modified by hyperglycemia (**Table 3)**. Under clamped hyperglycemia, empagliflozin was associated with significant increases in nitrogen metabolism and taurine and hypotaurine metabolism (**Figure 4**).

DISCUSSION

Disruptions to amino acid metabolism play a key role in the development of cardiorenal complications associated with diabetes (21). In the *post hoc* analysis of the ATIRMA trial, we found that acute, ambient hyperglycemia increased amino acid metabolism, specifically valine, leucine and isoleucine biosynthesis; alanine, aspartate and glutamate metabolism; arginine biosynthesis; and arginine and proline metabolism in patients with T1D. Under euglycemia, patients treated with 8 weeks of empagliflozin treatment reported increases in taurine and hypotaurine metabolism and cysteine and methionine metabolism, whereas under acute hyperglycemia, empagliflozin was associated with decreases in nitrogen metabolism. Despite initial increases to amino acid metabolism pathways with acute hyperglycemia, empagliflozin treatment mediated significant changes in plasma metabolites related to energy metabolism and mitochondrial function, implying shifts in metabolic pathways and substrate utilization in the context of T1D.

Branched chain amino acids (BCAAs) are protein-building groups of amino acids consisting of valine, leucine and isoleucine. We demonstrate that acute, ambient hyperglycemia in patients with T1D increased BCAA and decreased alanine levels. BCAAs are involved in various cellular signaling pathways, both anabolic and catabolic, that can lead to changes in cellular function and organismal phenotype (39). Notably, BCAAs are recognized for their role in enhancing protein synthesis by regulating protein translation. However, increased levels of plasma BCAAs have been linked to the severe insulin resistance (40). It is postulated that this increase in BCAAs may stem from impaired BCAA degradation processes, contributing to the development and progression of insulin resistance (41). Moreover, given that T1D is characterized by increased amino acid oxidation, it is proposed that when reduction-oxidation balance is disrupted, the influx of BCAAs from dietary sources and muscle protein breakdown

overwhelms the capacity of the body to metabolize them (42). This results in a significant elevation of plasma BCAA levels in patients with T1D and inadequately controlled glycemic levels (43-45). The primary acceptor of the amino nitrogen from BCAAs is α-ketoglutarate, which is subsequently converted into glutamate. This conversion is accompanied by the conversion of pyruvate to alanine. When BCAA metabolism and conversion to α-ketoglutarate is disrupted in patients with T1D, alanine levels decrease. Accordingly, our findings are consistent with the hypothesis that suggests elevated BCAA levels in T1D arise from disruptions in glycolysis and fatty acid oxidation due to hyperglycemia, which lead to insufficient BCAA catabolism in muscles (46).

Introduction of 8 weeks of empagliflozin therapy in a state of hyperglycemia attenuated the increase in cysteine and methionine metabolism observed under euglycemia. Despite evidence from at least one other analysis validating this finding (47), the resulting physiological implications in the kidney and in circulation, especially in patients with T1D, are not well known. Specifically, in people with diabetes, methionine is reported to have a significantly inverse relationship with hemoglobin A1c (48), suggesting that any increase in methionine and its metabolism should be beneficial for patients with T1D. However, amino acids, such as methionine (49) and cysteine (50, 51), have evolved to enhance their resistance to oxidation by modulating the content of sulfur-containing residues which reflect the reduction-oxidation status of the body. Therefore, adaptive reductions in methionine and cysteine levels have evolved as mechanisms in long-lived animal species. Beyond their structural and functional roles in proteins, methionine and cysteine participate in complex methionine metabolism (52, 53). As such, emerging evidence suggests that regulation of methionine metabolism may play a critical role in determining longevity (54), supporting our findings that the attenuation in the increase in

297 cysteine and methionine metabolism associated with empagliflozin under hyperglycemic
298 conditions may be protective. Additional research is needed to clarify this potential mechanism.

299 As highlighted in our previous findings across several patient cohorts, urine adenine may
300 potentially serve as a mechanistic biomarker for end-stage kidney disease (27). Specifically,
301 patients with the highest tertile of urine adenine-to-creatinine ratio were associated with an
302 increased risk of kidney failure and mortality. Notably however, we have previously showed that
303 empagliflozin effectively reduced elevated urine adenine-to-creatinine levels in patients with
304 T1D in the ATIRMA trial by 36% (from 70.9 to 44.8 nM/mM) (27). In our current analysis, we
305 found that plasma adenine levels decreased with empagliflozin therapy by 13% (from 6.8 to 5.9
306 nM), however this was exclusively under an euglycemic clamp, as corroborated by the
307 significant treatment by glycemia interaction. This parallel reduction in both plasma and urinary
308 adenine suggests a broader systemic shift in adenine handling, possibly involving renal transport
309 processes. Recent studies have postulated that SGLT2 inhibition may influence the activity of
310 other renal transporters, including solute carriers involved in urate, amino acid, and vitamin
311 transport, as well as organic anion transporters on the basolateral membrane (55-57). SGLT2
312 inhibition may indirectly modulate the expression or function of these ancillary transporters that
313 handle purine derivatives or related metabolites (55, 58, 59). Understanding the interplay
314 between SGLT2 inhibition and purine metabolism at the level of renal transport may yield new
315 insights into the pathogenesis and treatment of DKD. In our previous work, adenine was found to
316 stimulate matrix production in tubular cells by activating the mTOR signaling pathway (27).
317 Kidney biopsies from participants with T2D and healthy controls showed that T2D is associated
318 with upregulation of the mTORC1 pathway in each tubular segment of the nephron (27, 60).
319 SGLT2 inhibition is thought to attenuate aberrant mTOR signaling and restore proper mTOR

cycle regulation through the reduction of blood glucose levels (60, 61). As such, this upregulation of urine adenine was subsequently reduced with SGLT2 inhibition (27), which we were able to replicate in plasma adenine within the current analysis. Although more work is required to delineate the potential interplay between adenine, methionine and mTOR pathway in diabetic kidney disease, our data lend support that hyperglycemia drives glucose metabolism to the mTOR cycle and SGLT2 inhibition mitigates the effects of the mTOR pathway by way of adenine, methionine and cysteine.

Similar to our findings in plasma, in our prior work, we found that hyperglycemia augmented grouped urine amino acid pathways, including BCAAs, aromatic amino acids, and arginine biosynthesis (28). Notably, we found that empagliflozin was associated with an increase in urine BCAAs under clamped hyperglycemia (28). Although no changes to plasma BCAAs were demonstrated in response to empagliflozin, we observed an increase in plasma BCAAs in response to acute hyperglycemia. It appears that, while reduction-oxidation balance plays a role in modulating BCAA expression, the primary drivers are hyperglycemia and severe insulin resistance (40). These metabolic conditions may have a greater impact on BCAAs, suggesting that the altered glucose and insulin dynamics in such states override the effects of reduction-oxidation balance on BCAA metabolism. This provides insight into why an increase in BCAA was observed under ambient hyperglycemia with or without empagliflozin treatment, and not observed under euglycemic clamp. Moreover, in our prior work, we investigated other plasma metabolites involved in fatty acid metabolism, and we observed similar findings, particularly the increase in alanine, aspartate, and glutamate metabolism during acute hyperglycemia, prior to treatment (62). While we evaluated a range of different metabolites, the results reinforced the same overall conclusion. Furthermore, we did not observe an increase in plasma kynurenine

levels as seen in urine (28), however observed an increase under clamped euglycemia and clamped hyperglycemia with treatment, inconsistent with the hypothesized potential protective effect of empagliflozin against oxidative stress (63) and inflammation (64) in this patient population. Overall, the complex interplay between glucose metabolism, insulin resistance, and amino acid regulation should be further investigated, especially in patients with T1D.

This *post hoc* exploratory analysis has several limitations. Given that this analysis was exploratory and post hoc, and that the intervention trial lacked a control group, causality should not be directly inferred from these associations. Additionally, the small sample size and the relatively short 8-week treatment period limit the ability to generalize the long-term effects of SGLT2 inhibition to the broader population. Due to the limited duration of the glycemic clamp, the observed effects of hyperglycemia are confined to acute and ambient changes in metabolism. While the changes in plasma metabolites levels likely reflect metabolic alterations in the kidney, it remains possible that these findings may be attributed to broader systemic changes in energy metabolism, as we did not conduct kidney biopsies to confirm structural and functional alterations in renal mitochondria. Moreover, while our metabolomics data suggest altered amino acid metabolism, we did not perform metabolic flux analysis, which would provide direct evidence of amino acid utilization in energy metabolism. Therefore, future studies using stable isotope tracer-based flux analysis are warranted to mechanistically validate the metabolic roles of amino acids suggested by our findings. A key limitation of this study is the restricted coverage of metabolites, which constrains the depth of mechanistic insight that can be drawn from pathway-level analyses. The targeted metabolomics platform used in this work quantified a focused panel of amino acids and related metabolites, but did not include key intermediates in many of the metabolic pathways identified by enrichment tools such as MetaboAnalyst. As a result, pathway

assignments, including those related to folate metabolism, glyoxylate metabolism, BCAA metabolism and biosynthetic pathways for essential amino acids, may not reflect actual biochemical activity in vivo and should be interpreted with caution. Notably, intermediates such as alpha-ketoglutarate, which play a central role in transamination and TCA cycle integration, were not quantified. These computational pathway outputs are based on statistical overlap with canonical pathways, rather than biological plausibility or direct evidence of pathway flux. Finally, it is possible that some of the changes observed in this study could be linked to variations in total daily insulin during the study period. However, despite a 16.4% reduction in total daily insulin following drug initiation to mitigate hypoglycemia risk (29, 32), such adjustments are a standard part of care for individuals with T1D who are also receiving other anti-diabetic treatments.

PERSPECTIVES AND SIGNIFICANCE

Our findings highlight the metabolic changes induced by SGLT2 inhibition and acute hyperglycemia, particularly in the context of plasma amino acid metabolism. The increase in amino acid metabolism was mitigated by empagliflozin treatment and likely served as protection against dysfunctional systemic and renal metabolic alterations involving energy utilization and regulation. This provides further evidence for the concept of a ‘pseudo-fasting state’ induced by SGLT2 inhibition (22, 65, 66), to optimize energy substrate efficiency, particularly in energy-demanding organs like the heart and kidneys. Given the known cardiovascular and kidney risks in individuals with T1D, this adaptive metabolic response could have potential therapeutic implications. By improving energy utilization and supporting mitochondrial function, SGLT2 inhibition may offer protective benefits against diabetic complications, including cardiovascular

disease and kidney failure. These findings emphasize the importance of further investigating the long-term impact of SGLT2 inhibitors on metabolic processes and organ function in T1D.

DATA AVAILABILITY

Source data for this study (https://doi.org/10.1161/CIRCULATIONAHA.113.005081) are not publicly available due to privacy or ethical restrictions. The source data are available to verified researchers upon request by contacting the corresponding author. The authors confirm that the data supporting the findings of this study are openly available in figshare at https://figshare.com/s/d3cedb654cf80a2d8766.

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COMPETING INTEREST

KS has served as a consultant and received honoraria from Boehringer Ingelheim, Janssen and Sanofi. KS has received research support from Boerhinger Ingelheim. DZIC has received honoraria from Boehringer Ingelheim-Lilly, Merck, AstraZeneca, Sanofi, Mitsubishi-Tanabe, Abbvie, Janssen, Bayer, Prometic, BMS, Maze, Gilead, CSL-Behring, Otsuka, Novartis, Youngene, Lexicon and Novo-Nordisk and has received operational funding for clinical trials from Boehringer Ingelheim-Lilly, Merck, Janssen, Sanofi, AstraZeneca, CSL-Behring and Novo-

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AUTHORS CONTRIBUTIONS

All authors contributed to collection of the data and data interpretation. L.K. performed the statistical analysis. L.K. wrote the first draft of the manuscript. D.Z.I.C was the principal investigator involved in the ATIRMA study design. All authors provided critical revision for important intellectual content and approved the final version of the manuscript for submission. The corresponding author K.S. takes full responsibility for the work and/or conduct of the study, had access to the data, and controlled the decision to publish.

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FIGURE LEGEND

Figure 1. Study design for the ATIRMA trial. Empagliflozin 25mg oral once daily was administered for 8 weeks to 40 participants with T1D.

Figure 2. Heat map of plasma metabolites significantly changed by hyperglycemia and empagliflozin. **3-4** – baseline, clamped euglycemia vs clamped hyperglycemia; **3-12** – clamped euglycemia, baseline vs post-treatment; **4-13** – clamped hyperglycemia, baseline vs post-treatment; **12-13** – post-treatment, clamped euglycemia vs clamped hyperglycemia. Two-tailed hypothesis testing was utilized at a significance level of 5% to compare the treatment effects on metabolites. For the individual metabolites, we applied the Benjamini-Hochberg procedure to correct for multiple comparisons, assuming independence and defining significant results as an FDR of ≤ 0.05.

Figure 3. Plasma metabolite pathways altered by acute hyperglycemia at baseline is reflected as a Z-score of mean ± standard deviations. a) Cysteine and methionine metabolites, b) one carbon pool by folate metabolites, c) arginine metabolites, d) valine, leucine, and isoleucine metabolites, e) arginine and proline metabolites, f) alanine, aspartate and glutamate metabolites, g) glyoxylate and dicarboxylate metabolites, h) glycine, serine and threonine metabolites, i) nitrogen metabolites and j) taurine and hypotaurine metabolites. Metabolomic pathway compositions were identified by MetaboAnalyst 6.0 software to accurately group 35 metabolites measured in plasma in this *post hoc* analysis with an FDR ≤ 0.1 (**Supplemental Table S1**). Metabolites from each pathway were projected onto first principal component using PCA to represent the pathway composition (**Supplemental Table S4**). Repeated measures linear regression and *post hoc* least squares mean estimates were performed. Significant p-values are stated.

Figure 4. Plasma metabolite pathways at baseline and post-treatment (8 weeks of 25 mg empagliflozin once daily) are reflected as a Z-score of mean ± standard deviations. This is under both clamped euglycemic (blue) and hyperglycemia (red). a) Cysteine and methionine metabolites, b) one carbon pool by folate metabolites, c) arginine metabolites, d) valine, leucine, and isoleucine metabolites, e) alanine, aspartate, and glutamate metabolites, f) arginine and proline metabolites, g) glyoxylate and dicarboxylate metabolites, h) glycine, serine and threonine metabolites, i) nitrogen metabolites and j) taurine and hypotaurine metabolites. Metabolomic pathway compositions were identified by MetaboAnalyst 6.0 software to accurately group 35 metabolites measured in plasma in this *post hoc* analysis with an FDR ≤ 0.1 (**Supplemental Table S1**). Metabolites from each pathway were projected onto first principal component using PCA to represent the pathway composition (**Supplemental Table S4**). Repeated measures linear regression and *post hoc* least squares mean estimates were performed. A treatment (T_x) by glycemia interaction was analyzed for each metabolic pathway. Only significant p-values are stated. *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$.

Metabolic Shifts in Plasma Amino Acids and Related Metabolites in Response to SGLT2 Inhibition and Hyperglycemia in Type 1 Diabetes

Luxcia Kugathasan BSc[1,2,3*], Nagarjunachary Ragi PhD[4,5*], Subrata Debnath MBBS PhD[4,5], Vikas S. Sridhar MD[6], Soumya Maity PhD[4,5], Tianqing Feng BSc[4,5], Esmeralda Treviño BSc[4,5], Bruce A. Perkins MD MPH[7,8], David Z.I. Cherney MD PhD[1,2,3†], Kumar Sharma MD[4,5†]

Affiliations:

[1]Department of Medicine, Division of Nephrology, University Health Network, Toronto, Ontario, Canada
[2]Department of Physiology, University of Toronto, Toronto, Ontario, Canada
[3]Cardiovascular Sciences Collaborative Specialization, University of Toronto, Toronto, Canada
[4]Center for Precision Medicine, University of Texas Health San Antonio, Texas, USA
[5]Division of Nephrology, Department of Medicine, University of Texas Health San Antonio, Texas, USA
[6]Division of Nephrology, University of British Columbia, Vancouver, British Columbia, Canada
[7]Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, ON, Canada
[8]Division of Endocrinology and Metabolism, Department of Medicine, University of Toronto, Toronto, Ontario, Canada.

*Co-first authors
[†]Co-senior authors

Corresponding author:

Luxcia Kugathasan, BSc
University Health Network
585 University Avenue, 1N128
Toronto, ON M5G 2N2, Canada
Telephone: +1 (416) 340 4800 ext. 8508
Email: luxcia.kugathasan@uhn.ca

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ABSTRACT

Objective: Regulated kidney function is dependent on maintaining efficient energy utilization. Our aim in this study was to determine the effects of acute, ambient hyperglycemia and sodium-glucose cotransporter-2 (SGLT2) inhibition on plasma amino acid metabolism in patients with type 1 diabetes (T1D).

Methods: The ATIRMA trial, a single-arm study, evaluated the effects of 8 weeks of oral empagliflozin (25 mg/day) in 40 young adults with T1D. The study involved consecutive two-day assessments of clamped euglycemia and hyperglycemia at both baseline and post-treatment. MetaboAnalyst 6.0 categorized 35 metabolites into significant pathways, which were statistically compared using principal component analysis.

Findings: Acute hyperglycemia induced changes to ten metabolic pathways, including but not limited to, increases in cysteine and methionine metabolism (0.52±0.12, $p<0.0001$), valine, leucine and isoleucine biosynthesis (0.31±0.10, $p=0.002$); and nitrogen metabolism (0.11±0.03, $p=0.003$). Introduction of empagliflozin was associated with a decrease in adenine, and increase in cysteine and methionine metabolism (0.31±0.13, $p=0.02$) when maintained under euglycemia and a decrease in nitrogen metabolism under hyperglycemia (–0.07±0.04, $p=0.04$).

Conclusion: Our findings show that SGLT2 inhibition counteracts the hyperglycemia-induced changes in plasma amino acid metabolism, potentially improving energy efficiency and metabolic health, though more research is needed to confirm these metabolic effects.

INTRODUCTION

The glycemic benefits of sodium-glucose cotransporter-2 (SGLT2) inhibitors in individuals with type 1 diabetes (T1D) has been consistently reported in the few randomized controlled trials conducted to date, namely the DEPICT (1-4), EASE (5) and inTandem (6-9) trials. However, growing safety concerns, particularly regarding the heightened risk of diabetic ketoacidosis (DKA) (10-12), have raised questions about their long-term use, leading to a reconsideration of their role in T1D glycemic management. SGLT2 inhibitors have proven cardiovascular and kidney benefits in patients with type 2 diabetes (T2D) (13-15). Considering the substantial residual risk for cardiovascular disease and kidney failure in individuals with T1D despite guideline-directed therapy (16, 17), the use of SGLT2 inhibitors may improve the overall benefit-risk balance in patients with T1D.

The development of cardiovascular and kidney events follows similar pathophysiological pathways in both T1D and T2D (18, 19). Emphasizing the common pathways targeted by SGLT2 inhibitors provides a strong rationale for their clinical investigation and potential use in T1D management. Impaired amino acid metabolism is a distinct metabolic hallmark of diabetes, likely developed as a consequence of insulin resistance, changes in protein turnover, and kidney dysfunction (20). This bioenergetic mismatch accelerates a pathological shift in mitochondrial substrate utilization, affecting multiple organ systems (21). It is hypothesized that a key metabolic effect of SGLT2 inhibition is the induction of a 'pseudo-fasting' state, where increased glucosuria drives a shift in metabolism from glucose utilization to the enhanced use of circulating amino acids, ketones and lipids for energy (22, 23). This metabolic shift suggests that SGLT2 inhibition modulates mitochondrial energetics by enhancing the utilization of amino acid substrates at a systemic level (24). Moreover, many non-amino acid metabolites (e.g., adenine),

which are intermediates or byproducts of pathways linked to amino acid catabolism (25, 26), accompany the changes in amino acid levels due to SGLT2 inhibition (27), particularly in processes related to energy production and cellular function (28). Whether this metabolic benefit of SGLT2 inhibition extends to patients with T1D is unknown.

Our current analysis evaluated the changes in individual and grouped plasma amino acid levels and related metabolites in a cohort of patients with T1D in response to 1) hyperglycemia, 2) SGLT2 inhibitor empagliflozin under clamped euglycemia and 3) empagliflozin under clamped hyperglycemia. We hypothesized that SGLT2 inhibition will attenuate any increase in plasma metabolite levels in response to hyperglycemia.

MATERIALS AND METHODS

Research Design and Study Participants

We performed a secondary exploratory analysis of the 8-week Adjunctive-To-Insulin and Renal Mechanistic (ATIRMA) trial (NCT01392560) (29) to examine changes in plasma metabolites associated with empagliflozin and acute hyperglycemia. The ATIRMA study was an open-label, single-arm, mechanistic trial with the primary objective to evaluate the physiological effects of 8 weeks of 25 mg empagliflozin on glomerular hyperfiltration in 40 young adults with T1D who were normotensive and normoalbuminuric (30-35). Of the 40 patients, 27 were classified as hyperfiltering (with an estimated glomerular filtration rate [eGFR] $\geq$ 135 mL/min/1.73 m^2) (36), while the remaining 13 were considered normofiltering (with an eGFR of 90-134 mL/min/1.73 m^2). Of the 40 participants, 20 were female and 20 were male. Patient characteristics for this cohort are published elsewhere (29).

The ATIRMA trial was structured into three phases: a 2-week placebo run-in period, an 8-week treatment phase with a daily dose of 25 mg of empagliflozin, and a 2-week follow-up period (as depicted in **Figure 1**). Boehringer Ingelheim Ltd. Canada was the supplier of empagliflozin drug. After the run-in and treatment phases, glycemic exposure was assessed over two consecutive days: Day 1 involved clamped euglycemia (plasma glucose of 4-6 mmol/L), and Day 2 involved clamped hyperglycemia (plasma glucose of 9-11 mmol/L). To mitigate the risk of hypoglycemia associated with empagliflozin, both prandial and basal insulin doses were initially reduced by 30%. This initial reduction was subsequently adjusted under the guidance of the investigator, resulting in an average decrease of 16.4% (29). The study was approved by the Research Ethics Board at the University Health Network in Toronto, Canada, and informed consent was obtained from all participants prior to the start of the trial.

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Measurement of Plasma Amino Acids and Related Metabolites

Amino acids and related metabolites were measured from plasma samples by bulk liquid chromatography (LC) and mass spectroscopy (MS) analysis (**Supplemental Table S1**). The plasma (10 µL) samples were transferred into a fresh 96-well plate and extracted with the 80% methanol. The plate was vortexed for 1 min followed by centrifuge the samples for 10 minutes at 9000 RPM at 4^0C. Next, 5 µL of supernatant was transferred into 96-well plate for LC/MS analysis. Analysis was performed on Thermo Q Exactive HF-X Orbitrap mass spectrometer (Thermo, San Jose, CA) interfaced with heated electrospray ionization source (HESI) and coupled with Thermo Vanquish HPLC system. An aliquot of 2.5 µL of the sample was injected into the instrument using an autosampler. The chromatographic separation took place on an Agilent ZORBAX HILIC PLUS column with 3.5 µm particle size and with the dimensions 2.1

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×100 mm with a phase composition of 10 mM ammonium formate, 0.05% formic acid in Millipore water (component A), and 0.05% formic acid in acetonitrile (component B) at 0.3 mL/ min flow rate (37). Data acquisition and the data processing was carried out by using Thermo Xcalibur Quant Browser software version 4.2.47.

Retention time of metabolites were tabulated in the **Supplemental Table S2** and for assignment of metabolite identities, commercially available pure standards (≥90–99% purity; primarily from Sigma-Aldrich and MedChemExpress) were analyzed under identical LC-MS conditions as the experimental samples. Standards included, but were not limited to, nicotinic acid, 5-methylthioadenosine, SAM, SAH, ADMA, SDMA, MMA, GABA, and a comprehensive amino acid mix (MSK-A2-US-1.2, CIL Inc.); full details including CAS numbers, vendors, and purities are provided in **Supplemental Table S3**. Metabolite identities were confirmed by matching the retention time (±0.05 min) and accurate mass (±5 ppm) to those of the standards. MS/MS fragmentation spectra were acquired for selected metabolites and compared to spectra from reference standards. Retention times and signal intensities of internal standards and quality control samples were monitored across runs to ensure consistency. Any drift in retention time or changes in peak shape triggered manual review of the assignment. Peak integration was checked and adjusted as needed to maintain accuracy in quantification. By combining accurate mass, retention time, and MS/MS fragmentation approach ensures robust metabolite identification and facilitates reproducibility of results.

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Mapping Amino Acid Metabolic Networks

MetaboAnalyst 6.0 software (38) was employed to classify 19 plasma amino acids and 14 related metabolites into physiologically relevant metabolic pathways. Pathway analysis was

conducted according to standardized human metabolic pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. To evaluate the likelihood of random occurrences within specific metabolic pathways, we performed an overrepresentation analysis using hypergeometric distributions. A false discovery rate (FDR) threshold of ≤ 0.1 was established to select pathways that were significantly represented by the metabolites.

Thirty-five measured plasma metabolites were grouped into thirteen metabolic pathways by MetaboAnalyst 6.0: cysteine and methionine metabolites; arginine metabolites; valine, leucine, and isoleucine metabolites; alanine, aspartate, and glutamate metabolites; arginine and proline metabolites; glyoxylate and dicarboxylate metabolites; glycine, serine and threonine metabolites; one carbon pool by folate metabolites; glutathione metabolism; taurine and hypotaurine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; phenylalanine metabolism; and nitrogen metabolites (~~**Supplemental Table S3**~~**Supplemental Table S4**).

To reduce dimensionality, principal component analysis (PCA) was used to project the metabolites from each pathway onto the first principal component, resulting in Eigenvalues that indicated the contribution of each metabolite group to their respective pathways. It is noteworthy that the phenylalanine metabolic pathway and the phenylalanine, tyrosine and tryptophan biosynthesis metabolic pathway contained identical amino acid compositions, leading to a combined PCA for both pathways.

Statistical Analyses

We conducted linear regression analyses for repeated measures to evaluate changes in individual plasma metabolite levels and grouped metabolites following PCA. The results were expressed as Z-scores derived from linear mixed-effects models. We specifically examined

differences across four conditions: baseline clamped euglycemia, baseline clamped hyperglycemia, post-treatment clamped euglycemia, and post-treatment clamped hyperglycemia. This was done using two-tailed hypothesis testing based on a least squares means model with an unstructured random-effects covariance matrix for the intercept and independent errors. Each visit was treated as a repeated measure for the respective subject.

To enhance the normal distribution of residuals and mitigate skewness, the data underwent log transformation before PCA. To analyze the interaction effects of empagliflozin treatment and acute hyperglycemia, we conducted an interaction analysis for both individual and grouped metabolites. Changes in individual plasma amino acids and grouped pathways with empagliflozin and acute hyperglycemia were reported as differences in least squares means estimates of log-transformed values. Two-tailed hypothesis testing was utilized at a significance level of 5% to compare the treatment effects on grouped metabolites. For the individual metabolites, we applied the Benjamini-Hochberg procedure to correct for multiple comparisons, assuming independence and defining significant results as an FDR of ≤ 0.05. The adjusted p-values from the Benjamini-Hochberg procedure are reported. This approach was chosen to reduce the likelihood of false positives in our exploratory analysis of individual metabolites. All analyses were carried out using SAS System Version 9.4 (SAS Institute, Cary, NC).

RESULTS

Baseline Characteristics

The baseline characteristics of the ATIRMA trial have been previously reported (29, 32). This secondary analysis included 27 individuals with hyperfiltration (estimated glomerular filtration rate [eGFR] $\geq$ 135 mL/min/1.73m^2) and 13 with normofiltration (eGFR 90-134

mL/min/1.73m^2), all with T1D. The mean age of participants was 24.3 ± 5.1 years, with a mean diabetes duration of 17.1 ± 7.1 years (33). The cohort was equally distributed by sex and had a mean body-mass index of 24.5 ± 3.2 kg/m^2. None were taking renin-angiotensin system blockers, statins, or metformin.

Plasma Amino Acid Metabolism in Response to Acute Hyperglycemia

Under acute hyperglycemia, the level of six individual amino acids were significantly altered compared to euglycemic levels (**Figure 2**). Specifically, hyperglycemia was associated with decreases in methionine (–0.20, p=0.004), serine (–0.14, p=0.004), threonine (–0.15, p=0.01), glutamine (–0.07, p=0.02), proline (–0.15, p=0.03), and alanine (–0.19, p=0.004) (**Table 1**).

Of the grouped pathways identified using the 19 available plasma amino acids and 14 related metabolites, hyperglycemia significantly changed ten out of the twelve pathways. This includes increases in cysteine and methionine metabolism; one carbon pool by folate; arginine biosynthesis; valine, leucine and isoleucine biosynthesis; arginine and proline metabolism; alanine, aspartate and glutamate metabolism; glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; and nitrogen metabolism, as well as decreases in taurine and hypotaurine metabolism (**Figure 3; Table 2**).

Plasma Amino Acid Metabolism in Response to SGLT2 inhibition under Euglycemia

Of the 35 measured plasma metabolites, empagliflozin under clamped euglycemia significantly changed the levels of six individual amino acids and adenine metabolite (**Figure 2**). Specifically, empagliflozin was associated with increases in alanine (0.14, p=0.03), kynurenine

(0.07, p=0.03), and mono-methyl arginine (0.40, p=0.004) as well as decreases in L-alpha-aminobutyric acid (–0.26, p=0.004), citrulline (–0.15, p=0.03) and acetyl-lysine (–0.12, p=0.04) (**Table 3**). Empagliflozin was also associated with a decrease in adenine (–0.43, p=0.04). Under clamped euglycemia, empagliflozin was associated with significant decreases in taurine and hypotaurine metabolism and significant increases in cysteine and methionine metabolism (**Table 4; Figure 4**).

Plasma Amino Acid Metabolism in Response to SGLT2 inhibition under Hyperglycemia

Empagliflozin under clamped hyperglycemia significantly changed the levels of twelve individual amino acids (**Table 3**). Specifically, empagliflozin was associated with increases in phenylalanine (0.006, p=0.01), tryptophan (0.07, p=0.04), kynurenine (0.15, p=0.046), mono-methyl arginine (0.32, p=0.004) and alanine (0.16, p=0.009), whereas decreases in cysteine (–0.29, p=0.009), acetyl-lysine (–0.19, p=0.004), symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) (–0.29, p=0.04), citrulline (–0.15, p=0.01), taurine (–0.13, p=0.04), cystathionine (–0.28, p=0.046) and L-alpha-Aminobutyric acid (–0.18, p=0.009) was observed. Moreover, of the 35 individual metabolites, adenine was identified to have significant treatment by glycemia interaction where the effect of empagliflozin on adenine was modified by hyperglycemia (**Table 3)**. Under clamped hyperglycemia, empagliflozin was associated with significant increases in nitrogen metabolism and taurine and hypotaurine metabolism (**Figure 4**).

DISCUSSION

Disruptions to amino acid metabolism play a key role in the development of cardiorenal complications associated with diabetes (21). In the *post hoc* analysis of the ATIRMA trial, we found that acute, ambient hyperglycemia increased amino acid metabolism, specifically valine, leucine and isoleucine biosynthesis; alanine, aspartate and glutamate metabolism; arginine biosynthesis; and arginine and proline metabolism in patients with T1D. Under euglycemia, patients treated with 8 weeks of empagliflozin treatment reported increases in taurine and hypotaurine metabolism and cysteine and methionine metabolism, whereas under acute hyperglycemia, empagliflozin was associated with decreases in nitrogen metabolism. Despite initial increases to amino acid metabolism pathways with acute hyperglycemia, empagliflozin treatment mediated significant changes in plasma metabolites related to energy metabolism and mitochondrial function, implying shifts in metabolic pathways and substrate utilization in the context of T1D.

Branched chain amino acids (BCAAs) are protein-building groups of amino acids consisting of valine, leucine and isoleucine. We demonstrate that acute, ambient hyperglycemia in patients with T1D increased BCAA and decreased alanine levels. BCAAs are involved in various cellular signaling pathways, both anabolic and catabolic, that can lead to changes in cellular function and organismal phenotype (39). Notably, BCAAs are recognized for their role in enhancing protein synthesis by regulating protein translation. However, increased levels of plasma BCAAs have been linked to the severe insulin resistance (40). It is postulated that this increase in BCAAs may stem from impaired BCAA degradation processes, contributing to the development and progression of insulin resistance (41). Moreover, given that T1D is characterized by increased amino acid oxidation, it is proposed that when reduction-oxidation balance is disrupted, the influx of BCAAs from dietary sources and muscle protein breakdown

overwhelms the capacity of the body to metabolize them (42). This results in a significant elevation of plasma BCAA levels in patients with T1D and inadequately controlled glycemic levels (43-45). The primary acceptor of the amino nitrogen from BCAAs is α-ketoglutarate, which is subsequently converted into glutamate. This conversion is accompanied by the conversion of pyruvate to alanine. When BCAA metabolism and conversion to α-ketoglutarate is disrupted in patients with T1D, alanine levels decrease. Accordingly, our findings are consistent with the hypothesis that suggests elevated BCAA levels in T1D arise from disruptions in glycolysis and fatty acid oxidation due to hyperglycemia, which lead to insufficient BCAA catabolism in muscles (46).

Introduction of 8 weeks of empagliflozin therapy in a state of hyperglycemia attenuated the increase in cysteine and methionine metabolism observed under euglycemia. Despite evidence from at least one other analysis validating this finding (47), the resulting physiological implications in the kidney and in circulation, especially in patients with T1D, are not well known. Specifically, in people with diabetes, methionine is reported to have a significantly inverse relationship with hemoglobin A1c (48), suggesting that any increase in methionine and its metabolism should be beneficial for patients with T1D. However, amino acids, such as methionine (49) and cysteine (50, 51), have evolved to enhance their resistance to oxidation by modulating the content of sulfur-containing residues which reflect the reduction-oxidation status of the body. Therefore, adaptive reductions in methionine and cysteine levels have evolved as mechanisms in long-lived animal species. Beyond their structural and functional roles in proteins, methionine and cysteine participate in complex methionine metabolism (52, 53). As such, emerging evidence suggests that regulation of methionine metabolism may play a critical role in determining longevity (54), supporting our findings that the attenuation in the increase in

297 cysteine and methionine metabolism associated with empagliflozin under hyperglycemic
298 conditions may be protective. Additional research is needed to clarify this potential mechanism.
299 As highlighted in our previous findings across several patient cohorts, urine adenine may
300 potentially serve as a mechanistic biomarker for end-stage kidney disease (27). Specifically,
301 patients with the highest tertile of urine adenine-to-creatinine ratio were associated with an
302 increased risk of kidney failure and mortality. Notably however, we have previously showed that
303 empagliflozin effectively reduced elevated urine adenine-to-creatinine levels in patients with
304 T1D in the ATIRMA trial by 36% (from 70.9 to 44.8 nM/mM) (27). In our current analysis, we
305 found that plasma adenine levels decreased with empagliflozin therapy by 13% (from 6.8 to 5.9
306 nM), however this was exclusively under an euglycemic clamp, as corroborated by the
307 significant treatment by glycemia interaction. This parallel reduction in both plasma and urinary
308 adenine suggests a broader systemic shift in adenine handling, possibly involving renal transport
309 processes. Recent studies have postulated that SGLT2 inhibition may influence the activity of
310 other renal transporters, including solute carriers involved in urate, amino acid, and vitamin
311 transport, as well as organic anion transporters on the basolateral membrane (55-57). SGLT2
312 inhibition may indirectly modulate the expression or function of these ancillary transporters that
313 handle purine derivatives or related metabolites (55, 58, 59). Understanding the interplay
314 between SGLT2 inhibition and purine metabolism at the level of renal transport may yield new
315 insights into the pathogenesis and treatment of DKD. In our previous work, adenine was found to
316 stimulate matrix production in tubular cells by activating the mTOR signaling pathway (27).
317 Kidney biopsies from participants with T2D and healthy controls showed that T2D is associated
318 with upregulation of the mTORC1 pathway in each tubular segment of the nephron (27, 60).
319 SGLT2 inhibition is thought to attenuate aberrant mTOR signaling and restore proper mTOR

cycle regulation through the reduction of blood glucose levels (60, 61). As such, this upregulation of urine adenine was subsequently reduced with SGLT2 inhibition (27), which we were able to replicate in plasma adenine within the current analysis. Although more work is required to delineate the potential interplay between adenine, methionine and mTOR pathway in diabetic kidney disease, our data lend support that hyperglycemia drives glucose metabolism to the mTOR cycle and SGLT2 inhibition mitigates the effects of the mTOR pathway by way of adenine, methionine and cysteine.

Similar to our findings in plasma, in our prior work, we found that hyperglycemia augmented grouped urine amino acid pathways, including BCAAs, aromatic amino acids, and arginine biosynthesis (28). Notably, we found that empagliflozin was associated with an increase in urine BCAAs under clamped hyperglycemia (28). Although no changes to plasma BCAAs were demonstrated in response to empagliflozin, we observed an increase in plasma BCAAs in response to acute hyperglycemia. It appears that, while reduction-oxidation balance plays a role in modulating BCAA expression, the primary drivers are hyperglycemia and severe insulin resistance (40). These metabolic conditions may have a greater impact on BCAAs, suggesting that the altered glucose and insulin dynamics in such states override the effects of reduction-oxidation balance on BCAA metabolism. This provides insight into why an increase in BCAA was observed under ambient hyperglycemia with or without empagliflozin treatment, and not observed under euglycemic clamp. Moreover, in our prior work, we investigated other plasma metabolites involved in fatty acid metabolism, and we observed similar findings, particularly the increase in alanine, aspartate, and glutamate metabolism during acute hyperglycemia, prior to treatment (62). While we evaluated a range of different metabolites, the results reinforced the same overall conclusion. Furthermore, we did not observe an increase in plasma kynurenine

levels as seen in urine (28), however observed an increase under clamped euglycemia and clamped hyperglycemia with treatment, inconsistent with the hypothesized potential protective effect of empagliflozin against oxidative stress (63) and inflammation (64) in this patient population. Overall, the complex interplay between glucose metabolism, insulin resistance, and amino acid regulation should be further investigated, especially in patients with T1D.

This *post hoc* exploratory analysis has several limitations. Given that this analysis was exploratory and post hoc, and that the intervention trial lacked a control group, causality should not be directly inferred from these associations. Additionally, the small sample size and the relatively short 8-week treatment period limit the ability to generalize the long-term effects of SGLT2 inhibition to the broader population. Due to the limited duration of the glycemic clamp, the observed effects of hyperglycemia are confined to acute and ambient changes in metabolism. While the changes in plasma metabolites levels likely reflect metabolic alterations in the kidney, it remains possible that these findings may be attributed to broader systemic changes in energy metabolism, as we did not conduct kidney biopsies to confirm structural and functional alterations in renal mitochondria. Moreover, while our metabolomics data suggest altered amino acid metabolism, we did not perform metabolic flux analysis, which would provide direct evidence of amino acid utilization in energy metabolism. Therefore, future studies using stable isotope tracer-based flux analysis are warranted to mechanistically validate the metabolic roles of amino acids suggested by our findings. A key limitation of this study is the restricted coverage of metabolites, which constrains the depth of mechanistic insight that can be drawn from pathway-level analyses. The targeted metabolomics platform used in this work quantified a focused panel of amino acids and related metabolites, but did not include key intermediates in many of the metabolic pathways identified by enrichment tools such as MetaboAnalyst. As a result, pathway

assignments, including those related to folate metabolism, glyoxylate metabolism, BCAA metabolism and biosynthetic pathways for essential amino acids, may not reflect actual biochemical activity in vivo and should be interpreted with caution. Notably, intermediates such as alpha-ketoglutarate, which play a central role in transamination and TCA cycle integration, were not quantified. These computational pathway outputs are based on statistical overlap with canonical pathways, rather than biological plausibility or direct evidence of pathway flux. Finally, it is possible that some of the changes observed in this study could be linked to variations in total daily insulin during the study period. However, despite a 16.4% reduction in total daily insulin following drug initiation to mitigate hypoglycemia risk (29, 32), such adjustments are a standard part of care for individuals with T1D who are also receiving other anti-diabetic treatments.

PERSPECTIVES AND SIGNIFICANCE

Our findings highlight the metabolic changes induced by SGLT2 inhibition and acute hyperglycemia, particularly in the context of plasma amino acid metabolism. The increase in amino acid metabolism was mitigated by empagliflozin treatment and likely served as protection against dysfunctional systemic and renal metabolic alterations involving energy utilization and regulation. This provides further evidence for the concept of a ‘pseudo-fasting state’ induced by SGLT2 inhibition (22, 65, 66), to optimize energy substrate efficiency, particularly in energy-demanding organs like the heart and kidneys. Given the known cardiovascular and kidney risks in individuals with T1D, this adaptive metabolic response could have potential therapeutic implications. By improving energy utilization and supporting mitochondrial function, SGLT2 inhibition may offer protective benefits against diabetic complications, including cardiovascular

389 disease and kidney failure. These findings emphasize the importance of further investigating the
390 long-term impact of SGLT2 inhibitors on metabolic processes and organ function in T1D.
391

~~**SUPPLEMENTAL MATERIAL**~~

~~Supplemental Table S1–S3: https://doi.org/10.6084/m9.figshare.29403677~~

DATA AVAILABILITY

Source data for this study (https://doi.org/10.1161/CIRCULATIONAHA.113.005081) are not publicly available due to privacy or ethical restrictions. The source data are available to verified researchers upon request by contacting the corresponding author. The authors confirm that the data supporting the findings of this study are openly available in figshare at https://figshare.com/s/d3cedb654cf80a2d8766.

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COMPETING INTEREST

KS has served as a consultant and received honoraria from Boehringer Ingelheim, Janssen and Sanofi. KS has received research support from Boerhinger Ingelheim. DZIC has received honoraria from Boehringer Ingelheim-Lilly, Merck, AstraZeneca, Sanofi, Mitsubishi-Tanabe, Abbvie, Janssen, Bayer, Prometic, BMS, Maze, Gilead, CSL-Behring, Otsuka, Novartis,

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AUTHORS CONTRIBUTIONS

All authors contributed to collection of the data and data interpretation. L.K. performed the statistical analysis. L.K. wrote the first draft of the manuscript. D.Z.I.C was the principal investigator involved in the ATIRMA study design. All authors provided critical revision for important intellectual content and approved the final version of the manuscript for submission. The corresponding author K.S. takes full responsibility for the work and/or conduct of the study, had access to the data, and controlled the decision to publish.

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FIGURE LEGEND

Figure 1. Study design for the ATIRMA trial. Empagliflozin 25mg oral once daily was administered for 8 weeks to 40 participants with T1D.

Figure 2. Heat map of plasma metabolites significantly changed by hyperglycemia and empagliflozin. **3-4** – baseline, clamped euglycemia vs clamped hyperglycemia; **3-12** – clamped euglycemia, baseline vs post-treatment; **4-13** – clamped hyperglycemia, baseline vs post-treatment; **12-13** – post-treatment, clamped euglycemia vs clamped hyperglycemia. Two-tailed hypothesis testing was utilized at a significance level of 5% to compare the treatment effects on metabolites. For the individual metabolites, we applied the Benjamini-Hochberg procedure to correct for multiple comparisons, assuming independence and defining significant results as an FDR of ≤ 0.05.

Figure 3. Plasma metabolite pathways altered by acute hyperglycemia at baseline is reflected as a Z-score of mean ± standard deviations. ~~This is under clamped euglycemic (blue) and hyperglycemia (red).~~ a) Cysteine and methionine metabolites, b) one carbon pool by folate metabolites, c) arginine metabolites, d) valine, leucine, and isoleucine metabolites, e) arginine and proline metabolites, f) alanine, aspartate and glutamate metabolites, g) glyoxylate and dicarboxylate metabolites, h) glycine, serine and threonine metabolites, i) nitrogen metabolites and j) taurine and hypotaurine metabolites. Metabolomic pathway compositions were identified by MetaboAnalyst 6.0 software to accurately group 35 metabolites measured in plasma in this *post hoc* analysis with an FDR ≤ 0.1 (**Supplemental Table S1**). Metabolites from each pathway were projected onto first principal component using PCA to represent the pathway composition (**Supplemental Table S4~~3~~**). Repeated measures linear regression and *post hoc* least squares mean estimates were performed. Significant p-values are stated.

Figure 4. Plasma metabolite pathways at baseline and post-treatment (8 weeks of 25 mg empagliflozin once daily) are reflected as a Z-score of mean ± standard deviations. This is under both clamped euglycemic (blue) and hyperglycemia (red). a) Cysteine and methionine metabolites, b) one carbon pool by folate metabolites, c) arginine metabolites, d) valine, leucine, and isoleucine metabolites, e) alanine, aspartate, and glutamate metabolites, f) arginine and proline metabolites, g) glyoxylate and dicarboxylate metabolites, h) glycine, serine and threonine metabolites, i) nitrogen metabolites and j) taurine and hypotaurine metabolites. Metabolomic pathway compositions were identified by MetaboAnalyst 6.0 software to accurately group 35 metabolites measured in plasma in this *post hoc* analysis with an FDR ≤ 0.1 (**Supplemental Table S1**). Metabolites from each pathway were projected onto first principal component using PCA to represent the pathway composition (**Supplemental Table S4~~3~~**). Repeated measures linear regression and *post hoc* least squares mean estimates were performed. A treatment (T_x) by glycemia interaction was analyzed for each metabolic pathway. Only significant p-values are stated. *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$.

Table 1. Change in least square means estimate of log transformed plasma metabolites between euglycemia and acute hyperglycemia at baseline. Ranked by Benjamini Hochberg adjusted p values.

	Euglycemia vs Hyperglycemia, baseline (3-4)	
Metabolite	**Estimate**	***p* value**
Alanine	-0.1853	0.0035
Methionine	-0.1996	0.0035
Serine	-0.1402	0.0035
Threonine	-0.1495	0.0105
Glutamine	-0.0737	0.0245
Proline	-0.1473	0.0289
Asparagine	-0.0919	0.0588
Taurine	0.1152	0.0858
Kynurenine	-0.0251	0.0858
Arginine	-0.1151	0.0858
Adenine	-0.2651	0.3002
Lysine	-0.0529	0.3157
L-alpha-Aminobutyric acid	-0.0852	0.3157
Tryptophan	-0.0387	0.4066
Tyrosine	-0.2472	0.4440
Isoleucine	-0.1048	0.5650
Glycyl-histidine	0.1515	0.5650
Acetyl-Lysine	0.0520	0.5650
Glycine	-0.0467	0.5650
Glutamic Acid	0.0718	0.5650
Ornithine	-0.0626	0.5650
S-adenosylmethionine	0.0123	0.5650
Carnitine	0.0292	0.5650
SDMA+ADMA	0.0960	0.5732
Cysteine	0.0709	0.5732
Cystathionine	0.0941	0.5732
Histidine	-0.0164	0.6077
Nicotinic acid	0.0699	0.7035
Phenylalanine	-0.0407	0.7684
S-adenosylhomocysteine	0.1651	0.7748
5-Methylthioadenosine	-0.1225	0.7748
Mono-Methyl Arginine	-0.0248	0.8597
Citrulline	-0.0124	0.8936
Pipecolate	-0.0146	0.8936
Valine	-0.0076	0.9030

3-4 – baseline, clamped euglycemia vs clamped hyperglycemia.
All *p* values were adjusted with Benjamini Hochberg multiple comparisons.

Table 2. Least square means change in grouped plasma metabolites levels for comparison between euglycemia and hyperglycemia timepoints. Data reported as mean ± standard deviations.

Group	**3-4**
Cysteine and methionine metabolism	0.52 ± 0.12 $p<0.0001$
One carbon pool by folate	0.53 ± 0.14 $p=0.0002$
Arginine biosynthesis	0.34 ± 0.13 $p=0.0104$
Valine, leucine and isoleucine biosynthesis	0.31 ± 0.10 $p=0.0021$
Arginine and proline metabolism	0.27 ± 0.11 $p=0.0160$
Alanine, aspartate and glutamate metabolism	0.49 ± 0.13 $p=0.0003$
Glutathione metabolism	0.11 ± 0.11 $p=0.2848$
Glyoxylate and dicarboxylate metabolism	0.43 ± 0.13 $p=0.0012$
Phenylalanine, tyrosine and tryptophan biosynthesis	0.02 ± 0.01 $p=0.0705$
Glycine, serine and threonine metabolism	0.48 ± 0.13 $p=0.0004$
Nitrogen metabolism	0.11 ± 0.03 $p=0.0026$
Taurine and hypotaurine metabolism	–0.14 ± 0.06 $p=0.0149$

3-4 – baseline, clamped euglycemia vs clamped hyperglycemia.

Table 3. Change in least square means estimate of log transformed plasma metabolites with empagliflozin treatment. Ranked by Benjamini Hochberg adjusted p values.

	Baseline vs Post-treatment, at euglycemia (3-12)		Baseline vs Post-treatment, at hyperglycemia (4-13)		Treatment by glycemia
Metabolite	**Estimate**	***p* value**	**Estimate**	***p* value**	**Interaction**
Adenine	-0.4263	0.0378	0.1312	0.6580	0.0127
Carnitine	0.0257	0.5588	-0.0467	0.2755	0.0895
Tyrosine	0.0329	0.1828	0.0124	1.0000	0.1247
Phenylalanine	-0.0591	0.3589	0.0062	0.0123	0.2199
Cysteine	-0.1512	0.2044	-0.2851	0.0088	0.2377
Acetyl-Lysine	-0.1213	0.0379	-0.1935	0.0035	0.249
L-alpha-Aminobutyric acid	-0.2566	0.0035	-0.1820	0.0088	0.3068
Glutamine	0.0131	0.7798	0.0468	0.1461	0.3189
Asparagine	-0.0469	0.3747	-0.0001	1.0000	0.341
Cystathionine	-0.1353	0.4373	-0.2844	0.0455	0.3467
SDMA+ADMA	-0.1539	0.3698	-0.2948	0.0368	0.3509
5-Methylthioadenosine	-0.07712	0.8701	0.2647	0.5865	0.3663
Glutamic Acid	0.0236	0.8408	-0.0584	0.6580	0.3827
Glycyl-histidine	0.1773	0.3747	0.0225	1.0000	0.3915
Serine	-0.0383	0.5049	0.0021	1.0000	0.4258
Nicotinic acid	-0.1211	0.5049	0.0023	1.0000	0.4323
Arginine	0.0261	0.7798	0.0783	0.2755	0.4516
Mono-Methyl Arginine	0.4031	0.0035	0.3242	0.0035	0.4577
Valine	0.0076	0.9321	-0.0346	0.6580	0.4695
Methionine	-0.0163	0.8370	0.0250	0.7643	0.4832
Tryptophan	0.0485	0.2044	0.0732	0.0368	0.5098
Ornithine	0.0437	0.6468	-0.0106	1.0000	0.5199
Pipecolate	-0.01628	0.0580	-0.1053	0.2665	0.524
Taurine	-0.0892	0.2044	-0.1294	0.0368	0.5462
Proline	-0.0025	0.9876	-0.0388	0.6617	0.6065

Kynurenine	0.0715	0.0280	0.1548	0.0455	0.6316
Lysine	-0.0441	0.3747	-0.0244	0.6617	0.6574
S-adenosylhomocysteine	-0.1803	0.7798	-0.0266	1.0000	0.6643
S-adenosylmethionine	0.0122	0.5049	0.0186	0.2755	0.707
Alanine	0.1379	0.0280	0.1606	0.0088	0.7254
Histidine	0.0215	0.5049	0.0296	0.3381	0.787
Threonine	0.1051	0.0669	0.0979	0.0751	0.9055
Isoleucine	-0.1403	0.9321	-0.1106	1.0000	0.9439
Glycine	-0.0452	0.5049	-0.0470	0.5277	0.9771
Citrulline	-0.1482	0.0280	-0.1497	0.0140	0.9825

3-12 – clamped euglycemia, baseline vs post-treatment.
4-13 – clamped hyperglycemia, baseline vs post-treatment.

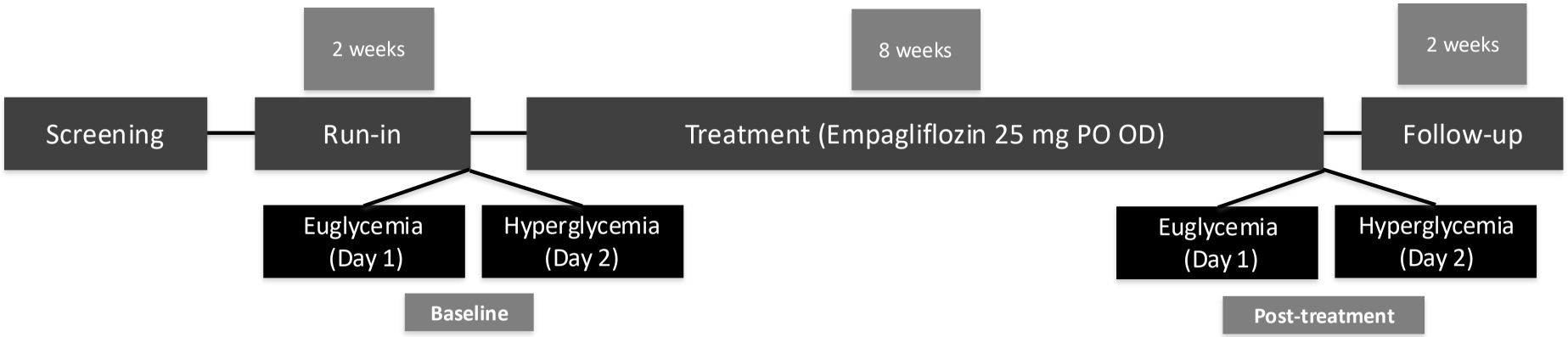
All visit change *p* values were adjusted with Benjamini Hochberg multiple comparisons.
Interaction – *p* value for treatment by glycemia interaction.

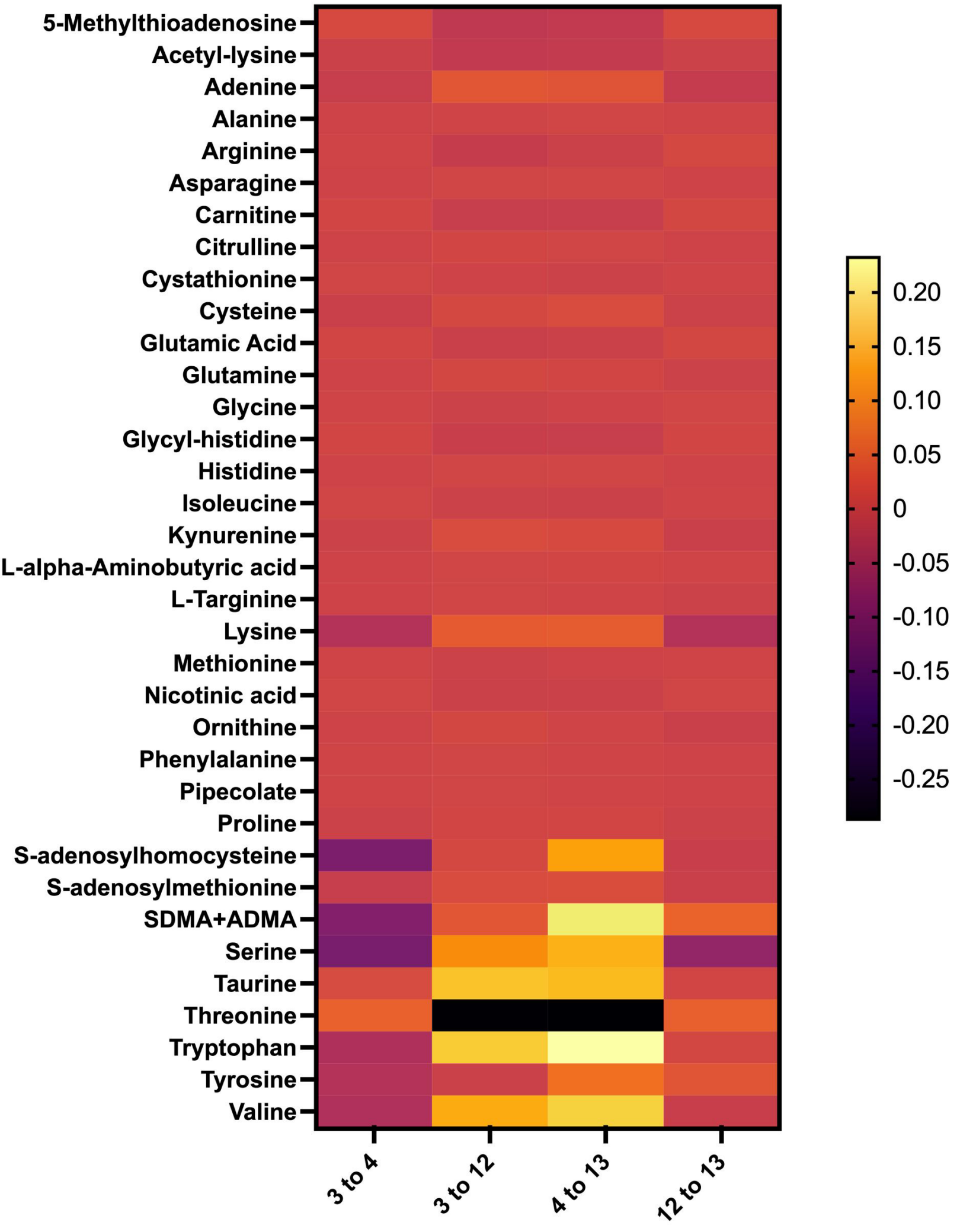
Table 4. Least square means change in grouped plasma metabolite levels for comparison between timepoints. Data reported as mean ± standard deviations.

Group	**3-12**	**4-13**	**Interaction**
Cysteine and methionine metabolism	0.31 ± 0.13 *p*=0.0152	0.20 ± 0.13 *p* =0.1155	0.5364
One carbon pool by folate	0.16 ± 0.14 *p*=0.2561	0.05 ± 0.14 *p*=0.7237	0.5804
Arginine biosynthesis	0.01 ± 0.13 *p*=0.9599	0.10 ± 0.13 *p*=0.4473	0.6168
Valine, leucine and isoleucine biosynthesis	0.11 ± 0.10 *p*=0.2715	0.09 ± 0.10 *p*=0.3657	0.8950
Arginine and proline metabolism	–0.06 ± 0.11 *p*=0.5686	–0.004 ± 0.11 *p*=0.9726	0.6700
Alanine, aspartate and glutamate metabolism	–0.06 ± 0.13 *p*=0.6680	–0.25 ± 0.13 *p*=0.0631	0.3118
Glutathione metabolism	–0.02 ± 0.11 *p*=0.8423	–0.11 ± 0.11 *p*=0.3126	0.5670
Glyoxylate and dicarboxylate metabolism	0.08 ± 0.13 *p*=0.5160	0.05 ± 0.13 *p*=0.7238	0.4806
Phenylalanine, tyrosine and tryptophan biosynthesis	–0.01 ± 0.01 *p*=0.3659	0.002 ± 0.01 *p*=0.8470	0.4406
Glycine, serine and threonine metabolism	0.06 ± 0.13 *p*=0.6439	0.01 ± 0.13 *p*=0.9462	0.7807
Nitrogen metabolism	–0.005 ± 0.04 *p*=0.8974	–0.07 ± 0.04 *p*=0.0399	0.1755
Taurine and hypotaurine metabolism	0.16 ± 0.06 *p*=0.0070	0.26 ± 0.06 *p*<0.0001	0.2017

3-12 – clamped euglycemia, baseline vs post 8-week treatment.
4-13 – clamped hyperglycemia, baseline vs post 8-week treatment.

Interaction – p value for Treatment by glycemia interaction


2 weeks
8 weeks
2 weeks
Screening
Run-in
Treatment (Empagliflozin 25 mg PO OD)
Follow-up
Euglycemia (Day 1)
Hyperglycemia (Day 2)
Euglycemia (Day 1)
Hyperglycemia (Day 2)
Baseline
Post-treatment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Adenine
Alanine
Arginine
Asparagine
Carnitine
Citrulline
Cystathionine
Cysteine
Glutamic Acid
Glutamine
Glycine
Glycyl-histidine
Histidine
Isoleucine
Kynurenine
L-alpha-Aminobutyric acid
L-Targinine
Lysine
Methionine
Nicotinic acid
Ornithine
Phenylalanine
Pipecolate
Proline
S-adenosylhomocysteine
S-adenosylmethionine
SDMA+ADMA
Serine
Taurine
Threonine
Tryptophan
Tyrosine
Valine
3 to 4
3 to 12
4 to 13
12 to 13
0.20
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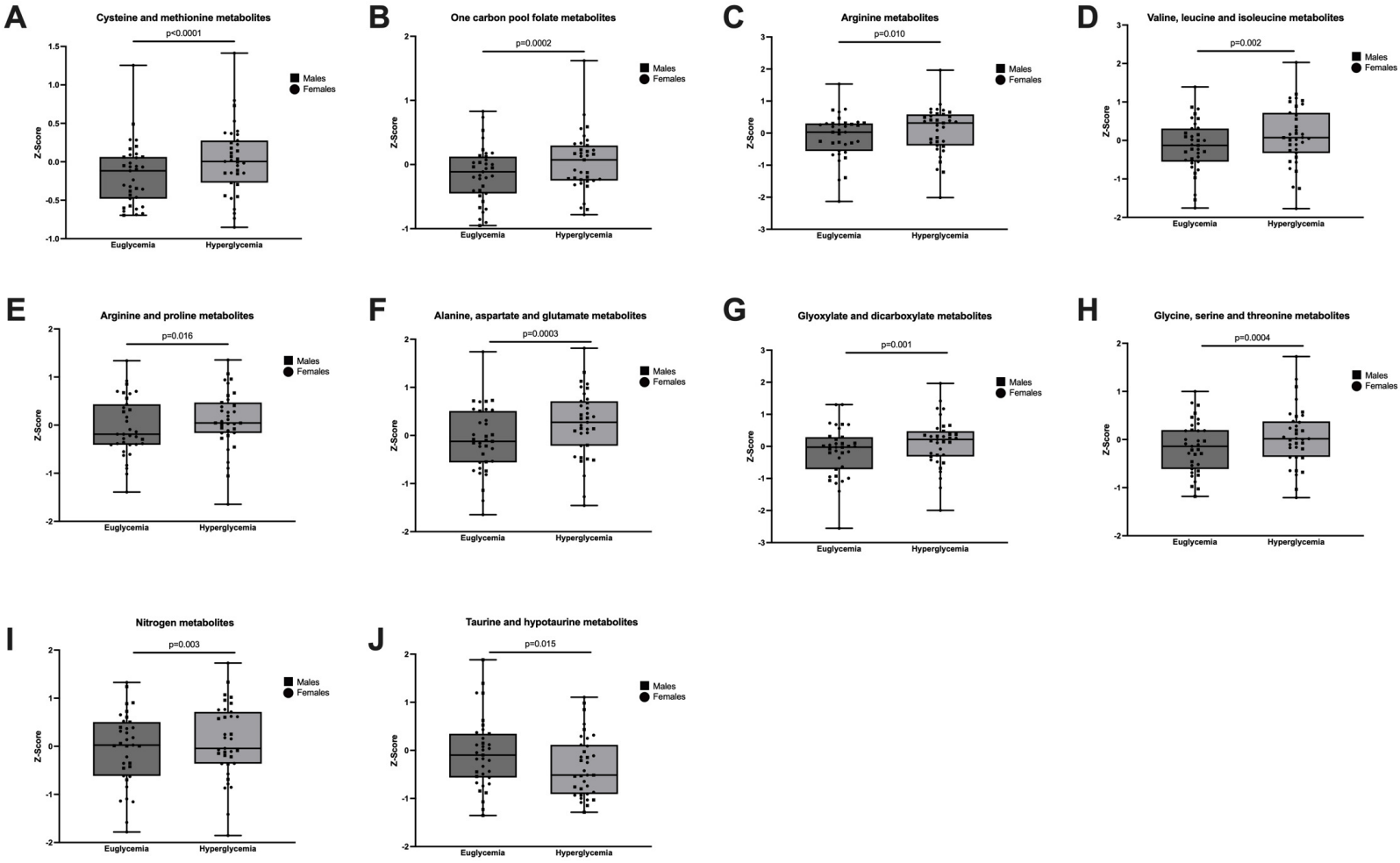
0
-0.05
-0.10
-0.15
-0.20
-0.25


A
Cysteine and methionine metabolites
p<0.0001
Z-Score
Euglycemia
Hyperglycemia
Males
Females
B
One carbon pool folate metabolites
p=0.0002
C
Arginine metabolites
p=0.010
D
Valine, leucine and isoleucine metabolites
p=0.002
E
Arginine and proline metabolites
p=0.016
F
Alanine, aspartate and glutamate metabolites
p=0.0003
G
Glyoxylate and dicarboxylate metabolites
p=0.001
H
Glycine, serine and threonine metabolites
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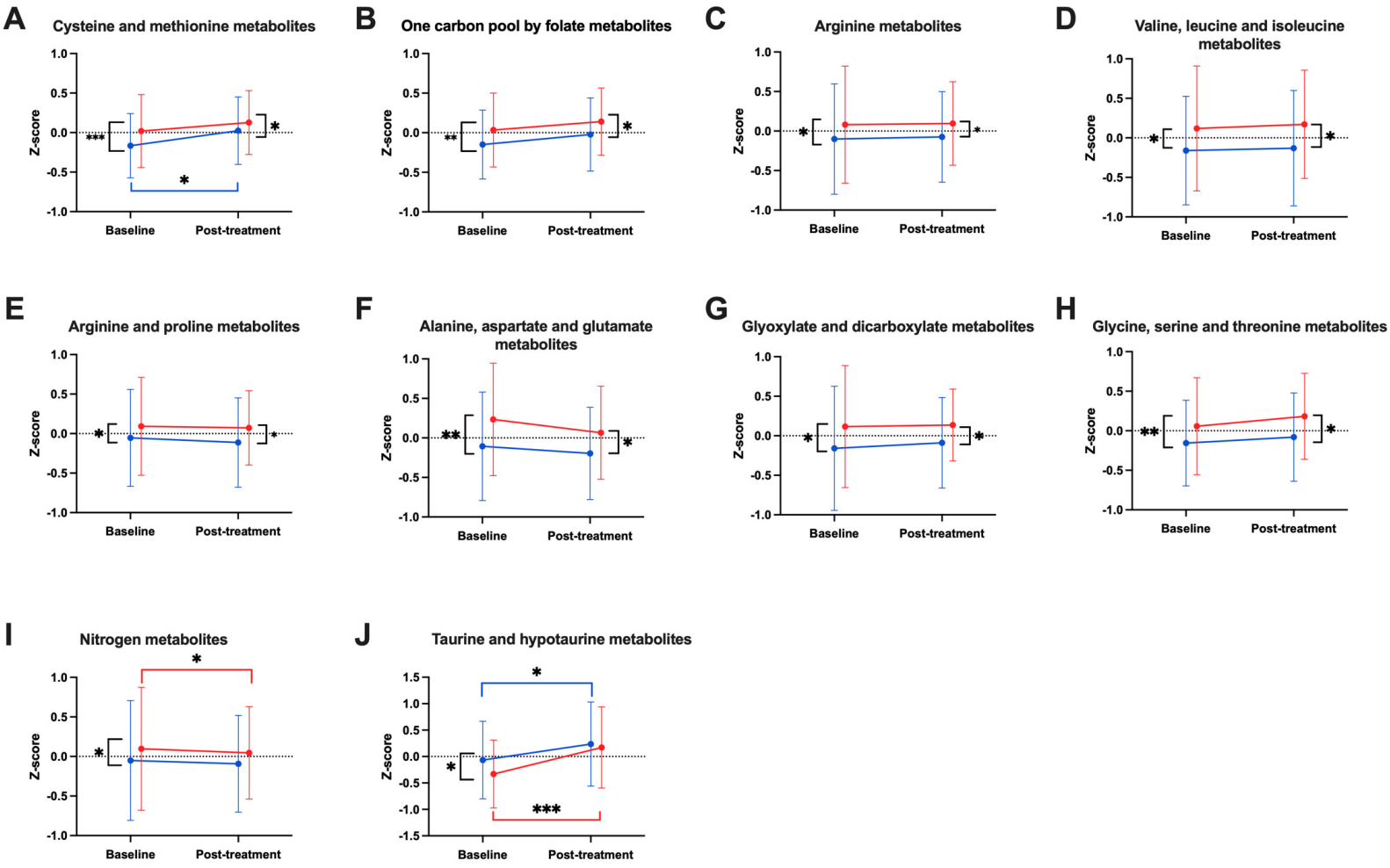
I
Nitrogen metabolites
p=0.003
J
Taurine and hypotaurine metabolites
p=0.015


A
Cysteine and methionine metabolites
B
One carbon pool by folate metabolites
C
Arginine metabolites
D
Valine, leucine and isoleucine metabolites
E
Arginine and proline metabolites
F
Alanine, aspartate and glutamate metabolites
G
Glyoxylate and dicarboxylate metabolites
H
Glycine, serine and threonine metabolites
I
Nitrogen metabolites
J
Taurine and hypotaurine metabolites
Z-score
Baseline
Post-treatment

SGLT2 Inhibition Attenuates Hyperglycemia-Induced Amino Acid Metabolism in T1D

METHODS

ATIRMA trial:

n=40
Diagnosed with T1D
Normotensive
Normoalbuminuric

- Quantification of 35 metabolites in plasma
- Grouped metabolites into key amino acid pathways using MetaboAnalyst 5.0
- Compare effect of hyperglycemia and empagliflozin on metabolic pathways using PCA
- Four timepoints assessed:
  - Baseline, euglycemia
  - Baseline, hyperglycemia
  - Post 8-week treatment, euglycemia
  - Post 8-week treatment, hyperglycemia

OUTCOME

Acute hyperglycemia significantly altered amino acid metabolism, increasing levels of methionine, serine, threonine, proline, and asparagine, and activated multiple metabolic pathways including branched-chain amino acid and nitrogen metabolism.

Empagliflozin treatment mitigated hyperglycemia-induced amino acid alterations, attenuating the increase in cysteine and methionine metabolism under hyperglycemia and decreasing nitrogen metabolism.

CONCLUSION Together, these findings highlight the potential of SGLT2 inhibition to optimize amino acid metabolism and protect cardiac and renal function in type 1 diabetes.