The metabolic and proteomic signatures of hypoglycemia in type 2 diabetes.

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Abstract:

Aims. Hypoglycemia is often related to insulin therapy in both type 1 and type 2 diabetes (T2D) patients. It may result in cardiovascular and neurological sequelae, and increased mortality. The molecular processes underlying the body's response to hypoglycemia, however, require further understanding. Our objective was to determine biochemical changes under hypoglycemia in healthy controls and T2D.

Materials and Methods Here we report a hypoglycemic clamp study with seven healthy controls and ten subjects with T2D. Blood was withdrawn at four time points: baseline after an overnight fast, clamping to euglycemia at 90mg/dl, hypoglycemia at 50mg/dl, and 24 hours later after overnight fast. Deep molecular phenotyping using non-targeted metabolomics and the Somalogic aptamer-based proteomics platforms was performed on collected samples.

Results. 955 metabolites and 1,125 proteins were identified, with significant alterations in over 90 molecules. A number of metabolites significantly increased during hypoglycemia, but only cortisol, adenosine-3',5'-cyclic monophosphate (cyclic AMP), and pregnenolone sulfate, were independent of insulin. By contrast, identified protein changes were triggered by hypoglycemia rather than insulin. T2D had significantly higher levels of fatty acids including 10−nonadecenoate, linolenate, and

dihomo−linoleate during hypoglycemia compared to the control. Molecules contributing to cardiovascular complications such as fatty-acid-binding protein-3 and pregnenolone sulfate were altered in T2D subjects during hypoglycemia. Almost all molecules returned to baseline at 24 hours.

Conclusions. Our study provides a comprehensive description of molecular events that are triggered by insulin-induced hypoglycemia. We identified deregulated pathways in T2D that may play a role in the pathophysiology of hypoglycemia-induced cardiovascular complications.

Introduction

In healthy individuals, blood glucose levels are maintained within a narrow range by mechanisms that dynamically respond to starvation, food intake, physical exercise and physiological stresses. Following a meal, insulin secretion restores normoglycemia by simultaneously decreasing hepatic glucose production and stimulating glucose uptake by skeletal muscle and adipose tissue [1]. Conversely, during fasting, regulatory hormonal responses are activated to prevent hypoglycemia (defined as blood glucose below 70mg/dl: 3.9 mmol/l) [2]. Decreasing glucose levels to 65-70mg/dl (3.9 - 3.6 mmol/l) stimulates epinephrine secretion, hepatic glucose release (glycogenolysis) and glucose formation (gluconeogenesis) [3]. Epinephrine inhibits insulin secretion and decreases utilization of glucose by peripheral tissues, as well as increasing the release of

gluconeogenic substrates. Hypoglycemia results in cortisol secretion that limits glucose utilization whilst simultaneously enhancing hepatic glucose production [3].

In advanced diabetes, medical control of glycemia is reached with insulin therapy or drugs stimulating insulin release (sulfonylureas), which is frequently associated with an increased risk of hypoglycemic episodes [4]. These hypoglycemic events appear more commonly in type 1 diabetes (T1D), but have increasingly been reported in T2D [5] as well, where the risk of hypoglycemia increases with the duration of insulin therapy and the progression of pancreatic β-cell failure [5]. In both patients with T1D and T2D, a reduction in the glycogen and epinephrine responses to hypoglycemia has been correlated with treatment duration [5].

Hypoglycemic episodes have been associated with cardiovascular complications, including myocardial ischemia [6] and cardiac arrhythmias [7]. They are also related to development of atherosclerosis through increased inflammation and endothelial dysfunction [8], and were observed to be associated with neurological deficits [4]. These cardiovascular and neurological complications associated with hypoglycemia are thought to occur, in part, due to diabetes-related biochemical dysregulations. Therefore, a greater insight into the physiological responses to insulin-induced hypoglycemia in healthy controls as compared to insulin naïve patients with T2D may improve our understanding of those processes.

A global characterization of biochemical process in an organism may be achieved through metabolic analysis, which enables quantitative or semi quantitative identification of small molecule composition in the sample [9]. Plasma proteome profiles may be investigated with highly multiplexed, aptamer-based, affinity proteomics platform SOMA(scan)[10]. Metabolic and proteomic changes associated with T2D have been well characterized in previous studies[11-13]. For instance, elevated levels of branched chain and aromatic amino acids were associated with insulin resistance as well as with early onset of diabetes[14]. Alterations in lipid metabolism including fatty acid metabolism, carnitines and glycerophospholipids have been identified in T2D[15,16]. Alterations in proteome levels of Fibroblast growth factor 23, Vascular endothelial growth factor A, and C-reactive protein were previously associated with T2D progression[17]. However, the impact of hypoglycemia on the metabolome and proteome in T2D patients has not been previously investigated.

The objective of our study was to determine, for the first time, biochemical changes occurring in healthy individuals and patients with T2D under insulin induced euglycemic and hypoglycemic conditions as well as 24 hours after the challenge, using state-of-the-art metabolomics based on a non-targeted profiling and proteomics approach enabling quantification of 1,125 proteins levels in blood plasma samples. Our experimental setup simulates the condition of patients treated with insulin therapy experiencing hypoglycemia, in which they experience opposing signaling from infused insulin,

"signaling" a well-fed state, and low glucose level, "signaling" starvation. In this study, we determined the molecular alterations in plasma of controls and individuals with T2D under insulin induced euglycemia and hypoglycemia.

Materials and methods:

Study design.

Seven healthy controls (5 male, 2 female; age 46.9± 6.3 SD; BMI 29.1± 4.2 SD) and 10 patients with T2D (7 male, 3 female; age 46.3± 5.6 SD; BMI 34.6 ± 7.2 SD) were recruited. The following inclusion criteria were applied for subject enrolment: 1) non-smoking men and women aged between 40 – 60 years; 2) no medical condition in control group subjects; 3) T2D for less than 10 years with no history of microvascular disease (retinopathy, nephropathy, and neuropathy), HbA1C ≤9.5% (80.3 mmol/mol), diet or metformin treatment in the T2D group. The exclusion criteria were as follows: pregnancy, lack of contraception in women with reproductive capacity, chronic medical conditions, smoking, evidence of ischemia on ECG, drop attacks, alcohol or drug abuse, psychiatric illness, or previous history of seizures.

The study participants underwent a euglycemic-hypoglycemic clamp as outlined in **Figure 1 A**. The study was started after an overnight (10h) fast, and the blood samples were collected at 4 time points, under the following conditions: 1) baseline, after an overnight (10h) fast; 2) after the subjects underwent a euglycaemic-hyperinsulinaemic

clamp to a blood glucose of 90mg/dl (5mmol/l; euglycemia) for 1 hour; 3) after the subjects underwent a hypoglycaemic-hyperinsulinaemic clamp, to a blood glucose of 50mg/dl (2.8mmol/l; hypoglycemia) for 1 hour; and 4) 24h after the challenge experiment and in an overnight (10h) fasting state.

The Yorkshire and Humber Research Ethics Committee approved this study (Clinical trial reg. no: NCT02205996). All experiments and procedures conformed to the 1975 Declaration of Helsinki. All experiments were conducted at the Diabetes Centre Hull Royal Infirmary. All study participants gave their signed informed consent.

Hyperinsulinaemic euglycaemic-hypoglycaemic clamp:

Three polyethylene catheters were inserted in the antecubital fossa and back of the hand veins enabling insulin/dextrose infusions, blood glucose measurements, and blood sampling. To arterialize the veins, the hand with the catheter was constantly warmed at 60°C, using a heat box. To guide the rate of the dextrose infusion, a small blood volume was withdrawn every 5 minutes from the catheter, and the blood was analyzed for the glucose level using HemoCue® Glucose 201+ (HemoCue AB, Angelholm, Sweden). The HemoCue's microcuvettes were stored and handled according to the manufacturer's protocol and the machine was calibrated before each session using manufacturer's control solutions.

The insulin infusion rate was constant throughout the euglycemic-hypoglycemic clamp at [60mU/body surface area (m^2)/min]. The body surface area (m^2) was calculated as previously described [0.007184 x (height(cm)^0.725) x (weight(kg)^0.425)] [18]. The blood glucose level was modulated by the infusion of 20% dextrose, which was adjusted every 5 minutes to achieve the targeted, stable blood glucose level of 90mg/dl (5 mmol/L) for 1 hour under the euglycaemic clamp, and 50mg/dl (2.8 mmol/L) for 1 hour under the hypoglycemic clamp. The blood glucose level was decreased gradually for around one hour per condition, and the overall duration of the euglycemic-hypoglycemic clamp was 4 hours.

Metabolomics measurements.

Non-targeted metabolic profiling

In total, 67 samples were submitted for metabolic profiling. The metabolite measurements were performed on the Metabolon platform deploying ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS) and gas chromatography-mass spectrometry (GC-MS) approaches, as previously described[19,20].

Briefly, recovery standards were added into each sample prior to extraction for quality control (QC) purposes. The sample extract was then divided into aliquots designated for the analysis using the following: 1) UPLC-MS/MS with positive ion mode

electrospray ionization (ESI); 2) UPLC-MS/MS with negative ion mode ESI; 3) hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS; 4) GC-MS.

The sample extract was dried under nitrogen flow and reconstituted in solvents compatible with each of the four analytical methods.

Metabolite measurements with UPLC–MS

Three of the sample aliquots were designated for LC-MS measurements. The samples were reconstituted in acidic or basic solvents. The first sample aliquot, reconstituted in acidic conditions, was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm) with water and methanol containing 0.1% formic acid (FA) and was analyzed using acidic positive ion conditions. The second sample aliquot, reconstituted in basic solvent, was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm) with water and methanol containing 6.5mM ammonium bicarbonate, and was analyzed using basic negative ion conditions. The third aliquot was gradient eluted from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using water and acetonitrile with 10mM ammonium formate. The flow rate was 350 μL/min. The sample injection volume was 5 μL.

The sample separation and measurements were performed on Waters ACQUITY UPLC in-line to Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and an Orbitrap mass

analyzer. In the MS analysis, the scan range varied between methods but fell within the range of 70-1000 m/z.

Metabolite measurements with GC–MS

Derivatization was performed using N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) subsequent to drying using nitrogen. Separation was achieved using a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 um film thickness), and helium was deployed as the carrier gas at a flow rate of 1 ml/min. The temperature ramp used for separation was from 60 to 340°C over a 17.5 min period.

The measurements were performed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI). The MS scan range was from 50-750 m/z.

Metabolite identification

Metabolon's hardware and software were deployed for raw data extraction. These systems are utilizing web-service platform relaying on Microsoft's.NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters. Spectral features were identified by comparison to entries in a library of purified standards. Metabolon's libraries also contain entries for features not identified through standard compound comparison. These were marked by "X-" followed by a numeric identifier and designated "unknown" in this manuscript.

We identified 975 molecules, including 581 known metabolites and 394 unknown compounds.

SOMA-scan measurements

The protein quantification was performed using a Slow Off-rate Modified Aptamer (SOMAmer)–based protein array, as previously described[21]. Briefly, EDTA plasma samples were diluted and the following assay steps were performed: 1) binding – analytes and primer beads (PB)-SOMAmers (fully synthetic fluorophore-labeled SOMAmer coupled to a biotin moiety through a photocleavable linker) are equilibrated; 2) *Catch I* - all analyte/SOMAmers complexes are immobilized on a streptavidin-substituted support. Washing steps remove proteins not stably bound to PB-SOMAmers and bound protein is biotinylated; 3) *Cleave* - long-wave ultraviolet light is applied to release analyte-SOMAmer complexes into the solution; 4) *Catch II* – analyte-SOMAmer complexes are selectively immobilized on streptavidin support via the introduced analyte-borne biotinylation. Further washing continues to select against non-specific analyte/SOMAmer complexes; 5) *Elution* – Denaturation disrupts analyte-SOMAmer complexes. Released SOMAmers serve as surrogates for quantification of analyte concentrations; 6) *Quantification* – hybridisation to custom arrays of SOMAmer-complementary oligonucleotides (**Supplementary Figure 1**).

Normalization of raw intensities, hybridization, median signal and calibration signal were performed based on the standard samples included on each plate, as previously described [21].

We used version 3.1 of the SomaScan Assay, which quantifies 1129 features. Four of them failed the SOMA feature QC (i.e. ColCheck value 'FAIL') and we removed those prior to further analysis.

Statistical data analysis.

Data analysis was performed using our in-house tool autonomics (https://github.com/bhagwataditya/autonomics). We first coded the interaction of two conditions and four time points as eight different subgroups. Then we fitted the general linear model log2(exprs) ~ 0 + subgroup | subject_id, and investigated the following contrasts: 1) T2D at euglycemia – T2D at baseline; 2) Control at euglycemia – Control at baseline; 3) T2D at hypoglycemia – T2D at baseline; 4) Control at hypoglycemia – Control at baseline; 5) T2D at 24h after hypoglycemia was induced – T2D at baseline; 6) Control at 24h after hypoglycemia was induced – Control at baseline, using the R package limma[22]. P-values (0.05) were corrected for multiple testing using Bonferroni.

Out of 975 detected metabolites, 20 metabolites absent in all samples of either subgroup were not computed. In total, we generated contrasts for 955 metabolites and 1125 proteins.

The Orthogonal Projections to Latent Structures (OPLS) analysis using condition and time as phenotypes was performed with SIMCA version 14 (Umetrics, Umea, Sweden).

The Venn diagram was created using an online tool: http://bioinformatics.psb.ugent.be/webtools/Venn/

The pathway analysis was conducted using MetaboAnalyst 4.0 [23] as well as Ingenuity Pathway Analysis software (IPA) (Ingenuity Systems, Redwood City, CA) for network analysis of proteins that were differentially regulated under euglycemic-hypoglycemic clamp.

Results:

Euglycemic-hypoglycemic clamp and normal physiological responses

To test whether individuals responded to the euglycemic-hypoglycemic clamp in the expected, physiological manner, we monitored insulin levels as well as levels of glucose and cortisol at each experimental point of the study (baseline, induced euglycemia, induced hypoglycemia, 24 h after hypoglycemia) (**Figure 1A**).

After overnight fasting (at the experimental baseline), the blood glucose in the controls was at the normal physiological level (4.8+/- 0.5 SD) and in individuals with T2D was above the normal level (6.2+/- 1.1 SD). The insulin infusion, was reflected in the plasma, as we observed a significant increase in levels of insulin under euglycemia in both

controls (p-value = 2.37 x 10^{-5}) and T2D (p-value = 4.87 x 10^{-5}) (**Figure 1B & C**). The glucose level under euglycemia remained unchanged in controls (p-value = 0.25) but was significantly decreased in T2D (p-value = 2.00 x 10^{-6}) (**Figure 1D & E**). At the condition of insulin-induced hypoglycemia, the blood glucose level was significantly decreased in both controls (p-value = 2.54 x 10^{-5}) and T2D (p-value = 2.52 x 10^{-13}), and the plasma insulin level remained significantly elevated in both groups (for controls p-value = 3.12 x 10^{-5}; for T2D p-value = 5.51 x 10^{-3}). A significant increase in cortisol level was observed under hypoglycemia in controls (p-value = 9.31 x 10^{-5}) and T2D (p-value = 2.39 x 10^{-8}) but not under insulin induced euglycemia (**Figure 1 F & G**). The levels of all investigated molecules returned to baseline at 24 h after hypoglycemia was induced.

Plasma metabolome and proteome responses to euglycemic-hypoglycemic clamp

We applied metabolome-wide profiling to improve the understanding of response to insulin-induced hypoglycemia in healthy and T2D subjects. We quantified relative levels of 955 distinct metabolites from eight primary pathways: the metabolism of amino acids, carbohydrates, lipids, nucleotides, cofactors, vitamins, peptides and xenobiotics. In addition, we quantified levels of 1,125 proteins in plasma. We used orthogonal partial least squares (OPLS) regression to examine whether metabolic, together with proteomic, profiling discriminates healthy controls from T2D patients at the baseline and at each experimental condition point. The OPLS analysis revealed four distinct clusters separating healthy controls from T2D at all examined condition points (**Figure 2A**). In

both control as well as T2D groups, we identified two distinct clusters: euglycemic and hypoglycemic samples clustered together that were clearly separated from the baseline and 24 hour time point samples. This data suggests health status to be a major determinant of the separation, and that insulin-induced hypoglycemia might trigger different responses, resulting in diverse molecular manifestations, between control and T2D patients.

We conducted statistical data analysis to assess the number of significantly altered metabolites and proteins under euglycemic-hypoglycemic clamp. Overall, 89 metabolites (**Supplementary Table 1**) and 13 proteins (**Supplementary Table 2**) showed Bonferroni significant alterations under examined conditions (for metabolites p-value ≤ 5.2×10^{-5}; for proteins p-value ≤ 4.4×10^{-5}). Under conditions of insulin-induced euglycemia, we observed Bonferroni significant alterations in 54 and 48 molecules in controls and T2D, respectively (for metabolites p-value ≤ 5.2×10^{-5}; for proteins p-value ≤ 4.4×10^{-5}). Insulin-induced hypoglycemia triggered 66 and 78 alterations at Bonferroni significant level in plasma of controls and T2D, respectively (for metabolites p-value ≤ 5.2×10^{-5}; for proteins p-value ≤ 4.4×10^{-5}).

We further investigated the overlap of significantly altered molecules between controls and T2D under euglycemia and hypoglycemia. Among 102 molecules (metabolites and proteins) showing Bonferroni significant alteration, 38 molecules, mainly metabolites involved in lipid metabolism, overlapped between all four groups; five molecules

(citrulline, and four lipid molecules) were unique for controls under euglycemia, one molecule (docosapentaenoate (n6 DPA; 22:5n6)) was unique for T2D under euglycemia, 9 molecules (Pancreatic hormone, three unknowns, uridine gamma-glutamyl-2-aminobutyrate, hexadecanedioate, docosadienoate (22:2n6), and 1-arachidonoylglycerophosphoinositol) were exclusive for controls under hypoglycemia and 28 molecules (10 proteins and 18 metabolites including steroids, amino acids, gamma-glutamyl amino acid, and fatty acids) were attributed to T2D under hypoglycemia (**Figure 2B** and **Supplementary Table 3**).

Fatty acid metabolism is predominantly regulated by insulin infusion.

To provide further insight into human physiology under euglycemic-hypoglycemic clamp conditions, we investigated the metabolites showing Bonferroni significant (p-value ≤ 5.23×10^{-5}) alterations after the insulin infusion induced euglycemia (5.0 ±0.3 SD mmol/L), in healthy and T2D individuals. Insulin infusion under normal blood glucose levels leads to a Bonferroni- significant (p-value ≤ 5.23×10^{-5}) decrease in 56 metabolites (53 in healthy controls and 45 in T2D patients), 77% overlap between controls and T2D subjects was observed. The metabolites that were not identified as overlapping showed the same trend of changes for all of these, apart from glucose.

The majority of decreased metabolites (29 out of 56 metabolites) were fatty acids with different chain characteristics, including long and medium chain, polyunsaturated, branched chain, mono- and di- hydroxylated.

We also observed a Bonferroni significant (p-value ≤ 5.23×10^{-5}) decrease in levels of amino acids, including isoleucine, leucine and methionine, as well as metabolites involved in branch chain amino acid metabolism (4-methyl-2-oxopentanoate and 3-methyl-2-oxovalerate), and six unknowns.

The metabolic alterations that we observed after insulin infusion, under physiological glucose levels, reflected the body's responses to insulin, which, under euglycemic clamp, were significantly altered in comparison to baseline in both control and T2D subjects (**Figure 1C**).

The insulin infusion under normal glucose levels had a minor effect on the blood proteome and only two proteins showing Bonferroni significant alterations (p-value ≤ 4.4×10^{-5}) were identified, namely insulin and Activated Protein C (**Supplementary Table 2**).

Steroids and amino acid metabolism is regulated under insulin induced hypoglycemia

Under insulin-induced hypoglycemia (blood glucose ~ 50mg/dl), we identified 82 significantly (p-value ≤ 4.4×10^{-5}) altered metabolites in comparison to baseline levels

(**Supplementary Table 1**). Among these 82 molecules, 50 metabolites showed already a Bonferroni significant decrease (p-value ≤ 4.4 x 10^{-5}) under euglycemia and 32 metabolites were altered at a Bonferroni significant level (p-value ≤ 4.4 x 10^{-5}) under hypoglycemia. Of these 32 metabolites, five molecules (isocaprate, corticosterone, cortisol, pregnelone sulfate and adenosine-3',5'-cyclic monophosphate) were increased and 27 decreased in comparison with the baseline level.

Only three of these 32 metabolites, namely cortisol, adenosine-3',5'-cyclic monophosphate (cyclic AMP), and pregnenolone sulfate (**Supplementary Figure 2 A, B, C**) appeared specific to hypoglycemia and did not show even a trend under euglycemic insulin infusion. Moreover, those metabolites showed an increase at a Bonferroni significant (p-value ≤ 4.4 x 10^{-5}) level only in individuals with T2D. Pregnenolone sulfate is synthetized from cholesterol, which leads to formation of isocaproate and pregnelone [24]. We identified significantly increased (p-value ≤ 4.4 x 10^{-5}) levels of isocaproate under hypoglycemia in T2D (**Supplementary Figure 2 D**).

With respect to the remaining 29 metabolites, mainly amino acids (serine, valine, lysine, proline, arginine, tyrosine, phenylalanine and asparagine) and gamma-glutamyl amino acids showed a nominally significant decrease under insulin infusion and euglycemia, and became Bonferroni significant under hypoglycemia. Noteworthy, asparagine, phenylalanine, tyrosine, arginine and proline showed Bonferroni significant decreases (p-value ≤ 4.4 x 10^{-5}) only in T2D (**Supplementary Table 1**).

Steroids and fatty acid metabolism pathways are enriched under euglycemic-hypoglycemic clamp

We conducted enrichment analysis to elucidate the metabolic pathways affected under euglycemic-hypoglycemic clamp in controls and T2D. This analysis revealed 21 metabolic pathways showing significantly relevant enrichment (**Figure 3A & Supplementary Table 4**). The strongest enrichment was observed in steroids (p-value = 1×10^{-5}) and fatty acid metabolism (poly unsaturated fatty acids PUFA (p-value = 1.2×10^{-4}) and long chain fatty acids (p-value = 1×10^{-5})) pathways. The enrichment in steroid metabolism was observed for controls and T2D already under euglycemia, whereas the pathway of fatty acid metabolism was specific only to hypoglycemia in both groups. Additionally, the metabolism of bile acid and gamma-glutamyl amino acid was significantly affected under euglycemic-hypoglycemic clamp as the enrichment was observed in both controls and T2D. The enrichment in glutathione metabolism and monoglycerols was observed only under hypoglycemia in T2D. The pathway enrichment analysis results further pinpoint the impact of the euglycemic-hypoglycemic clamp on steroids and fatty acid metabolism.

We further investigated the metabolic pathway enrichment in the context of metabolic profile of different diseases. Data analysis revealed that metabolic manifestations under hypoglycemia in T2D showed a significant association with refractory localization-related epilepsy[25], maple syrup urine disease[26] as well as ornithine transcarbamylase

deficiency[27] (**Supplementary Figure 3B**). We compared the directionality of metabolic alterations observed in those diseases with the metabolic manifestations in T2D under hypoglycemia. The metabolic alterations observed only in refractory localization-related epilepsy showed the same directionality as the one observed in our study. We did not observe a significant association of metabolic manifestation with any of diseases in the control group (**Supplementary Figure 3 A**).

Fatty acid metabolism differentiates T2D from healthy individuals under hypoglycemia

We identified 26 metabolites under hypoglycemia, which were differential between T2D and control. Five of them differentiate T2D from healthy controls at Bonferroni significance level (p-value ≤ 4.4×10^{-5}) (**Figure 4**) and 21 were nominally significant (p-value ≤ 0.05) (**Supplementary Table 1**). The majority of metabolites differentiating T2D from healthy controls were involved in lipid metabolism (20 out of 26), mainly long chain fatty acids and polyunsaturated fatty acids. The levels of the differentiating metabolites were elevated in T2D for almost all metabolites except gluconate.

Insulin induced hypoglycemia triggers inflammatory responses in plasma proteome.

The hypoglycemic clamp resulted in observed, significantly relevant (p-value ≤ 4.4×10^{-5}), changes in plasma levels of 13 proteins. These changes were apparently

independent of the infusion of insulin, as they appeared unaltered under euglycemia (**Supplementary Table 2**). The majority of the altered proteins were observed at Bonferroni significance levels only in T2D subjects; Ephrin-A5, prolactin, Myoglobin, Wnt inhibitory factor 1, R-spondin-2 and dynactin subunit 2. We also identified molecules including chordin-like protein 1, fatty acid-binding protein 3, and Repulsive guidance molecule A, which were altered at a Bonferroni significant level in T2D subject and showed a trend under euglycemia in healthy controls (**Supplementary Table 2**).

To provide further insight into identified proteins, we conducted pathway analysis using Ingenuity® Pathway Analysis (IPA®) software. Given the relatively small numbers of proteins showing a Bonferroni significant level, we also included proteins showing nominally significant (p-value ≤ 0.05) alterations under hypoglycemia. The number of nominally significant (p-value ≤ 0.05) proteins was 63 in controls and 292 in T2D (**Supplementary Table 5**). The data analysis revealed that the altered molecules could be linked to inflammatory responses, cellular movements related to inflammatory events as well as cardiovascular diseases (**Supplementary Figure 4 & 5**).

In the control group, molecules altered under insulin-induced hypoglycemia were linked to activation and movement (e.g. infiltration, migration and adhesion) of phagocytes, leukocytes, neutrophils and platelets, as well as with body and organ inflammation (**Supplementary Figure 4A**). Additionally, the analysis revealed that altered molecules in the control group can contribute to cardiovascular complications such as thrombosis,

abnormal cardiovascular system morphology, damage of endothelial tissue as well as ischemic stroke (**Supplementary Figure 4B**); there was a strong overlap between the molecules implicated in inflammation and cardiovascular pathways.

In the T2D group, the analysis revealed that, among nominally significant (p-value ≤ 0.05) molecules, 78 were associated with body inflammation and cellular processes involved in inflammatory responses such as activation, accumulation, movement, migration, recruitment and infiltration of various cells of the immune system including phagocytes, leukocytes, monocytes, lymphocytes, neutrophils and granulocytes (**Supplementary Figure 5A**). The altered molecules were also linked with inflammatory diseases, such as rheumatoid arthritis and psoriasis, as well as liver inflammation. Additionally, the analysis of the nominally significant molecules (p-value ≤ 0.05) implied a connection with cardiac infarction (**Supplementary Figure 5B**).

Hypoglycemia has minimal effects on the metabolome after 24 hours.

To determine if hypoglycemia results in extended metabolic changes, we sampled the subjects 24h after the induced hypoglycemia. Only three metabolites (**Supplementary Figure 6**) were altered significantly at this time point (1-stearoylglycerophosphoinositol, 1,6-anhydroglucose and X – 16938). We observed a Bonferroni significant decrease in the level of 1-stearoylglycerophosphoinositol in both T2D and healthy controls. The

remaining two metabolites, namely increased 1,6-anhydroglucose and increased X – 16938, were attributed to control and T2D, respectively.

Discussion:

This is the first study deploying metabolomics and proteomics (SOMA-scan) to comprehensively explore molecular responses of control as well as T2D subjects to insulin stimuli under euglycemia and hypoglycemia. This study shows that euglycemia followed by hypoglycemia triggers metabolome-wide alterations, but has a limited impact on the blood proteome, where only 13 proteins were identified as significantly altered. In contrast to metabolomics, the selected approach for proteomics was targeted, therefore we may have observed only 13 significantly altered proteins. Despite the small cohort size in this study, which limits statistical power, we found that more than 90 molecules were highly sensitive in their response to glucose/insulin stimuli.

Insulin infusion in euglycemia (90mg/dl) triggered a large spectrum of metabolic changes, mainly involved in lipid metabolism, in both healthy and T2D subjects. This reflects the role of insulin as a regulator of glucose intake and a suppressor of lipid catabolism, and is in agreement with previous reports [28]. Thus, a decrease in fatty acid levels after the insulin infusion in euglycemia indicated that lipid-catabolism and subsequent beta-oxidation was suppressed, and incorporation of fatty acids into

triglycerides was activated. Moreover, a recent study showed that fatty acids are essential signaling molecules in insulin secretion [29]. However, under conditions of decreased glucose levels caused by, for example, prolonged fasting, fatty acids are utilized as an energy source in the process of beta-oxidation [30]. A decrease in fatty acid levels under insulin-induced hypoglycemia suggests that fatty acids are utilized to produce the required energy. The differences between T2D and controls in fatty acid levels, which are higher in T2D, might therefore represent an impairment of those signaling mechanisms. Given that muscle cells of subjects with T2D feature a limited capacity for fatty acid oxidation the elevated levels of fatty acids in the plasma of T2D in comparison with the control group suggests that beta-oxidation is compromised. Therefore, under hypoglycemic conditions, T2D patients likely have limited access to energy. Because the heart is utilizing fatty acids as a main energy source [31], a decrease in the capacity of fatty acid utilization in T2D under hypoglycemia could contribute to cardiovascular complications. Thus, the abnormalities in fatty acid metabolism observed in our study are in agreement with a recent reports showing a delayed heart rate increment under hypoglycemic conditions as well as greater repolarization abnormalities, and vagal activity reactivation during sustained hypoglycemia in T2D subjects, but not in control group[32].

The insulin infusion under euglycemia triggered a decrease in methionine as well as branch chain amino acids (leucine and isoleucine), as well as products of their

metabolism. Leucine was suggested as one of the amino acids that enhances insulin secretion [33]; additionally BCAA and the products of their metabolism, together with lipids, were previously identified as factors contributing to insulin resistance [14]. Thus, a decrease in leucine, isoleucine and the products of their metabolism, which occur already under euglycemia, might suggest activation of mechanisms inhibiting signals enhancing insulin secretion. We observed the Bonferroni significant decrease in the levels of several amino acids only in T2D subjects under hypoglycemia. Amino acids can contribute to energy generation as well as glucose and ketone body formation[34]. Under limited glucose access, the brain relies upon ketone bodies as an energy source; thus, a decrease in levels of lysine, phenylalanine and tyrosine could suggest the brain's utilization of the ketone bodies required to maintain proper brain function. Additionally, a decrease in glucogenic amino acids, such as proline, arginine, serine, and asparagine, could suggest utilization of those molecules to counteract the overall energy depletion under hypoglycemia.

We identified only three metabolites that appeared to be responding to the low glucose level, namely cortisol, cyclic AMP, and pregnenolone sulfate. Increases in cortisol and cyclic AMP during hypoglycemia and their role in carbohydrate metabolism are well described in the literature [3,35,36]. An increase in cortisol level is a recognized physiological response to hypoglycemia [3], and the increase in cortisol level was observed in both control and T2D subjects, but was more pronounced in T2D, which is

possibly related to a more rapid decrease in the glucose level. Pregnenolone sulfate is a neurosteroid that acts in the nervous system by modulating neurotransmission, as well as in nutritional homeostasis by inducing a signaling cascade in insulinoma cells, resulting in similar responses to the signaling cascade induced by glucose in β-cells [37]. Pregnenolone sulfate is synthetized from cholesterol in the reaction catalyzed by cytochrome P450 side-chain cleavage enzyme (CYP11A1) leading to the formation of isocaproate, [24], which was also found significantly increased in hypoglycemia in T2D. Pregnenolone sulfate is known to impact many processes by modulating diverse molecular targets including GABA channels, N-methyl-d-aspartate receptor, as well as transient receptor potential melastatin (TRPM) channels [24]. The regulatory impact of pregnenolone sulfate, via TRPM4, on vascular smooth muscle secretion and contraction has been reported [38]. At high dosage, pregnenolone sulfate results in increased vascular tone [38], which is implicated in cardiovascular diseases and together with impaired fatty acid oxidation may be a factor contributing to hypoglycemia- induced cardiovascular complications in T2D patients. Increases in prolactin and FABP3, observed in our study, were also described previously and have been positively correlated to cardiovascular disease [29,39].

Both controls and T2D subjects responded similarly at the metabolite level to insulin stimuli under euglycemia. At the protein level, there was only an increase in Activated Protein C in T2D after insulin infusion and euglycemia. Activated Protein C is a natural

anticoagulant with anti-inflammatory and anti-apoptotic properties, recognized as beneficial in preventing β-cell destruction in type 1 diabetes [40]. Thus, elevated level of Activated Protein C, observed after insulin infusion, might suggest activation of protective mechanisms in T2D patients. However, under insulin-induced hypoglycemia, 13 proteins showed significant alteration and over 200 proteins showed nominally significant changes; the majority of those proteins were significantly relevant only in T2D. Overall, the analysis revealed that the proteins altered at nominally significant levels are linked predominantly with inflammation and inflammatory diseases such as rheumatoid arthritis or psoriasis. Additionally, some of the proteins were implicated in cardiac infraction. This is in agreement with a previous study showing that acute hypoglycemia triggers inflammatory and pro-atherothrombotic responses in type 1 diabetes patients [41]. Additionally, it has been shown that low glucose levels lead to inflammatory responses in monocytes from both T2D and healthy controls [42]. Our analysis revealed that altered molecules could be implicated in monocyte migration (including CCL1, CCL20, SEMA3A, IL-4, IL-2). Moreover, we identified several molecules involved in activation of immunoresponses, and this was more acute in the T2D group. This further suggests a higher susceptibility of T2D to cardiovascular complications mediated by inflammatory signals. Furthermore, we observed a Bonferroni significant decrease in myoglobin levels under induced hypoglycemia in T2D. Interestingly, lack of myoglobin was shown to contribute to a switch in cardiac substrate

utilization from fatty acid to glucose oxidation [43]. Thus, the decrease in myoglobin levels in T2D observed in our study suggests activation of potentially protective mechanisms in response to restricted fatty acid utilization. However, under hypoglycemia there is limited glucose access that might further contribute to an even more severe state of cardiac energy depletion and cardiovascular complications.

The levels of almost all investigated molecules returned to their baseline at 24 h following induced hypoglycemia, which might suggest a temporal rather than a long-term impact of molecular changes on cardiovascular function.

Conclusions

This study is the first to comprehensively explore molecular processes under euglycemia followed by hypoglycemic conditions and thus contribute to further understanding of human physiology. We have shown that insulin is the main trigger of the molecular responses, influencing predominantly fatty acid metabolism, which was impaired in T2D. We have also demonstrated that proteins altered under insulin-induced hypoglycemia play a significant role in inflammation and inflammatory responses. We further identified changes in levels of pregnenolone sulfate as well as prolactin and FABP3 under hypoglycemia. Collectively, limited capacity of fatty acid utilization in T2D together with accumulation of pregnenolone sulfate and alterations in proinflammatory proteins under insulin induced hypoglycemia, might be a co-players contributing to

hypoglycemia-induced cardiovascular complications. Our study suggests that insulin-induced hypoglycemia could contribute to cardiovascular complications via inflammatory pathways. The identified molecules could be considered as potential targets for development of therapeutic strategies for patients on insulin therapy, which are at risk of experiencing hypoglycemic events.

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Author Contributions

Conceptualization: SA; Investigation: H.K. TS, A.H.; Methodology: H.K., A.H., A.B., R.E, H.S., H.K.; Data analysis: A.H., J.Z, A.B.; Writing and Revisions: A.H., K.S., J.G., T.S., S.A.; Funding Acquisition, S.A., K.S.

Declarations of interest

None

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Figures

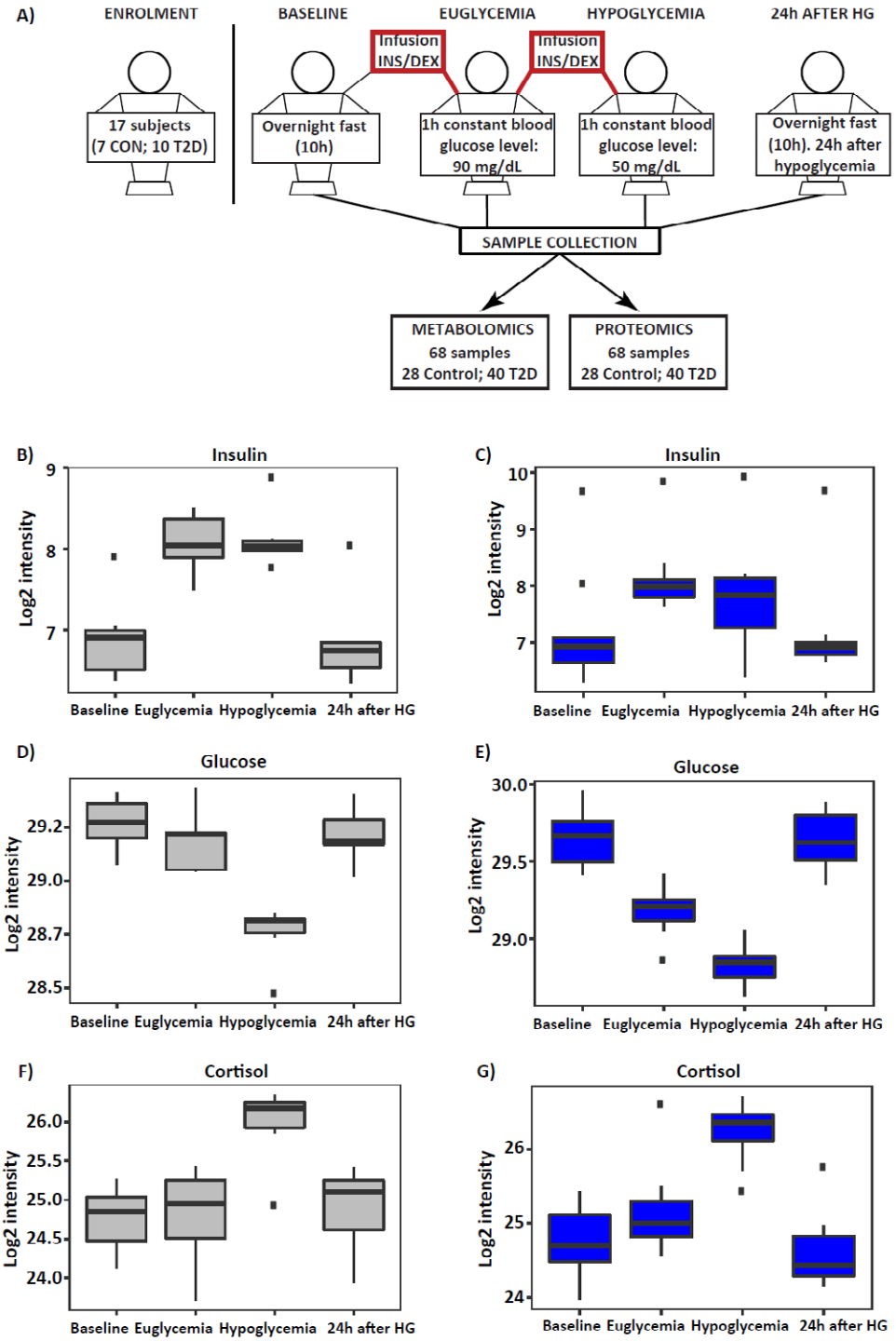
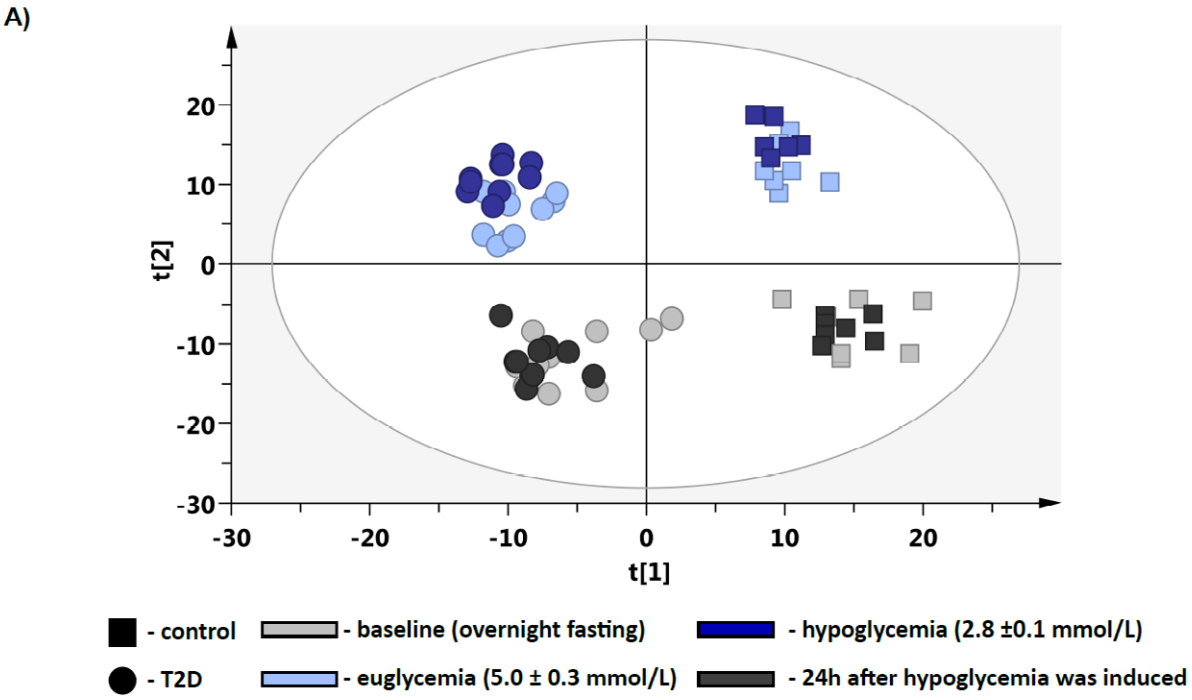


Figure 1. **Physiological responses of healthy controls (gray) and T2D (blue) to insulin/dextrose stimuli. A)** Study design. Seven healthy controls (CON) and ten subjects with type 2 diabetes (T2D) participated in the experiment. Samples were collected after an overnight fast (baseline), and the insulin/dextrose (INS/DEX) was infused to keep the blood glucose at a constant level of euglycemia of 90mg/dL (5 mmol/L) for one hour, when samples were collected. The blood glucose level was then reduced to 50mg/dl (2.8 mmol/L) for another hour, after which samples were again collected. Finally, samples were collected after overnight fast, 24h after the induced hypoglycemia. The collected samples were submitted for metabolomics and proteomics measurements. **B) – G)** Box plots presents alteration patterns of insulin, glucose and cortisol occurring at experimental time points: 1) baseline sampling performed after overnight fast; 2) euglycemia - glucose level kept at 5 mmol/L for 1h by insulin/dextrose infusion; 3) hypoglycemia – glucose level kept at 2.8 mmol/L for 1h by insulin /dextrose infusion; 4) 24h after HG – sampling performed after overnight fast 24h after hypoglycemia was induced in controls and T2D. Grey and blue indicate control and T2D, respectively.

A)



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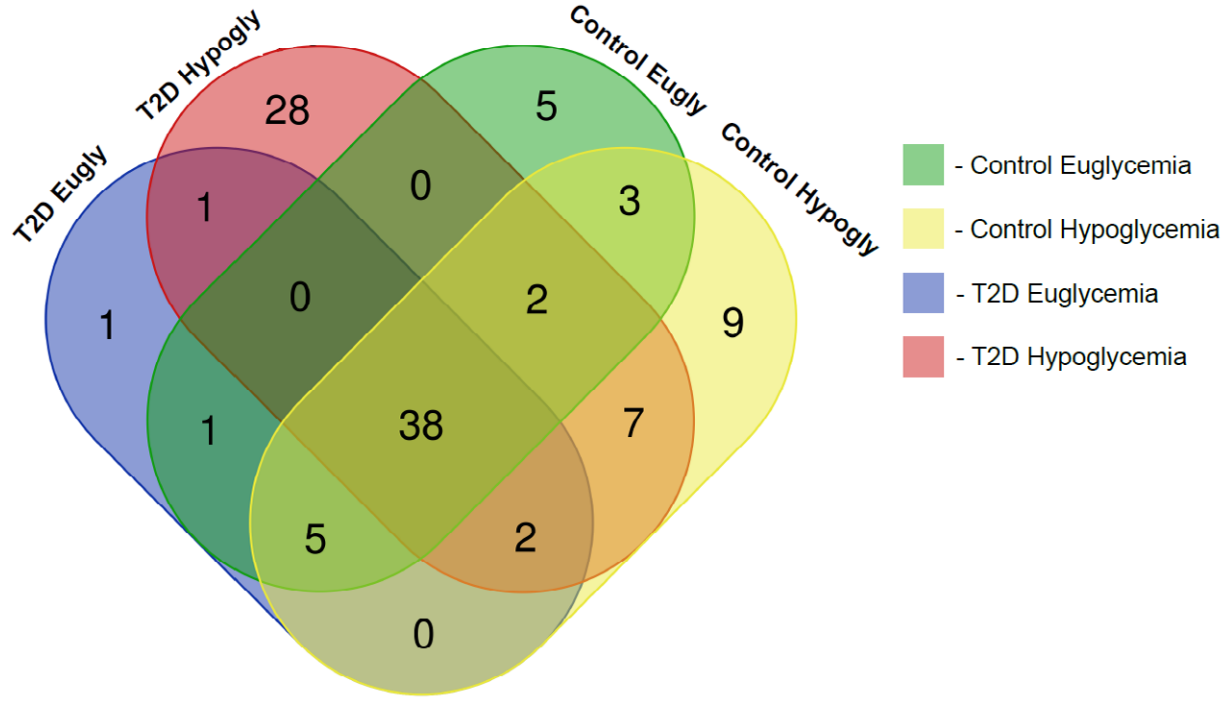

Figure 2. Metabolites and proteins differentiate healthy controls from T2D under euglycemic-hypoglycemic clamp. A) OPLS analysis reveals condition dependent clustering. **B)** A venn diagram showing the overlap of proteins and metabolites between controls and T2D under euglycemia and hypoglycemia.

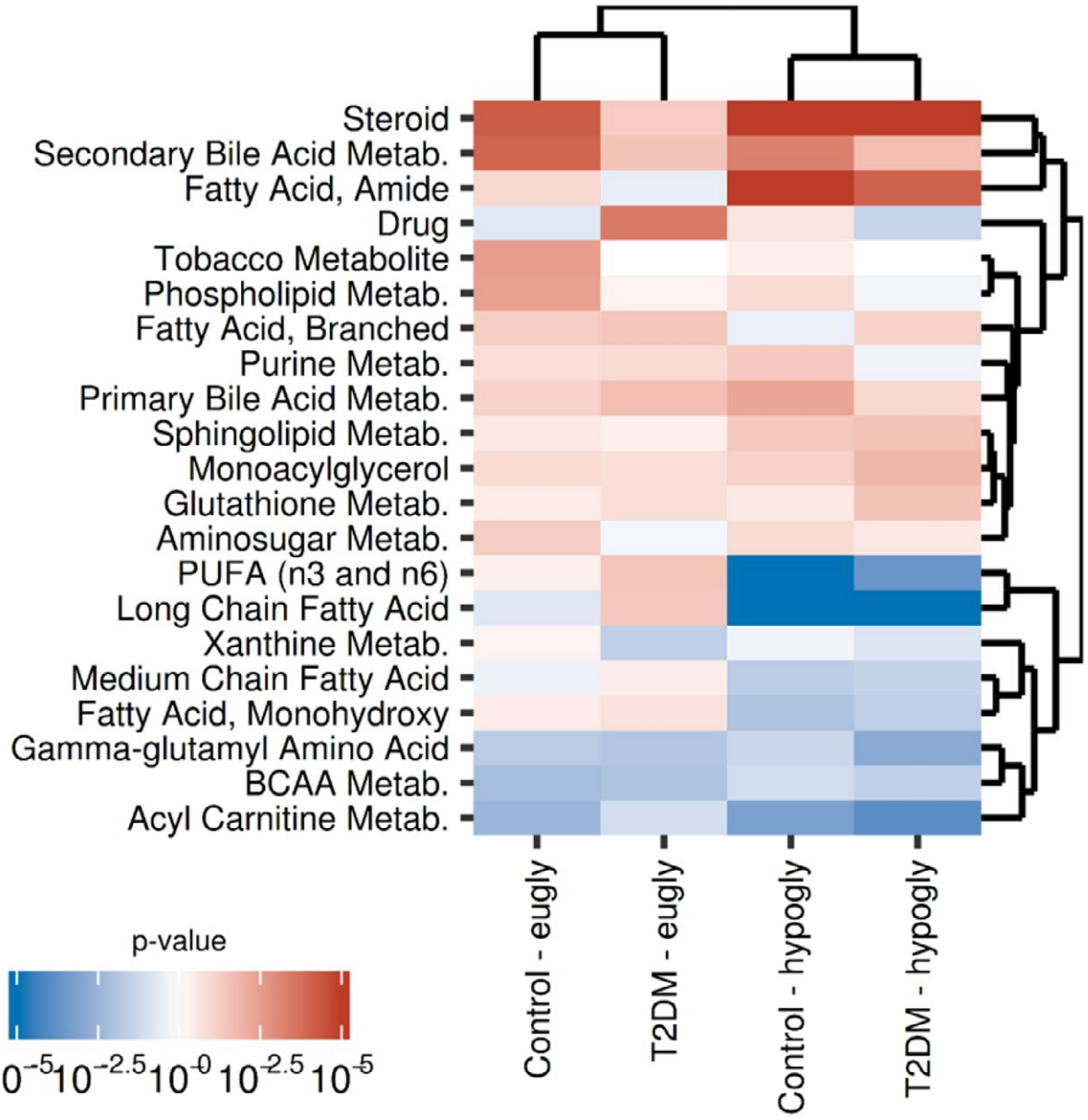

Figure 3. Enrichment analysis. A heatmap of the p-values of pathway enrichment. A color coding displays directionality (blue – decrease; red – increase). The pathway

enrichment was calculated for controls and T2D under euglycemia (- eugly) and hypoglycemia (- hypogly).

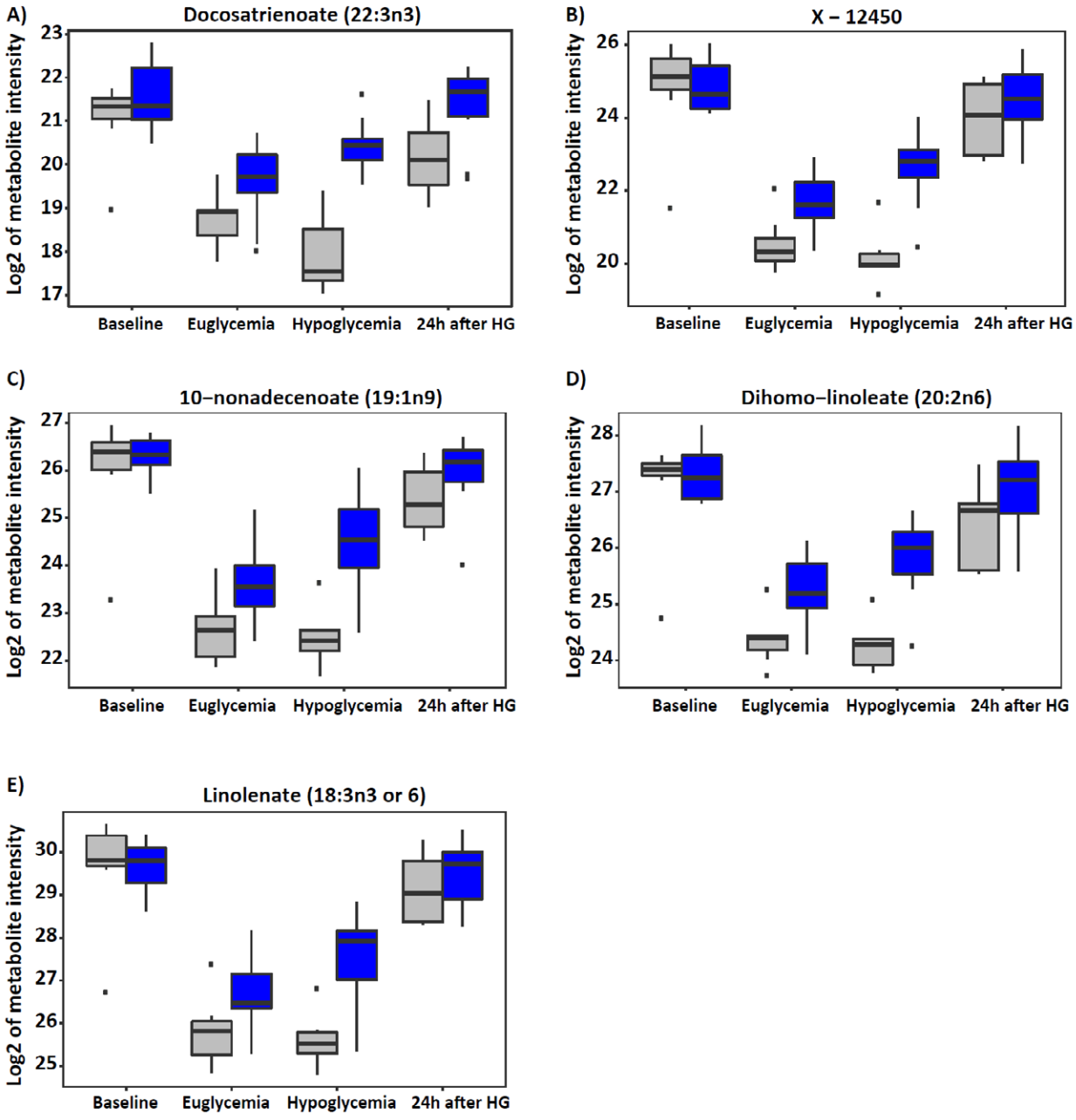

Figure 4. Fatty acid metabolism differentiates control from T2D in the responses to hypoglycemia. Five metabolites showed Bonferroni significant difference between control and T2D under hypoglycemia that was not observed under baseline. Gray – healthy control, blue – T2D.

Supplementary Tables.

Supplementary Table 1. Metabolic responses to euglycemic-hypoglycemic clamp.

The table include all metabolites showing Bonferroni significant alteration (p-value ≤ 5.2 x 10^{-5}) under euglycemic-hypoglycemic clamp either in controls or T2D. The directionality of alterations in comparison to baseline is depicted by "beta"; positive beta value indicate increase and negative beta value decrease.

Supplementary Table 2. Euglycemic-hypoglycemic clamp induced changes in blood proteome.

The table include all proteins showing Bonferroni significant alteration (p-value ≤ 4.4x 10-5) under euglycemic-hypoglycemic clamp either in controls or T2D. The directionality of alterations in comparison to baseline is depicted by "beta"; positive beta value indicate increase and negative beta value decrease.

Supplementary Table 3. Overlap of significantly altered metabolites across different groups.

Supplementary Table 4. Enrichment analysis. Pathway enrichment depicted by the p-value and directionality.

Supplementary Figures description.

Supplementary Figure 1. SOMAmer-based assay principles. 1) binding – analytes (Pi) and SOMAmers (S) (fully synthetic fluorophore-labeled SOMAmer coupled to a biotin moiety through a photocleavable linker) are equilibrated; 2) Catch I - all analyte/SOMAmers complexes are immobilized on a streptavidin-substituted support (SA). Washing steps remove proteins not stably bound to SOMAmers. The bound protein is biotinylated; 3) Cleave - long-wave ultraviolet light is applied to cleave the photocleavable linker and release analyte-SOMAmer complexes into the solution; 4) Catch II – analyte-SOMAmer complexes are selectively immobilized on streptavidin support via the introduced analyte-borne biotinylation. Further washing continues to select against non-specific analyte/SOMAmer complexes; 5) Elution – Denaturation disrupts analyte-SOMAmer complexes. Released SOMAmers serve as surrogates for quantification of analyte concentrations; 6) Quantification – hybridisation to custom arrays of SOMAmer-complementary oligonucleotides. Abbreviation: S – SOMAmer; Pi – protein; B – biotin.

Supplementary Figure 2. Metabolic signatures of hypoglycemia in human plasma. A) – D) Box plots presents alteration patterns of molecules altered under insulin-induced hypoglycemia at all four experimental time points. Gray – control; blue – T2D.

Supplementary Figure 3. Enrichment analysis of metabolites in context of metabolic patterns of diseases. Enrichment analysis conducted in control **A)** and T2D **B).**

Supplementary Figure 4. Proteins altered under hypoglycemia in controls are associated with inflammatory responses **A)** and cardiovascular complication **B)**. Green indicate decrease and orange increase.

Supplementary Figure 5. Proteins altered under hypoglycemia in T2D are associated with inflammatory responses **A)** and cardiovascular complication **B)**. Green indicate decrease and orange increase.

Supplementary Figure 6. **Long-term metabolic changes induced by hypoglycemia.** Three metabolites showed Bonferroni significant alteration 24h after hypoglycemia was induced. Gray – control; blue – T2D.