Metabolic and proteomic signatures of hypoglycaemia in type 2 diabetes

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Aims: To determine the biochemical changes that underlie hypoglycaemia in a healthy control group and in people with type 2 diabetes (T2D).

Materials and methods: We report a hypoglycaemic clamp study in seven healthy controls and 10 people with T2D. Blood was withdrawn at four time points: at baseline after an overnight fast; after clamping to euglycaemia at 5 mmol/L; after clamping to hypoglycaemia at 2.8 mmol/L; and 24 hours later, after overnight fast. Deep molecular phenotyping using non-targeted metabolomics and the SomaLogic aptamer-based proteomics platform was performed on collected samples.

Results: A total of 955 metabolites and 1125 proteins were identified, with significant alterations in >90 molecules. A number of metabolites significantly increased during hypoglycaemia, but only cortisol, adenosine-3',5'-cyclic monophosphate (cyclic AMP), and pregnenolone sulphate, were independent of insulin. By contrast, identified protein changes were triggered by hypoglycaemia rather than insulin. The T2D group had significantly higher levels of fatty acids including 10-nonaladecenoate, linolenate and dihomo-linoleate during hypoglycaemia compared with the control group. Molecules contributing to cardiovascular complications such as fatty-acid-binding protein-3 and pregnenolone sulphate were altered in the participants with T2D during hypoglycaemia. Almost all molecules returned to baseline at 24 hours.

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

KEYWORDS
hypoglycaemia, type 2 diabetes, clinical physiology, glucose metabolism

1 | 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. After a meal, insulin secretion restores normoglycaemia by simultaneously decreasing hepatic glucose production and stimulating glucose uptake by skeletal muscle and adipose tissue. Conversely, during fasting, regulatory hormonal responses are activated to prevent hypoglycaemia (defined as blood glucose <3.9 mmol/L [70 mg/dL]). Decreasing glucose levels to between 3.6 and 3.9 mmol/L (65 and 70 mg/dL) stimulates epinephrine secretion, hepatic glucose release (glycogenolysis) and glucose formation (gluconeogenesis). Epinephrine inhibits insulin secretion and decreases utilization of glucose by peripheral tissues, and increases the release of gluconeogenic substrates. Hypoglycaemia results in cortisol secretion that limits glucose utilization whilst simultaneously enhancing hepatic glucose production.

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In advanced diabetes, medical control of glycaemia is reached with insulin therapy or drugs stimulating insulin release (sulphonylureas), which is frequently associated with an increased risk of hypoglycaemic episodes. These hypoglycaemic events appear more commonly in type 1 diabetes (T1D), but have increasingly been reported in type 2 diabetes (T2D) as well, where the risk of hypoglycaemia increases with the duration of insulin therapy and the progression of pancreatic β-cell failure. Both in patients with T1D and in those with T2D, a reduction in the glycogen and epinephrine responses to hypoglycaemia has been correlated with treatment duration.

Hypoglycaemic episodes have been associated with cardiovascular complications, including myocardial ischaemia and cardiac arrhythmias. They are also related to development of atherosclerosis through increased inflammation and endothelial dysfunction, and were observed to be associated with neurological deficits. These cardiovascular and neurological complications associated with hypoglycaemia are thought to occur, in part, due to diabetes-related biochemical dysregulations; therefore, a greater insight into the physiological responses to insulin-induced hypoglycaemia 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. Plasma proteome profiles can be investigated with a highly multiplexed, aptamer-based, affinity proteomics platform, slow off-rate modified aptamer (SOMA) scan. Metabolic and proteomic changes associated with T2D have been well characterized in previous studies. For instance, elevated levels of branched chain and aromatic amino acids were associated with insulin resistance as well as with early onset of diabetes. Alterations in lipid metabolism including fatty acid metabolism, carnitines and glycerophospholipids have been identified in T2D. Alterations in proteome levels of fibroblast growth factor 23, vascular endothelial growth factor A and C-reactive protein were previously associated with T2D progression; however, the impact of hypoglycaemia on the metabolome and proteome in patients with T2D has not been previously investigated.

The aim of the present study was to determine, for the first time, biochemical changes occurring in healthy individuals and patients with T2D under insulin-induced euglycaemic and hypoglycaemic 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 levels of 1125 proteins in blood plasma samples. Our experimental set-up simulates the condition of patients treated with insulin therapy experiencing hypoglycaemia, in which they experience opposing signals from both infused insulin, signalling a well-fed state, and low glucose levels, signalling starvation. In this study, we determined the molecular alterations in plasma taken from a control group and from a group of individuals with T2D, under insulin-induced euglycaemic and hypoglycaemic conditions.

2 | MATERIALS AND METHODS

2.1 | Study design

Seven healthy controls (five men, two women; mean ± SD age 46.9 ± 6.3 years, body mass index 29.1 ± 4.2 kg/m²) and 10 participants with T2D (seven men, three women; mean ± SD age 46.3 ± 5.6 years, body mass index 34.6 ± 7.2 kg/m²) were recruited. Non-smoking men or women, aged between 40 and 60 years were eligible for inclusion in the study. In addition, participants in the control group were required to have no medical conditions and participants in the T2D group were required to have had T2D for <10 years, treated with diet or metformin, with no history of microvascular disease (retinopathy, nephropathy or neuropathy) and with a glycated haemoglobin (HbA1c) concentration ≤ 80.3 mmol/mol (9.5%). The exclusion criteria were as follows: pregnancy or lack of contraception in women with reproductive capacity; chronic medical conditions; smoking; evidence of ischaemia on ECG; drop attacks; alcohol or drug abuse; psychiatric illness; or previous history of seizures.

The study participants underwent a euglycaemic-hypoglycaemic clamp as outlined in Figure 1A. The study was started after an overnight (10-hour) fast, and the blood samples were collected at four time points, under the following conditions: (a) baseline, after an overnight (10-hour) fast, (b) after the participants had undergone a euglycaemic-hyperinsulinaemic clamp to a blood glucose of 5 mmol/L (90 mg/dL; euglycaemia) for 1 hour, (c) after the participants had undergone a hypoglycaemic-hyperinsulinaemic clamp to a blood glucose of 2.8 mmol/L (50 mg/dL; hypoglycaemia) for 1 hour, and (d) 24 hours after the challenge experiment and after an overnight (10-hour) fast.

The Yorkshire and Humber Research Ethics Committee approved the 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.

2.2 | 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 to 60 °C, using a heat box. To guide the rate of dextrose infusion, a small blood volume was withdrawn every 5 minutes from the catheter, and the blood was analysed 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 the manufacturer's control solutions.

The insulin infusion rate was constant throughout the euglycaemic-hypoglycaemic clamp (60 mU/body surface area [m²]/min). The body surface area (m²) was calculated as previously described (0.007184 × [height [cm] × 0.725] × [weight [kg] × 0.425]).

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 5 mmol/L (90 mg/dL) for 1 hour under the euglycaemic clamp, and 2.8 mmol/L (50 mg/dL) for 1 hour under the hypoglycaemic clamp. The blood glucose level was decreased gradually for ~1 hour per condition, and the overall duration of the euglycaemic-hypoglycaemic clamp was 4 hours.

FIGURE 1  Physiological responses of healthy control participants (grey) and participants with type 2 diabetes (T2D; blue) to insulin/dextrose stimuli. A, Study design. Seven healthy controls (CON) and 10 participants with 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 euglycaemia of 5 mmol/L (90 mg/dL) for 1 hour, when samples were collected. The blood glucose level was then reduced to 2.8 mmol/L (50 mg/dL) for another hour, after which samples were again collected. Finally, samples were collected after overnight fast, 24 hours after the induced hypoglycaemia. The collected samples were submitted for metabolomic and proteomic measurements. B–G, Box plots present alteration patterns of insulin, glucose and cortisol occurring at experimental time points: (a) baseline sampling performed after overnight fast, (b) euglycaemia: glucose level kept at 5 mmol/L for 1 hour by insulin/dextrose infusion, (c) hypoglycaemia: glucose level kept at 2.8 mmol/L for 1 hour by insulin/dextrose infusion, and (d) 24 hour after HG: sampling performed after overnight fast 24 hours after hypoglycaemia was induced in the control and T2D groups. Grey and blue indicate control and T2D, respectively.
2.3 | Metabolomic measurements

2.3.1 | 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 purposes. The sample extract was then divided into aliquots designated for the analysis using the following:

(a) UPLC-MS/MS with positive ion mode electrospray ionization,
(b) UPLC-MS/MS with negative ion mode electrospray ionization,
(c) hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS, and
(d) GC-MS.

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

2.3.2 | 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 (UPLC BEH C18-2.1 × 100 mm, 1.7 μm; Waters Corp., Milford, Massachusetts) with water and methanol containing 0.1% formic acid and was analysed using positive ion conditions. The second sample aliquot, reconstituted in basic solvent, was gradient-eluted from a C18 column (UPLC BEH C18-2.1 × 100 mm, 1.7 μm; Waters Corp.) with water and methanol containing 6.5 mM ammonium bicarbonate, and was analysed using basic negative ion conditions. The third aliquot was gradient-eluted from a HILIC column (UPLC BEH Amide 2.1 × 150 mm, 1.7 μm; Waters Corp.) using water and acetonitrile with 10 mM ammonium formate. The flow rate was 350 μL/min. The sample injection volume was 5 μL.

The sample separation and measurements were performed on ACQUITY UPLC (Waters Corp.) in-line to a Q-Exacte high resolution/accurate mass spectrometer (Thermo Scientific, Waltham, Massachusetts) interfaced with a heated electrospray ionization (HESI-II) source and an Orbitrap mass analyser. In the MS analysis, the scan range varied according to the method used, but fell within the range of 70 and 1000 m/z.

2.3.3 | 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 × 0.18 mm ID; 0.18-μm 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°C to 340°C over a 17.5-minute period.

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

2.3.4 | Metabolite identification

Metabolon (Morrisville, North Carolina) hardware and software were used for raw data extraction. These systems use web-service platform-relaying on Microsoft’s NET technologies, which run on high-performance application servers and fibre-channel storage arrays in clusters. Spectral features were identified by comparison with 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.

2.4 | SOMAscan measurements

The protein quantification was performed using a Slow Off-rate Modified Aptamer (SOMAmer)-based protein array (SomaLogic, Boulder, Colorado), as previously described.21 Briefly, EDTA plasma samples were diluted, and the following assay steps were performed:

(a) binding: analytes and primer bead–SOMAmers (fully synthetic fluorophore-labelled SOMAmer, coupled to a biotin moiety through a photocleavable linker) are equilibrated, (b) Catch I: all analyte–SOMAmer complexes are immobilized on a streptavidin-substituted support. Washing steps remove proteins not stably bound to primer bead–SOMAmers and bound protein is biotinylated. (c) Cleave: long-wave ultraviolet light is applied to release analyte–SOMAmer complexes into the solution, (d) 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, (e) Elution: denaturation disrupts analyte–SOMAmer complexes. Released SOMAmers serve as surrogates for quantification of analyte concentrations, and (f) Quantification: hybridization to custom arrays of SOMAmer-complementary oligonucleotides (Supporting Information Figure S1).

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 quality control (i.e. ColCheck value “FAIL”) and we removed those prior to further analysis.

2.5 | Statistical analysis

Data analysis was performed using our in-house tool autonics (https://github.com/bhagwatadiya/autonics). 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:

(a) T2D group at euglycaemia – T2D at baseline, (b) control group at euglycaemia – control group at baseline, (c) T2D group at hypoglycaemia – T2D group at baseline, (d) control group at hypoglycaemia – control group at baseline; (e) T2D group at 24 h after hypoglycaemia was induced – T2D group at baseline, and (f) control group at 24 hours after hypoglycaemia was induced – control group at
baseline, using the R package limma.\textsuperscript{22} P values (0.05) were corrected for multiple testing using Bonferroni correction.

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.

Orthogonal projections to latent structures (OPLS) analysis, using condition and time as phenotypes, was performed with SIMCA version 14 (Umetrics, Umeå, Sweden).

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

Pathway analysis was conducted using MetaboAnalyst 4.0,\textsuperscript{23} as well as Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, California), for network analysis of proteins that were differentially regulated under euglycaemic-hypoglycaemic clamp.

### 3 | RESULTS

#### 3.1 | Euglycaemic-hypoglycaemic clamp and normal physiological responses

To test whether individuals responded to the euglycaemic-hypoglycaemic 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 euglycaemia, induced hypoglycaemia, 24 hours after hypoglycaemia; Figure 1A).

After overnight fasting (at the experimental baseline), mean ± SD blood glucose in the control group was at the normal physiological level (4.8 ± 0.5 mmol/L) and in the T2D group was above the normal level (6.2 ± 1.1 mmol/L). Insulin infusion was reflected in the plasma samples; we observed a significant increase in levels of insulin under euglycaemia in both the control group ($P = 2.37 \times 10^{-5}$) and the T2D group ($P = 4.87 \times 10^{-5}$; Figure 1B,C). The glucose level under euglycaemia remained unchanged in the control group ($P = 0.25$) but was significantly decreased in the T2D group ($P = 2.00 \times 10^{-4}$; Figure 1D,E). Under insulin-induced hypoglycaemia, the blood glucose level was significantly decreased in both the control group ($P = 2.54 \times 10^{-5}$) and the T2D group ($P = 2.52 \times 10^{-13}$), and the plasma insulin level remained significantly elevated in both groups (control group, $P = 3.12 \times 10^{-5}$; T2D group, $P = 5.51 \times 10^{-3}$). A significant increase in cortisol level was observed under hypoglycaemia in the control group ($P = 9.31 \times 10^{-5}$) and the T2D group ($P = 2.39 \times 10^{-8}$), but not under insulin-induced hypoglycaemia (Figure 1F,G). Levels of all investigated molecules returned to baseline values 24 hours after hypoglycaemia was induced.

#### 3.2 | Plasma metabolome and proteome responses to euglycaemic-hypoglycaemic clamp

We applied metabolome-wide profiling to improve the understanding of response to insulin-induced hypoglycaemia in healthy participants and in those with T2D. 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 1125 proteins in plasma. We used orthogonal partial least squares (OPLS) regression to examine whether metabolic, together with proteomic, profiling discriminates healthy controls from those with T2D at baseline and at each experimental condition point. The OPLS analysis revealed four distinct clusters separating healthy controls from participants with T2D at all examined condition points (Figure 2A). In both the control and the T2D groups, we identified two distinct clusters: euglycaemic and hypoglycaemic samples clustered together that were clearly separated from the baseline and 24-hour time point samples. These data suggest health status to be a major determinant of the separation, and that insulin-induced hypoglycaemia might trigger different responses, resulting in diverse molecular manifestations, between the control and T2D groups.

We conducted statistical data analysis to assess the number of significantly altered metabolites and proteins under euglycaemic-hypoglycaemic clamp conditions. Overall, 89 metabolites (Supporting Information Table S1) and 13 proteins (Supporting Information Table S2) showed Bonferroni-significant alterations under examined conditions (metabolites, $P \leq 5.2 \times 10^{-5}$; proteins, $P \leq 4.4 \times 10^{-5}$). Under conditions of insulin-induced euglycaemia, we observed Bonferroni-significant alterations in 54 and 48 molecules in the control group and T2D group, respectively (metabolites $P \leq 5.2 \times 10^{-5}$; proteins $P \leq 4.4 \times 10^{-5}$). Insulin-induced hypoglycaemia triggered 66 and 78 Bonferroni-significant alterations in the plasma samples from the control and T2D groups, respectively (metabolites $P \leq 5.2 \times 10^{-5}$, for proteins $P \leq 4.4 \times 10^{-5}$).

We further investigated the overlap of significantly altered molecules between the control group and T2D group under euglycaemia and hypoglycaemia. Among 102 molecules (metabolites and proteins) showing Bonferroni-significant alteration, 38 molecules, mainly metabolites involved in lipid metabolism, overlapped among all four groups; five molecules (citrulline and four lipid molecules) were unique to the control group under euglycaemia, one molecule (docosapentaenoate [n6 DPA; 22:5n6]) was unique to the T2D group under euglycaemia, nine molecules (pancreatic hormone, three unknowns, uridine gamma-glutamyl-2-aminobutyrate, hexadecanediol, docosadienoate [22:2n6], and 1-arachidonoylglycerophosphoinositol) were exclusive for the control group under hypoglycaemia and 28 molecules (10 proteins and 18 metabolites including steroids, amino acids, gamma-glutamyl amino acid, and fatty acids) were attributed to the T2D group under hypoglycaemia (Figure 2B and Supporting Information Table S3).

#### 3.3 | Fatty acid metabolism is predominantly regulated by insulin infusion

To provide further insight into human physiology under euglycaemic-hypoglycaemic clamp conditions, we investigated the metabolites showing Bonferroni-significant ($P \leq 5.23 \times 10^{-5}$) alterations after the insulin infusion-induced euglycaemia ($5.0 \pm 0.3$ mmol/L), in healthy controls and participants with T2D. Insulin infusion under normal blood glucose levels led to a Bonferroni-significant ($P \leq 5.23 \times 10^{-5}$) decrease in 56 metabolites (53 in healthy controls and 45 in participants with T2D) and a 77% overlap between the control and T2D groups was observed. The metabolites that were not identified as...
overlapping showed the same trend in 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 \( P \leq 5.23 \times 10^{-5} \) 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-oxovale-rate), 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 euglycaemic clamp, were significantly altered in comparison to baseline in both the control and T2D groups (Figure 1C).

The insulin infusion under normal glucose levels had a minor effect on the blood proteome; only two proteins showing Bonferroni-significant alterations \( P \leq 4.4 \times 10^{-5} \) were identified, namely, insulin and activated protein C (Supporting Information Table S2).

### 3.4 Steroids and amino acid metabolism is regulated under insulin-induced hypoglycaemia

Under insulin-induced hypoglycaemia (blood glucose ~2.8 mmol/L), we identified 82 significantly \( P \leq 4.4 \times 10^{-5} \) altered metabolites in comparison with baseline levels (Supporting Information Table S1). Of these 82 molecules, 50 metabolites showed already a Bonferroni-significant decrease \( P \leq 4.4 \times 10^{-5} \) under euglycaemia and 32 metabolites were altered at a Bonferroni significant level \( P \leq 4.4 \times 10^{-5} \) under hypoglycaemia. Of these 32 metabolites, five molecules (isocaprate, corticosterone, cortisol, pregnelone sulphate

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**FIGURE 2**  Metabolites and proteins differentiate the healthy control group from participants with type 2 diabetes (T2D) under euglycaemic-hypoglycaemic clamp. A, Orthogonal projections to latent structures analysis showing condition-dependent clustering. B, Venn diagram showing the overlap of proteins and metabolites between the control group and the T2D group under euglycaemia and hypoglycaemia.
and adenosine-3',5'-cyclic monophosphate (cAMP) were increased and 27 decreased in comparison with the baseline level.

Only three of these 32 metabolites, namely cortisol, cAMP and pregnenolone sulphate (Supporting Information Figure S2A–C), appeared specific to hypoglycaemia and even a trend was not apparent under euglycaemic insulin infusion. Moreover, those metabolites showed an increase at a Bonferroni-significant level ($P \leq 4.4 \times 10^{-5}$) only in individuals with T2D. Pregnenolone sulphate is synthesized from cholesterol, which leads to formation of isocaproate and pregnenolone. We identified significantly increased ($P \leq 4.4 \times 10^{-5}$) levels of isocaproate under hypoglycaemia in T2D (Supporting Information Figure S2D).

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 euglycaemia, and became Bonferroni-significant under hypoglycaemia. Notably, asparagine, phenylalanine, tyrosine, arginine and proline showed Bonferroni-significant decreases ($P \leq 4.4 \times 10^{-5}$) only in T2D (Supporting Information Table S1).

3.5 Steroids and fatty acid metabolism pathways are enriched under euglycaemic-hypoglycaemic clamp

We conducted enrichment analysis to elucidate the metabolic pathways affected under euglycaemic-hypoglycaemic clamp conditions in the control group and T2D group. This analysis showed 21 metabolic pathways with significantly relevant enrichment (Figure 3A and Supporting Information Table S4). The strongest enrichment was observed in steroids ($P = 1 \times 10^{-5}$) and fatty acid metabolism pathways (polyunsaturated fatty acids [$P = 1.2 \times 10^{-4}$] and long-chain fatty acids [$P = 1 \times 10^{-5}$]). The enrichment in steroid metabolism was already observed in the control and the T2D group under euglycaemia, whereas the fatty acid metabolism pathway was specific only to hypoglycaemia in both groups. Additionally, the metabolism of bile acid and gamma-glutamyl amino acid was significantly affected under euglycaemic-hypoglycaemic clamp conditions, with enrichment observed in both the control group and the T2D group. The enrichment in glutathione metabolism and monoglycerols was observed only under hypoglycaemia in the T2D group. The results of the pathway enrichment analysis further pinpoint the impact of the euglycaemic-hypoglycaemic 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 showed that metabolic manifestations under hypoglycaemia in T2D had a significant association with refractory localization-related epilepsy, maple syrup urine disease and ornithine transcarbamylase deficiency (Supporting Information Figure S3B). We compared the directionality of metabolic alterations observed in those diseases with the metabolic manifestations in T2D under hypoglycaemia. The metabolic alterations observed only in refractory localization-related epilepsy showed the same directionality as that observed in the present study. We did not observe a significant association of metabolic manifestation with any of the diseases in the control group (Supporting Information Figure S3A).

3.6 Fatty acid metabolism differentiates people with T2D from healthy individuals under hypoglycaemia

We identified 26 metabolites under hypoglycaemia, which were differential between the T2D and control groups. Five of them differentiated T2D from healthy controls at a Bonferroni significance level ($P \leq 4.4 \times 10^{-5}$; Figure 4) and 21 were nominally significant ($P \leq 0.05$; Supporting Information Table S1). 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.

3.7 Insulin-induced hypoglycaemia triggers inflammatory responses in plasma proteome

The hypoglycaemic clamp resulted in observed significantly relevant ($P \leq 4.4 \times 10^{-5}$) changes in plasma levels of 13 proteins. These changes were apparently independent of the infusion of insulin, as they appeared unaltered under euglycaemia (Supporting Information Table S2). The majority of the altered proteins, ephrin-A5, prolactin, myoglobin, Wnt inhibitory factor 1, R-spondin-2 and dynactin subunit 2, were observed at Bonferroni significance levels only in participants with T2D. 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 participants with T2D and showed a nominally significant trend under euglycaemia in healthy controls (Supporting Information Table S2).
To provide further insight into identified proteins, we conducted pathway analysis using Ingenuity Pathway Analysis software. Given the relatively small numbers of proteins with Bonferroni-significant alterations, we also included proteins showing nominally significant (P \leq 0.05) alterations under hypoglycaemia. The number of nominally significant (P \leq 0.05) proteins was 63 in the control group and 292 in the T2D group (Supporting Information Table S5). The data analysis showed that the altered molecules could be linked to inflammatory responses, cellular movements related to inflammatory events, and cardiovascular diseases (Supporting Information Figure S4 and S5).

In the control group, molecules altered under insulin-induced hypoglycaemia were linked to activation and movement (e.g. infiltration, migration and adhesion) of phagocytes, leukocytes, neutrophils and platelets, as well as to body and organ inflammation (Supporting Information Figure S4A). The analysis showed that the altered molecules could be linked to inflammatory responses, cellular movements related to inflammatory events, and cardiovascular diseases (Supporting Information Figure S4 and S5).

In the T2D group, the analysis showed that, among nominally significant (P \leq 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 (Supporting Information Figure S5A). The altered molecules were also linked to inflammatory diseases, such as rheumatoid arthritis and psoriasis, as well as liver inflammation. Additionally, the analysis of the nominally significant molecules (P \leq 0.05) implied a connection with cardiac infarction (Supporting Information Figure S5B).

### 3.8 Hypoglycaemia has minimal effects on the metabolome after 24 hours

To determine if hypoglycaemia results in extended metabolic changes, we sampled the participants 24 hours after the induced hypoglycaemia. Only three metabolites (Supporting Information Figure S6) 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 the T2D and healthy control group. The remaining two

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**FIGURE 4** Fatty acid metabolism differentiates the healthy control group from participants with type 2 diabetes (T2D) in the responses to hypoglycaemia. Bonferroni-significant differences in five metabolites were observed between the control group and the T2D group under hypoglycaemia that were not observed at baseline. Grey, healthy control; blue, T2D.
metabolites, namely increased 1,6-anhydroglucose and increased X-16938, were attributed to the control and T2D groups, respectively.

4 | DISCUSSION

This is the first study deploying metabolomics and proteomics (SOMAscan) to explore comprehensively the molecular responses of a control group as well as a T2D group to insulin stimuli under euglycaemia and hypoglycaemia. The study shows that euglycaemia followed by hypoglycaemia 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 >90 molecules were highly sensitive in their response to glucose/insulin stimuli.

Insulin infusion in euglycaemia (5 mmol/L) triggered a large spectrum of metabolic changes, mainly involved in lipid metabolism, in both healthy participants and those with T2D. 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. Thus, a decrease in fatty acid levels after the insulin infusion in euglycaemia 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 signalling molecules in insulin secretion. However, under conditions of decreased glucose levels caused by, for example, prolonged fasting, fatty acids are used as an energy source in the process of beta-oxidation. A decrease in fatty acid levels under insulin-induced hypoglycaemia suggests that fatty acids are used to produce the required energy. The differences between the T2D and control groups in fatty acid levels, which are higher in T2D, might therefore represent an impairment of those signalling mechanisms. Given that muscle cells of people with T2D feature a limited capacity for fatty acid oxidation, the elevated levels of fatty acids in the plasma of participants in the T2D group in comparison with those in the control group suggests that beta-oxidation is compromised; therefore, under hypoglycaemic conditions, people with T2D probably have limited access to energy. Because the heart is utilizing fatty acids as a main energy source, a decrease in the capacity of fatty acid utilization in T2D under hypoglycaemia could contribute to cardiovascular complications; thus, the abnormalities in fatty acid metabolism observed in the present study are consistent with recent reports showing a delayed heart rate increment under hypoglycaemic conditions as well as greater repolarization abnormalities, and vagal activity reactivation during sustained hypoglycaemia in the T2D group, but not in the control group.

The insulin infusion under euglycaemia 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, additionally BCAA and the products of their metabolism, together with lipids, were previously identified as factors contributing to insulin resistance. A decrease in leucine, isoleucine and the products of their metabolism, which occur already under euglycaemia, might therefore suggest activation of mechanisms inhibiting signals enhancing insulin secretion. We observed the Bonferoni-significant decrease in the levels of several amino acids only in participants with T2D under hypoglycaemia. Amino acids can contribute to energy generation as well as glucose and ketone body formation. Under conditions of limited glucose access, the brain relies on ketone bodies as an energy source; thus, a decrease in levels of lysine, phenylalanine and tyrosine suggest that the brain utilizes the ketone bodies required to maintain proper brain function. Additionally, a decrease in glucogenic amino acids, such as proline, arginine, serine and asparagine, suggests utilization of those molecules to counteract the overall energy depletion under hypoglycaemia.

We identified only three metabolites that appeared to be responding to the low glucose level, namely cortisol, cAMP and pregnenolone sulphate. Increases in cortisol and cAMP during hypoglycaemia and their role in carbohydrate metabolism are well described in the literature. An increase in cortisol level is a recognized physiological response to hypoglycaemia, and the increase in cortisol level was observed in both the control and T2D groups, but was more pronounced in the T2D group, which is possibly related to a more rapid decrease in the glucose level. Pregnenolone sulphate is a neurosteroid that acts in the nervous system by modulating neurotransmission, as well as in nutritional homeostasis by inducing a signalling cascade in insulinoma cells, resulting in similar responses to the signalling cascade induced by glucose in β cells. Pregnenolone sulphate is synthetized from cholesterol in the reaction catalysed by cytochrome P450 side-chain cleavage enzyme (CYP11A1) leading to the formation of isocaproate, which was also found to be significantly increased in hypoglycaemia in people with T2D. Pregnenolone sulphate is known to affect many processes by modulating diverse molecular targets, including GABA channels, N-methyl-d-aspartate receptor, as well as transient receptor potential melastatin (TRPM) channels. The regulatory impact of pregnenolone sulphate, via TRPM4, on vascular smooth muscle secretion and contraction has been reported. At high dosage, pregnenolone sulphate results in increased vascular tone, which is implicated in cardiovascular disease and, together with impaired fatty acid oxidation, may be a factor contributing to hypoglycaemia-induced cardiovascular complications in people with T2D. Increases in prolactin and FABP3 observed in the present study, were also described previously and have been positively correlated to cardiovascular disease.

Both the control and T2D groups responded similarly at the metabolite level to insulin stimuli under euglycaemia. At the protein level, there was only an increase in activated protein C in T2D after insulin infusion and euglycaemia. Activated protein C is a natural anti-coagulant with anti-inflammatory and anti-apoptotic properties, recognized as beneficial in preventing β-cell destruction in T1D. Thus, elevated activated protein C levels, observed after insulin infusion, might suggest activation of protective mechanisms in people with T2D; however, under insulin-induced hypoglycaemia, 13 proteins showed significant alterations and > 200 proteins showed nominally significant changes; the majority of those proteins were significantly relevant only in T2D. Overall, the analysis showed 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 cardiovascular infarction. This is consistent with a previous study showing that low glucose levels lead to inflammatory responses in monocytes from both people with T2D and healthy controls. The present analysis showed that altered molecules could be implicated in monocyte migration (including CCL1, CCL20, SEMA3A, IL-4, IL-2). Moreover, we identified several molecules involved in the activation of immunoresponses, and this was more acute in the T2D group. This further suggests a higher susceptibility of people with T2D to cardiovascular complications mediated by inflammatory signals. Furthermore, we observed a Bonferroni-significant decrease in myoglobin levels under induced hypoglycaemia in T2D. Interestingly, lack of myoglobin was shown to contribute to a switch in cardiac substrate utilization from fatty acid to glucose oxidation. Thus, the decrease in myoglobin levels in T2D observed in the present study suggests activation of potentially protective mechanisms in response to restricted fatty acid utilization; however, under hypoglycaemia there is limited glucose access which might further contribute to an even more severe state of cardiac energy depletion and to cardiovascular complications.

The levels of almost all investigated molecules returned to their baseline levels 24 hours after induced hypoglycaemia, which suggests a temporal rather than long-term impact of molecular changes on cardiovascular function.

In conclusion, the present study is the first to explore comprehensively the molecular processes under euglycaemia followed by hypoglycaemic conditions and thus contributes 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 hypoglycaemia play a significant role in inflammation and inflammatory responses. We further identified changes in levels of pregnenolone sulphate as well as prolactin and FABP3 under hypoglycaemia. Collectively, limited capacity of fatty acid utilization in T2D, together with accumulation of pregnenolone sulphate and alterations in proinflammatory proteins under insulin-induced hypoglycaemia, might be co-players contributing to hypoglycaemia-induced cardiovascular complications. The present study suggests that insulin-induced hypoglycaemia could contribute to cardiovascular complications via inflammatory pathways. The identified molecules could be considered potential targets for the development of therapeutic strategies for patients on insulin therapy who are at risk of experiencing hypoglycaemic events.

**CONFLICTS OF INTEREST**

None declared.

**Author contributions**

Author contributions to the paper were as follows: conceptualization: S.A.; investigation: H.K., T.S. and A.H.; methodology: H.K., A.H., A.B., R.E., H.S. and H.K.; data analysis: A.H., J.Z. and A.B.; writing and revisions: A.H., K.S., J.G., T.S. and S.A.; funding acquisition: S.A. and K.S.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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