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# Hypoglycaemia in type 2 diabetes exacerbates amyloid-related proteins associated with dementia

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## Abstract

**Aims:** Hypoglycaemia in diabetes (T2D) may increase the risk of Alzheimer's disease (AD). We hypothesized that hypoglycaemia-induced amyloid-related protein changes would be exacerbated in T2D.

**Materials and methods:** A prospective, parallel study in T2D (n = 23) and controls (n = 23). Subjects underwent insulin-induced hypoglycaemia with blood sampling at baseline, hypoglycaemia and post-hypoglycaemia; proteomic analysis of amyloid-related proteins was undertaken.

**Results:** At baseline, amyloid-precursor protein (APP) ($P < .01$) was elevated and alpha-synuclein (SNCA) ($P < .01$) reduced in T2D. At hypoglycaemia, amyloid P-component ($P < .01$) was elevated and SNCA ($P < .05$) reduced in T2D; APP ($P < .01$) and noggin ($P < .05$) were elevated and SNCA ($P < .01$) reduced in controls. In the post-hypoglycaemia follow-up period, APP and microtubule-associated protein tau normalized in controls but showed a below-baseline decrease in T2D; noggin normalized in both; SNCA normalized in T2D, with a below-baseline decrease in controls.

**Conclusion:** The AD-associated protein pattern found in T2D, with basal elevated APP and reduced SNCA, was exaggerated by hypoglycaemia with increased APP and decreased SNCA. Additional AD-associated protein levels that changed in response to hypoglycaemia, particularly in T2D, included amyloid P-component, microtubule-associated protein tau, apolipoproteins A1 and E3, pappalysin and noggin. These results are in accordance with the reported detrimental effects of hypoglycaemia.

KEYWORDS

Alzheimer's disease, amyloid-related proteins, dementia, hypoglycaemia, type 2 diabetes

## 1 | INTRODUCTION

The prevalence of type 2 diabetes (T2D) has reached pandemic proportions. The International Diabetes Federation (IDF) currently estimates approximately 425 million individuals worldwide have diabetes,[1] a figure projected to rise to 642 million by the year 2040.

In addition to classical macro- and microvascular complications of diabetes, dementia is a recognized association.[2] The risk of dementia in individuals with T2D is strikingly increased (50-150%) relative to the general population.[3–5] Predictions suggest the worldwide prevalence of patients with dementia will increase from 35.6 million in 2010 to 115.4 million in 2050.[6] Given the association between T2D and dementia, and the dramatic predicted increases in each disease individually, the combined effect may exceed this, delivering a devastating global effect.

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Alzheimer's disease (AD) comprises 60-80% of all dementia cases.[7] Epidemiological studies indicate an increased risk for patients with T2D developing AD.[8–10] Elevation in plasma amyloid precursor protein (APP) has been reported in association with AD, levels tending to increase with increasing cognitive impairment.[11,12] While no difference in plasma levels of alpha-synuclein (SNCA) had previously been reported,[13] a recent study showed lower concentrations of SNCA in red blood cells in subjects with AD.[14]

Improved management of T2D involves stricter glucose control, increasing the risk and frequency of hypoglycaemic episodes. Hypoglycaemia itself is directly linked to cognitive dysfunction, complicating diabetic management and perhaps increasing the risk of dementia.[15]

We hypothesized that changes in hypoglycaemia-induced amyloid-related protein levels would be exacerbated during/following hypoglycaemia in T2D, providing a potential mechanistic link between T2D-related hypoglycaemia and AD. This study was specifically designed to mimic the physiological responses to hypoglycaemia as would be seen in patients with diabetes in clinical practice.[16] To this end, we analysed amyloid-related proteins levels following acute iatrogenic-induced hypoglycaemia in subjects with and without T2D.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

This was a prospective parallel study performed in 46 subjects (23 adults with T2D and 23 controls) at the Diabetes Centre at Hull Royal Infirmary; all were white and aged 40-70 years. The duration of diabetes was <10 years and all subjects with T2D were on a stable dose of medication (metformin, statin and/or angiotensin-converting enzyme inhibitor/angiotensin receptor blocker) over the previous 3 months. For those with T2D no medication for glycaemic control except metformin was allowed, haemoglobin A1c levels were <10% (86 mmol/mol) and none had either hypoglycaemic unawareness or hypoglycaemia within a 3-month period. In the control group, diabetes was excluded with an oral glucose tolerance test. All subjects had a body mass index between 18 and 49 kg/m$^2$, and all had normal renal and hepatic biochemical indices and no previous history of cancer or any contraindication to insulin infusion to achieve hypoglycaemia (ischaemic heart disease, epilepsy, seizure history, drop attacks, history of adrenal insufficiency and treated hypothyroidism).

### 2.2 | Study participants

Medical history, clinical examination, routine blood tests and an electrocardiogram were performed for all participants. A continuous insulin infusion was performed to induce hypoglycaemia as previously detailed[16] with blood samples taken at 0, 30, 60, 120 and 240 min post-hypoglycaemia. After 240 min, participants were provided lunch and were given their (morning) diabetes medications. Patients later took their evening medication as prescribed. Subjects reattended 24 h following the induction of hypoglycaemia; patients withheld their medications until they completed the blood tests in the fasted state, after which breakfast was provided. Before discharge, blood glucose was checked using a glucose analyser (HemoCue® glucose 201+) to ensure normal levels, together with other vital signs.

All participants provided written informed consent. The trial was approved by the North West-Greater Manchester East Research Ethics Committee (REC no. 16/NW/0518), registered at www.clinicaltrials.gov (NCT03102801) and conducted according to the Declaration of Helsinki.

### 2.3 | Insulin infusion

The insulin infusion was performed as previously detailed.[16] Following an overnight fast, bilateral antecubital fossa indwelling cannulas were inserted 30-60 min before the commencement of the test (08:30 h). To induce hypoglycaemia, soluble intravenous insulin (Humulin S; Lilly, Basingstoke, Hampshire, UK) was given in a pump starting at a dose of 2.5 mU/kg body weight/min with an increment of 2.5 mU/kg body weight/min every 15 min by hypoglycaemic clamp,[17] until two readings of capillary blood glucose measured by a glucose analyser (HemoCue® glucose 201+) ≤2.2 mmol/L (<40 mg/dL) or reading of ≤2.0 mmol/L (36 mg/dL).[17] The blood sample schedule was timed subsequently in respect to the timepoint that hypoglycaemia occurred. Following the identification of hypoglycaemia, intravenous glucose was given in the form of 150 mL of 10% dextrose and a repeat blood glucose check was performed after 5 min if blood glucose was still <4.0 mmol/L. The comparison of blood glucose levels at baseline, at hypoglycaemia and post-hypoglycaemia up to 24 h is shown in Figure 1A.

### 2.4 | Biochemical markers

Blood samples were separated immediately by centrifugation at 2000 *g* for 15 min at 4°C, and the aliquots were stored at −80°C, within 30 min of blood collection, until batch analysis. Fasting plasma glucose, total cholesterol, triglycerides and high-density lipoprotein cholesterol levels were measured enzymatically using a Beckman AU 5800 analyser (Beckman-Coulter, High Wycombe, UK).

### 2.5 | SOMA scan assay

The SOMAscan assay used to quantify proteins was performed on an in-house Tecan Freedom EVO liquid handling system (Tecan Group, Maennedorf, Switzerland) utilizing buffers and SOMAmers from the SOMAscan HTS Assay 1.3K plasma kit (SomaLogic, Boulder, CO, USA) according to the manufacturer's instructions and as described previously.[18,19] The assay was performed in 96-well plates containing up to 85 plasma samples, three quality control and five calibrator plasma

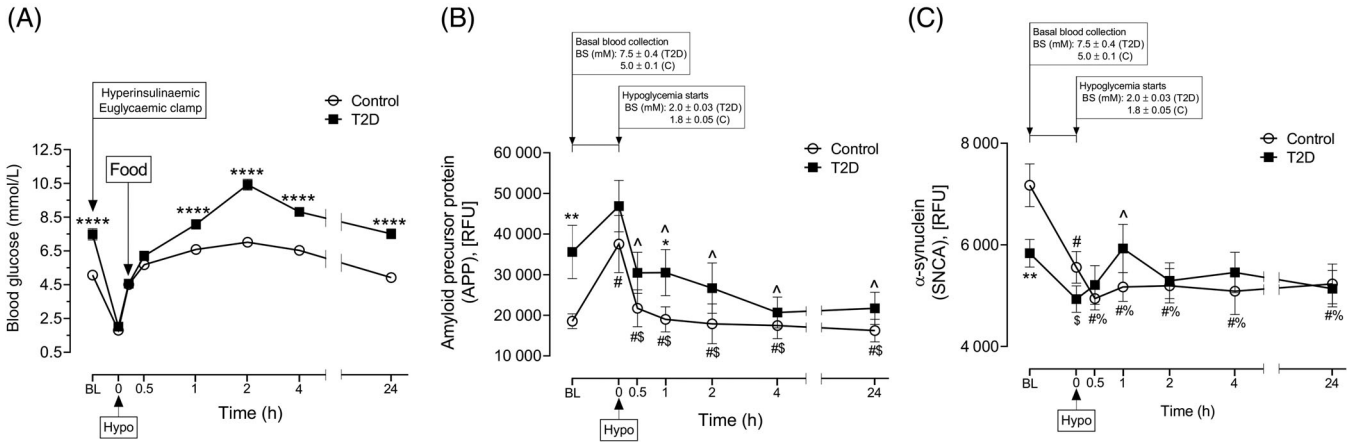


**FIGURE 1** Comparison of blood glucose levels and the Alzheimer-related proteins, amyloid precursor protein (APP) and α-synuclein (SNCA), in plasma before, during and after iatrogenic induction of hypoglycaemia. A, Blood sampling was performed at baseline (BL), at hypoglycaemia (0 min) and post-hypoglycaemia (30 min, 1, 2, 4 and 24 h) for controls (white circles) and for type 2 diabetes (T2D) (black squares). At BL, blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 mM (for control, C). At point of hypoglycaemia, BS was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 mM (for control). B,C, Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for APP and SNCA. Statistics: $^{*}P < .05$ or $^{**}P < .01$, $^{****}P < .0001$, control vs. T2D; #$P < .01$, control baseline vs. control hypoglycaemia; \$$P < .05$, T2D baseline vs. T2D hypoglycaemia; #\$$P < .05$, control hypoglycaemia vs. control post-hypoglycaemia timepoints; ^$P < .05$, T2D hypoglycaemia vs. T2D post-hypoglycaemia timepoints; #%$P < .01$, control baseline vs. control post-hypoglycaemia timepoints. RFU, relative fluorescent units

samples. Briefly, EDTA plasma samples were diluted into bins of 40%, 1% and 0.05% and incubated with streptavidin-coated beads immobilized with dilution-specific SOMAmers via a photocleavable linker and biotin. After washing, bound proteins were first biotinylated and then released from beads by photocleaving the SOMAmer-bead linker. The released SOMAmer-protein complex was treated with a polyanionic competitor to disrupt unspecific interactions and recaptured on the second set of streptavidin-coated beads. Thorough washing was performed before 5′ Cy3 fluorophore-labelled SOMAmers were released under denaturing conditions, hybridized on microarray chips with SOMAmer-complementary sequences and scanned using the SureScan G2565 Microarray Scanner (Agilent, Santa Clara, CA, USA).

## 2.6 | Data processing and analysis

Initial relative fluorescent units (RFUs) were obtained from microarray intensity images using the Agilent Feature Extraction Software (Agilent). Raw RFUs were normalized and calibrated using the software pipeline provided by SomaLogic. This included: (i) microarray hybridization normalization based on spiked-in hybridization controls, (ii) plate-specific intensity normalization, (iii) median signal normalization, and (iv) median calibrator scaling of single RFU intensities according to calibrator reference values. Samples with a high degree of haemolysis (haptoglobin log RFU <10) were excluded from the analysis.

Statistical analyses were performed on $\log_2$ RFU values using R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) including base R package. Data handling and differential protein expression were analysed using the autonomics and limma[20] packages. For differential protein analysis, we applied limma models containing contrasts between timepoints, as well as contrasts between healthy subjects and patients with diabetes at single timepoints. In both models, blocking by patient ID was performed to account for random effects. Batch effect correction was performed by adding batch as a covariate to the model. Limma obtained *P* values were corrected using the Benjamini-Hochberg method.[21]

## 2.7 | Statistical analysis

There are no studies detailing the changes in Alzheimer proteins in response to hypoglycaemia on which to base a power calculation. Sample size for pilot studies has been reviewed by Birkett and Day.[22] They concluded that a minimum of 20 degrees-of-freedom was required to estimate effect size and variability. Hence, we needed to analyse the samples from a minimum of 20 patients per group. Data trends were visually evaluated for each parameter and non-parametric tests were applied on data that violated the assumptions of normality when tested using the Kolmogorov-Smirnov test. Comparison between groups was performed at each timepoint using Student's t-test. $P < .05$ was considered statistically significant. Within-group comparisons are as follows: changes from baseline, and from hypoglycaemia, to each subsequent timepoint were compared using Student's t-test. The sample size was too small to adjust for baseline covariates. Statistical analysis was performed using Graphpad Prism (San Diego, CA, USA).

For the proteomic analysis we fitted an intercept-free general linear model as a function of a subgroup (i.e. condition:timepoint), while taking the patient ID as a random effect using the R package limma.

**TABLE 1** Demographic and clinical characteristics of the study participants

| Baseline | Type 2 diabetes (n = 23) | Controls (n = 23) | *P*-value |
|---|---|---|---|
| Age (years) | 64 ± 8 | 60 ± 10 | <.0001 |
| Sex (M/F) | 12/11 | 11/12 | .77 |
| Weight (kg) | 90.9 ± 11.1 | 79.5 ± 8.8 | <.0001 |
| Height (cm) | 167 ± 14 | 169 ± 5 | .64 |
| BMI (kg/m$^2$) | 32 ± 4 | 28 ± 3 | <.0001 |
| Systolic BP (mmHg) | 132 ± 8 | 122 ± 8 | .001 |
| Diastolic BP (mmHg) | 81 ± 7 | 75 ± 6 | .003 |
| Duration of diabetes (years) | 4.5 ± 2.2 | N/A | |
| HbA1c (mmol/mol) | 51.2 ± 11.4 | 37.2 ± 2.2 | <.0001 |
| HbA1c (%) | 6.8 ± 1.0 | 5.6 ± 0.2 | <.0001 |
| Total cholesterol (mmol/L) | 4.2 ± 1.0 | 4.8 ± 0.77 | .014 |
| Triglyceride (mmol/L) | 1.7 ± 0.7 | 1.34 ± 0.6 | .055 |
| HDL-cholesterol (mmol/L) | 1.1 ± 0.3 | 1.5 ± 0.4 | .001 |
| LDL-cholesterol (mmol/L) | 2.23 ± 0.8 | 2.7 ± 0.87 | .051 |
| CRP (mg/L) | 3.0 ± 2.7 | 5.1 ± 10.3 | .33 |

*Note:* Data are presented as mean ± SD.
Abbreviations: BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; HbA1c, Haemoglobin A1c; HDL, high-density lipoprotein; LDL, low density lipoprotein.

Subsequently, we computed the *P* value for two contrasts: baseline to hypoglycaemia for both T2D and controls, and false discovery rate corrected at a value of <.05 as the cut-off for significance.

### 2.8 | Protein-protein interaction tools

STRING 11.0 (Search Tool for the Retrieval of Interacting Genes) was used to visualize the known and predicted protein-protein interactions for proteins identified by SOMAscan assay in plasma of T2D versus control subjects (https://string-db.org/). Interactions between proteins are evidence-based and collated from databases, experiments, neighbourhood, gene fusion, co-occurrence, text mining, co-expression and homology. Here, we determined the relationships between the amyloid-associated proteins presented in this study.

Ingenuity pathway analysis (IPA) software (Qiagen, Germantown, MD, USA) enables analysis, integration and visual representation of data from a wide array of experimental datasets (gene expression, miRNA, single nucleotide polymorphism microarrays, metabolomics, proteomics and RNA sequencing); it is supported by the Ingenuity Knowledge Base, an extensive repository for biological interactions and functional annotations based on relationships between proteins, genes, complexes, cells, tissues, drugs and diseases. Here, we utilized IPA to show the canonical pathways related to key amyloid-related proteins presented in this study.

## 3 | RESULTS

In total, 46 subjects were recruited (23 people with T2D, 23 controls).[16] Demographic and clinical characteristics of the subjects are presented in Table 1. Ten amyloid-related proteins were included in the analysis: APP, amyloid P component (APCS), noggin, SNCA, microtubule-associated protein tau (MAPT), pappalysin (PAPPA) and serum amyloid A1 (SAA1), apolipoprotein (APO) A1, APOE3 and APOE4.

### 3.1 | Differences between type 2 diabetes and controls

In the T2D cohort, baseline levels of APP were elevated ($P < .01$) (Figure 1B) and SNCA decreased ($P < .01$) (Figure 1C). Baseline levels of the other proteins did not differ between cohorts (Figures 2A-D and 3A-D). At hypoglycaemia, APCS was elevated in T2D ($P < .05$) (Figure 2B). Post-hypoglycaemia, APOA1 was reduced in T2D at 4 h ($P < .05$) and 24 h ($P < .01$).

### 3.2 | Within cohort changes at the point of hypoglycaemia

Significant changes occurred in the control group in response to hypoglycaemia with APP and noggin being elevated [APP $P < .01$ (Figure 1A); noggin $P < .05$ (Figure 2C)] and SNCA levels decreasing ($P < .01$; Figure 1B).

In the T2D cohort, APP levels were already elevated as compared with controls, so while there was a further increase in response to hypoglycaemia, this did not reach significance. While SNCA levels were already relatively decreased in T2D, as in controls, SNCA levels were further depressed in T2D in response to hypoglycaemia ($P < .05$; Figure 1B).

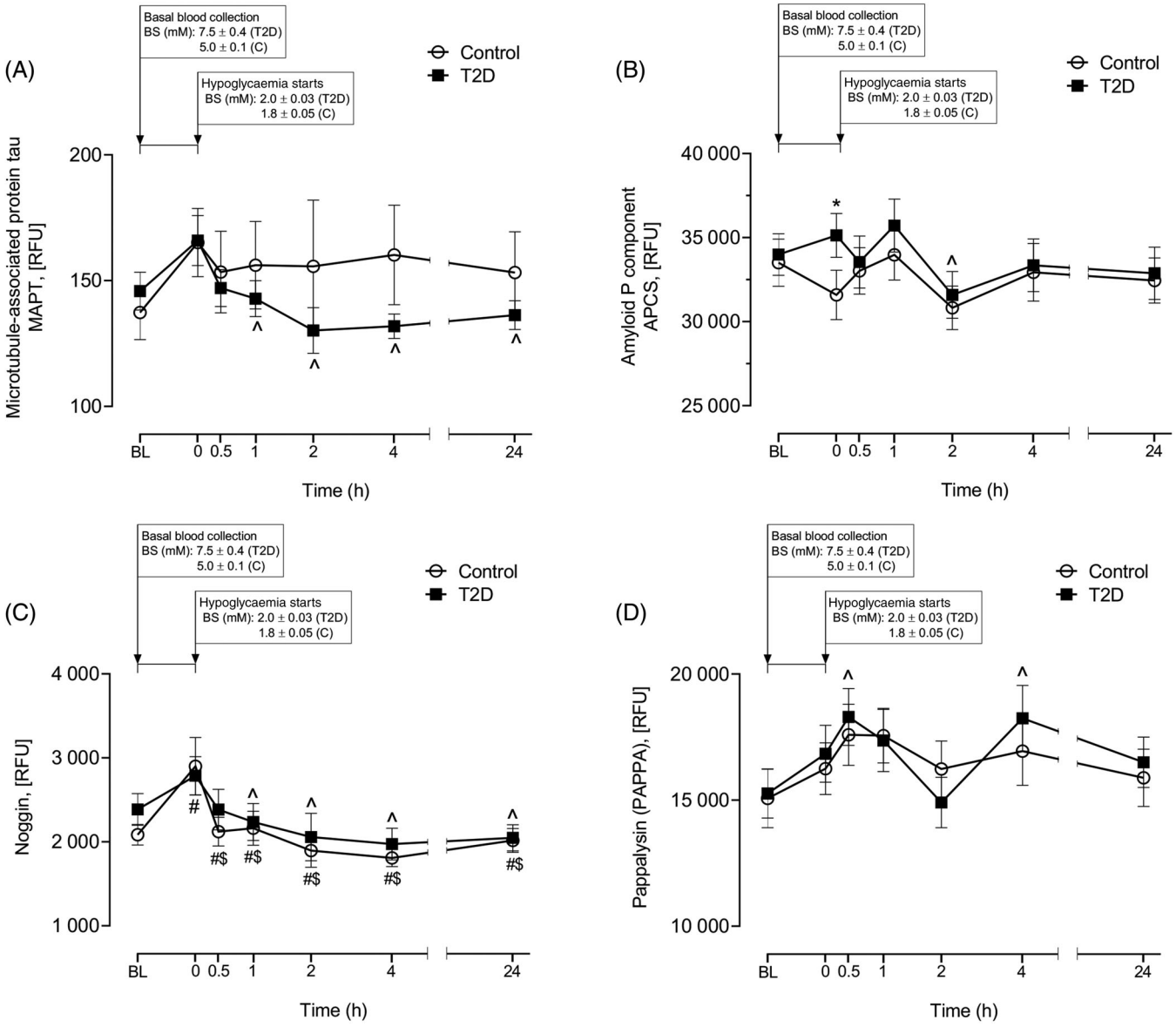


**FIGURE 2** Comparison of Alzheimer-related proteins, microtubule associated protein (MAPT), amyloid P component (APCS), noggin and papplysin (PAPPA) in plasma before, during and after iatrogenic induction of hypoglycaemia. Blood sampling was performed at baseline (BL), at hypoglycaemia (0 min) and post-hypoglycaemia (30 min, 1, 2, 4 and 24 h) for controls (white circles) and for type 2 diabetes (T2D) (black squares). At BL, blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 mM (for control, C). At point of hypoglycaemia, blood sugar (BS) was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 mM (for control). Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for A, MAPT, B, APCS, C, noggin and D, PAPPA. Statistics: **P* < .05, control vs. T2D; #*P* < .01, control baseline vs. control hypoglycaemia; ^*P* < .05, T2D hypoglycaemia vs. T2D post-hypoglycaemia timepoints; #$*P* < .05, control hypoglycaemia vs. control post-hypoglycaemia timepoints. RFU, relative fluorescent units

### 3.3 | Changes in protein levels in the 24-h follow-up period

In the post-hypoglycaemia period, the elevated hypoglycaemia-induced APP levels decreased to baseline during the 24-h follow-up period in controls (controls: 30 min vs. hypoglycaemia, *P* < .05; 1 h vs. hypoglycaemia, *P* < .01; 2 h vs. hypoglycaemia, *P* < .05; 4 h vs. hypoglycaemia, *P* < .01; 24 h vs. hypoglycaemia, *P* < .01). In T2D, APP levels were depressed below baseline levels in the follow-up period (T2D: 30 min vs. hypoglycaemia, *P* < .05; 1 h vs. hypoglycaemia, *P* < .05; 2 h vs. hypoglycaemia, *P* < .05; 4 h vs. hypoglycaemia, *P* < .001; 24 h vs. hypoglycaemia, *P* < .001) (Figure 1A).

Following a hypoglycaemia-induced decrease in SNCA, levels normalized to baseline in the T2D cohort. However, in the control group, levels continued to decrease further during the follow-up period relative to baseline (control: 30 min vs. baseline, *P* < .0001; 1 h vs. hypoglycaemia, *P* = .0001; 2 h vs. hypoglycaemia, *P* < .001; 4 h vs. hypoglycaemia, *P* < .001; 24 h vs. hypoglycaemia, *P* < .001) (Figure 1B).

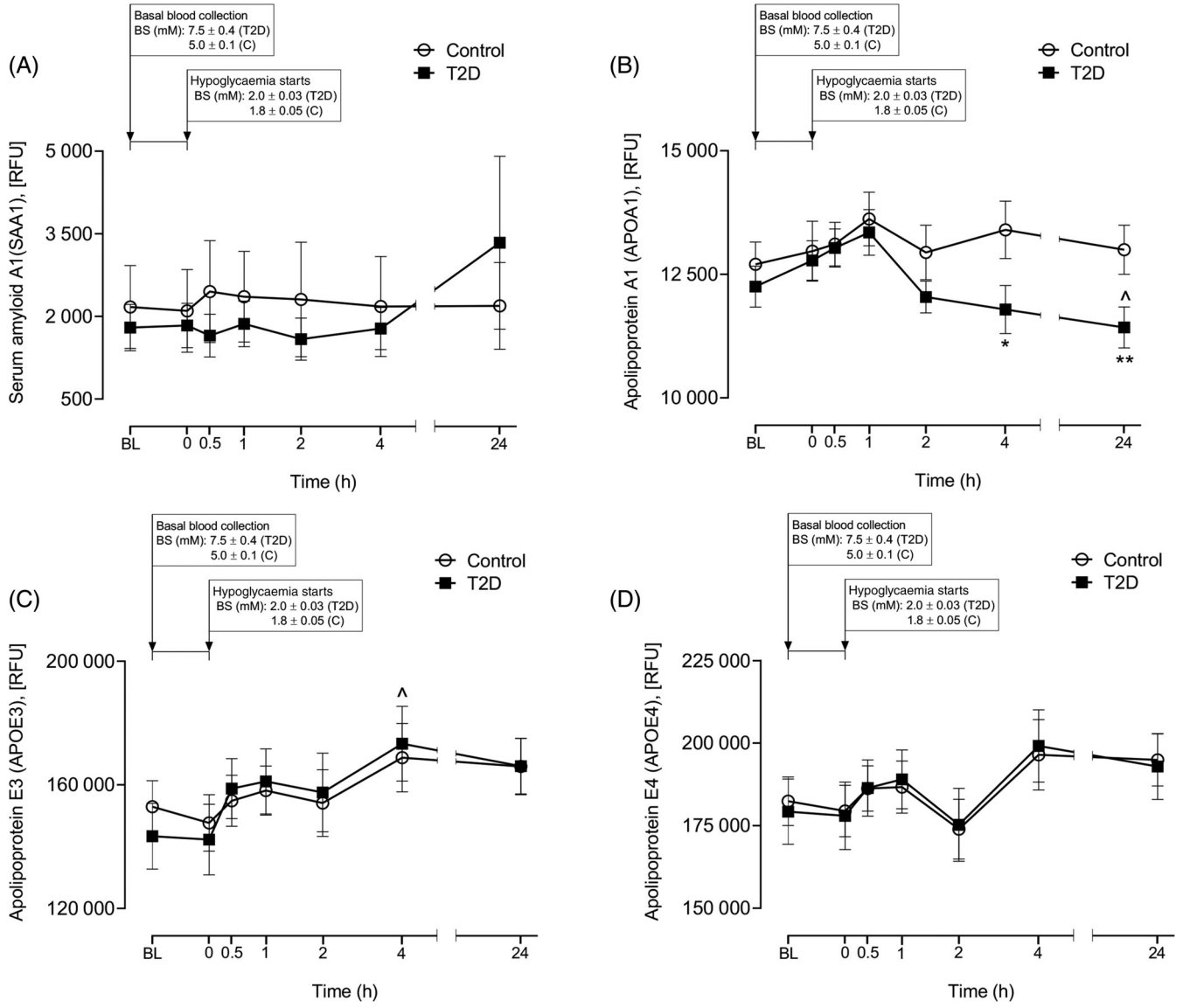


**FIGURE 3** Comparison of Alzheimer-related proteins, serum amyloid A1 (SAA1), apolipoprotein (APO) A1 (APOA1), APOE3 and APOE4 in plasma before, during and after iatrogenic induction of hypoglycaemia. Blood sampling was performed at baseline (BL), at hypoglycaemia (0 min) and post-hypoglycaemia (30 min, 1-, 2, 4 and 24 h) for controls (white circles) and for type 2 diabetes (T2D) (black squares). At BL, blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 mM (for control, C). At point of hypoglycaemia, BS was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 mM (for control). Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for A, SAA1, B, APOA1, C, APOE3 and D, APOE4. Statistics: *$P$ < .05, **$P$ < .01 control vs. T2D; ^$P$ < .05, T2D hypoglycaemia vs. T2D post-hypoglycaemia timepoints. RFU, relative fluorescent units

In both cohorts, MAPT levels showed a non-significant increasing trend in response to hypoglycaemia that, in controls, normalized to baseline in the follow-up period. In T2D, the MAPT level decreased below baseline in the follow-up period (T2D: 30 min vs. hypoglycaemia, $P$ = NS; 1 h vs. hypoglycaemia, $P$ < .05; 2 h vs. hypoglycaemia, $P$ < .01; 4 h vs. hypoglycaemia, $P$ < .01; 24 h vs. hypoglycaemia, $P$ < .01) (Figure 2A).

APCS levels were unchanged in the control group and largely unchanged in the T2D cohort, with only the 2-h timepoint being decreased relative to hypoglycaemia (T2D: 2 h vs. hypoglycaemia, $P$ < .05) (Figure 2B).

Hypoglycaemia-induced elevations in noggin also returned to baseline in both the control and T2D cohorts during the follow-up period (control: 30 min vs. hypoglycaemia, $P$ < .05; 1 h vs. hypoglycaemia, $P$ < .05; 2 h vs. hypoglycaemia, $P$ < .01; 4 h vs. hypoglycaemia, $P$ < .01; 24 h vs. hypoglycaemia, $P$ < .05) (T2D: 30 min vs. hypoglycaemia, $P$ = NS; 1 h vs. hypoglycaemia, $P$ < .05; 2 h vs. hypoglycaemia, $P$ < .05; 4 h vs. hypoglycaemia, $P$ < .001; 24 h vs. hypoglycaemia, $P$ < .001) (Figure 2C).

PAPPA was unchanged during the study in the control subjects; in the subjects with T2D, PAPPA tended to increase in the follow-up period relative to baseline, specifically at the 30-min and 4-h timepoints (T2D: 30 min vs. baseline, $P$ < .05; 1 h vs. hypoglycaemia,

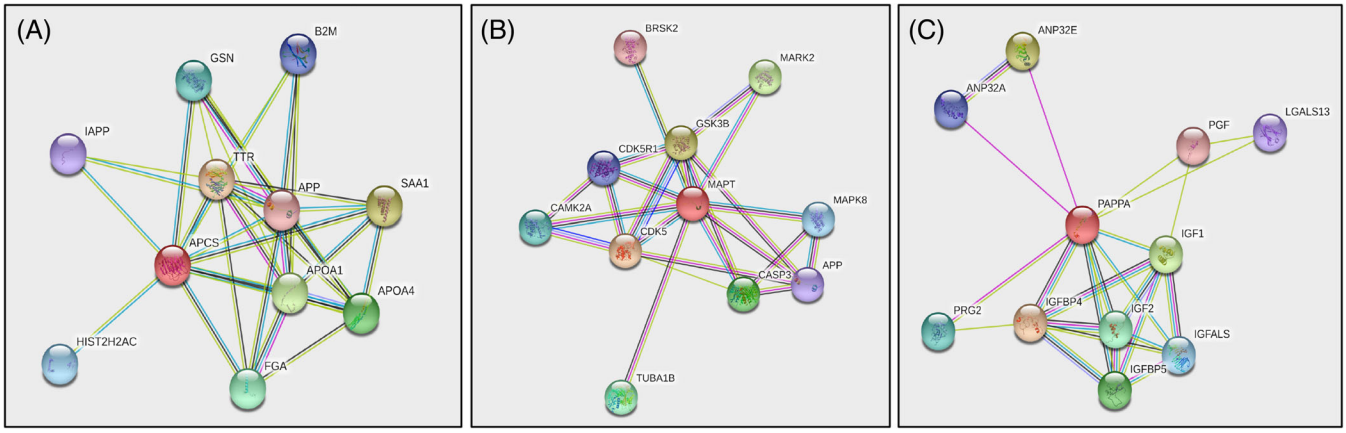


**FIGURE 4** STRING interaction network. STRING version 11.0 interaction network showing the interactions of: A, amyloid P component (APCS), amyloid precursor protein (APP), serum amyloid A1 (SAA1) and apolipoprotein (APO) A1; B, microtubule-associated protein tau (MAPT) and APP; and C, pappalysin (PAPPA). STRING 11.0 (Search Tool for the Retrieval of Interacting Genes) was used to visualize the known and predicted protein-protein interactions for proteins identified by SOMAscan assay in plasma of type 2 diabetes vs. control subjects (https://string-db.org/). Network nodes represent proteins and the lines reflect physical and/or functional interactions of proteins. Empty nodes represent the proteins of unknown three-dimensional structure, and filled nodes represent the proteins with some three-dimensional structure, either known or predicted. Different coloured lines between the proteins represent the various types of interaction evidence in STRING (databases, experiments, neighbourhood, gene fusion, co-occurrence, text mining, co-expression and homology): here, known interactions are shown in light blue (from curated databases) and pink (experimentally determined); predicted interactions are shown in dark blue (gene co-occurrence); relationships gleaned from text mining (lime green), co-expression (black) and protein homology (mauve) are also shown

*P* = NS; 2 h vs. hypoglycaemia, *P* = NS; 4 h vs. hypoglycaemia, *P* < .05; 24 h vs. hypoglycaemia, *P* = NS) (Figure 2D).

SAA1 levels did not change during the study in either cohort (Figure 3A). APOA1, a protein central to the interaction network of APCS, APP and SAA1, was unchanged at hypoglycaemia or at any timepoint in the 24-h follow-up period in control subjects; in the subjects with T2D, the level of APOA1 trended downwards in the follow-up period, reaching significance at 24 h (T2D: 24 h vs. hypoglycaemia, *P* < .05) (Figure 3B). APOE3 levels were raised at 4 h post-hypoglycaemia in the T2D cohort only (T2D: 4 h vs. baseline, *P* < .05; 4 h vs. hypoglycaemia, *P* < .05) (Figure 3C).

APOE4 levels were no different between control and T2D subjects and remained unchanged throughout the study (Figure 3D).

STRING analysis revealed that plasma AD-related proteins (APCS, APP, SAA1, APOA1, MAPT and PAPPA), that showed differences in response to hypoglycaemia, are interconnected, suggesting linkage between hypoglycaemia and induction of AD in T2D (Figure 4A-C).

IPA of the AD-related proteins revealed the possible cellular pathways and mechanistic linkage of four proteins (APCS, APP, PAPPA, SAA1) in hypoglycaemia-mediated development of AD (Figure 5). Canonical pathways that were affected by key AD-related proteins showed a direct relevance to the induced hypoglycaemia reported here.

### 3.4 | Correlation between age and body mass index and Alzheimer's disease-related proteins

Basal levels of APP increased with age in subjects with T2D, but not in controls (Figure S1A; see Supporting Information); however, basal levels of SNCA did not differ with age in either group (Figure S1B; see Supporting Information). Basal APOE proteins (AE, APOE2, APOE3, APOE4) decreased with age in subjects with T2D, but not in controls (Figure S1C-S1F; see Supporting Information). There were no correlations found between body mass index and basal levels of AD-related proteins in controls or subjects with T2D (Figure S2; see Supporting Information).

## 4 | DISCUSSION

There have been no previous studies looking at Alzheimer-related proteins in plasma in response to hypoglycaemia. Here, we show changes in plasma Alzheimer-related proteins in response to iatrogenic-induced hypoglycaemia in T2D and control subjects. Basal APP was higher and SNCA was lower in T2D, a pattern reflecting those findings reported for basal levels in AD.[11,12,14] These changes were also observed in the post-hypoglycaemia period for subjects with T2D and controls, with an elevation of APP that subsequently and significantly reduced in both controls and T2D, while SNCA significantly reduced in both controls and T2D following hypoglycaemia, indicating that hypoglycaemia induced further changes of these AD-related proteins. Similarly, noggin appeared to be increased by hypoglycaemia, then fell significantly afterwards to baseline levels. The AD-related proteins MAPT, PAPPA, APOA1 and APOE3 remained unchanged in controls, whereas in T2D, MAPT and APOA1 were reduced following hypoglycaemia but returned to baseline levels, while PAPPA and APOE3 increased in response to hypoglycaemia over the following 4-h time course but returned to

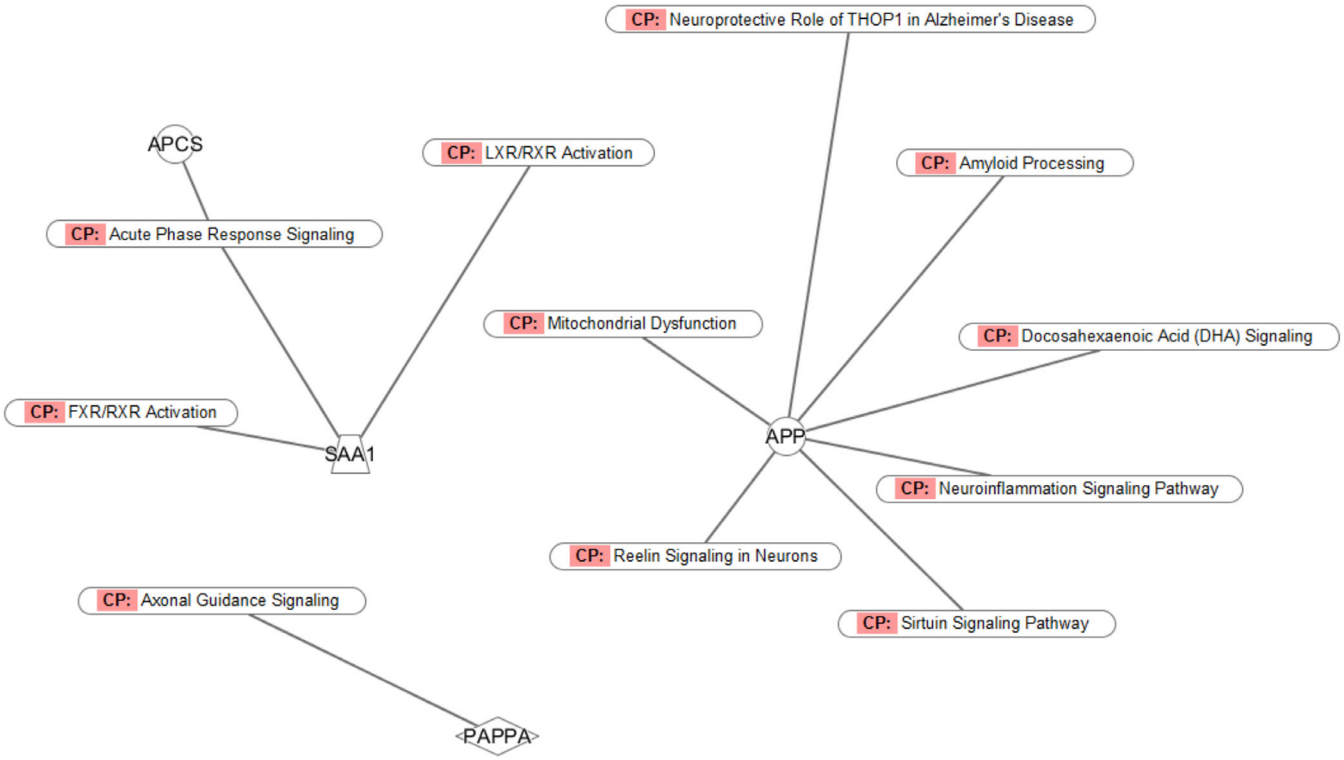


**FIGURE 5** Ingenuity pathway analysis. Ingenuity pathway analysis (IPA) of four amyloid-related protein genes, amyloid P component (APCS), amyloid precursor protein (APP), pappalysin (PAPPA) and serum amyloid A1 (SAA1). IPA demonstrates the most significantly affected pathways relating to the proteins in question. Of particular relevance to the induction of hypoglycaemia reported here, canonical pathways (CPs) affected by APP include the neuroprotective role of Thimet oligopeptidase (THOP1, an enzyme able to degrade the amyloid-beta precursor protein and generate amyloidogenic fragments) in Alzheimer's disease and the neuroinflammation signaling pathway; CPs affected by APCS and SAA1 include acute phase response signaling

baseline at 24 h. Some proteins, such as SAA1, remained unchanged throughout the study period in both cohorts.

The network of amyloid-related protein-protein interactions shown here using the STRING analysis tool demonstrates the close interconnectedness of these proteins, and therefore it would be anticipated that they act in concert in response to the stress of hypoglycaemia. Of interest are the most significantly affected canonical pathways relating to key amyloid-related proteins, as revealed by IPA. Of particular relevance to the induction of hypoglycaemia, canonical pathways affected by APP include the neuroprotective role of Thimet oligopeptidase (THOP1, an enzyme able to degrade the amyloid-beta precursor protein and generate amyloidogenic fragments) in AD, the neuroinflammation signalling pathway and amyloid processing; canonical pathways affected by APCS and SAA1 include acute phase response signalling.

AD and T2D are both diseases characterized by increased prevalence with ageing, genetic predisposition and comparable pathological features of amyloid deposition in the brain[23–25] and pancreatic islets,[26,27] respectively. The pathogenesis of amyloid diseases involving, for example, hippocampal cells in AD, dopaminergic neurons in Parkinson's disease and beta-cells in T2D, involves abnormal interactions of amyloidogenic proteins with cellular machinery and membranes.[28] Thus, these diverse conditions are all considered diseases of protein misfolding.[29–31] Much evidence points to the prefibrillar oligomers as being the primary cytotoxic form of these proteins.[32–35]

AD is characterized by an accumulation of β-amyloid (Aβ) and tau proteins in the brain. Evidence points to the generation of Aβ from APP as the critical step in the development of AD[36] and elevated serum levels have been reported,[11,12] as are reported here. In this study, it can be seen that the APP levels continue to fall after the hypoglycaemic event in those with T2D. The comparison of blood glucose in post-hypoglycaemic timepoints between control and T2D revealed a significant increase in blood glucose level occurring 1 h after the hypoglycaemic event and continuing up to 24 h in T2D compared with controls. This elevation of blood glucose in T2D occurred in response to a meal that the patients received 10 min post-hypoglycaemia (Figure 1A). This elevation of blood glucose post-hypoglycaemia is concurrent with the decrease of APP in T2D. A previous report demonstrated the same, where an increase in blood insulin concentration, followed by an increase in blood glucose concentration, was associated with a decrease in plasma APP concentration.[37] Moreover, previous reports demonstrated that an increase in insulin stimulates the synthesis of the lipoprotein receptor-related protein (LRP), within minutes, in vitro in hepatic cells.[38] As APP binds directly to LRP, which in turn mediates its uptake into cells,[39] and we observed a rapid rise of insulin just after starting the insulin clamp

(data not shown), it is possible that an insulin-induced decrease in plasma APP occurred in our study. Taken together, hyperinsulinaemia and postprandial hyperglycaemia probably drive the depression of plasma APP levels in subjects with T2D at post-hypoglycaemic timepoints (up to 24 h), and this action is probably mediated by rapid uptake of APP by the lipoprotein-binding protein LRP.

It is also possible that the decreasing APP levels in the T2D cohort post-hypoglycaemia reflect a general stress response. A study assessing proteomic changes in military personnel following moderate blast exposure resulting in traumatic brain injury found an acute decrease in APP lasting for up to 3 days post-injury and suggested that this was a protective mechanism.[40] IPA in that study revealed that the APP network was central in the response to traumatic brain injury.[40] Of relevance here, acute hypoglycaemia has been suggested to result in a form of traumatic brain injury and patients with T2D may be more severely affected than their non-diabetic counterparts,[41] which may help to explain the differences in protein levels in the peripheral circulation between the two groups. Given that diabetes may exacerbate changes in traumatic brain injury,[42] this may also account for the differences seen between controls and subjects with T2D for MAPT and for the changes in noggin.

Plasma MAPT levels had also fallen post-hypoglycaemia in T2D compared with control. Another possibility for the gradual decrease of MAPT might also be the elevation of blood glucose levels in response to the post-hypoglycaemia meal in patients with T2D. Previously, a hyperglycaemia-induced TAU cleavage has been reported in vitro and in diabetic mice, suggesting a possible link between diabetes and AD.[43] According to our data, post-hypoglycaemia MAPT levels were lower at 2 h post-hypoglycaemia induction (Figure 2A) and, at the same timepoint, the post-hypoglycaemia postprandial hyperglycaemia in the T2D cohort was highest (Figure 1A), suggesting hyperglycaemia-induced cleavage of MAPT.

APCS has been found in the senile plaques and neurofibrillary tangles in the brain of subjects with AD[44] and may hinder proteolysis of Aβ deposits, thereby promoting plaque formation[45]; serum levels have been reported to be decreased,[44] in accordance with our findings in the current study. SNCA induces the fibrillization of MAPT and while mostly associated with the pathophysiology of synucleinopathies such as Parkinson's disease and Lewy body-associated dementia, it is also involved in AD-related brain pathology through its interactions with Aβ.[46] Tau is a microtubule-associated protein in neurons encoded by the *MAPT* gene; in tauopathies, aberrant assembly and deposition of tau is accompanied by synaptic dysfunction and death of neurons[47] and increases in plasma tau have been associated with AD.[48] In this study, there was a transient increase in MAPT levels in response to hypoglycaemia, with levels in the follow-up period returning to baseline.

PAPPAs are known to cleave insulin-like growth factor binding proteins, and overexpression of PAPPA-2 has been shown to play a role in Aβ peptide accumulation in AD.[49] Increased serum levels of PAPPA-1 have been reported in subjects with T2D[50] and this is in accord with their suggested shared pathophysiology.[51] Elevated levels of PAPPA were only seen in patients with T2D after the hypoglycaemic event and were increased for up to 4 h, as levels returned to baseline at 24 h. Studies are inconclusive as regards circulating APOE levels in AD,[52] with some reporting an increase,[53] some a decrease[54] and some no change.[55] In this study, we found an increase in APOE3 levels in T2D following hypoglycaemia and lasting until the 4-h timepoint, but with no change in controls. APOE and APOE4 levels were unchanged throughout the study in either cohort.

Higher circulating APOA1 levels are associated with a lower risk of AD[56] and dementia.[57] In this study, APOA1 showed an overall decrease, still present at 24 h, following hypoglycaemia in T2D but not in controls. SAA1 is an acute-phase protein that may play a housekeeping role in healthy tissue, but increased expression has been shown in the brain in AD.[58] There were no changes in SAA in either the controls or T2D in response to hypoglycaemia. That so many of the proteins changed in response to induced hypoglycaemia is not surprising given the interrelated protein interactions shown clearly in the STRING analysis that were in accord with other reports[11,12,44,48,50,56]; however, the differential effect of the protein response in T2D compared with controls was marked.

It can be seen in this study that basal APP levels increased with age only in the T2D cohort, and a decrease in the basal APOE proteins, that have been reported to be protective,[59,60] was also seen only in the T2D cohort. Taken together, these results suggest that an AD pattern of proteins may be seen at baseline for patients with T2D and the changes in APP and APOE proteins are more pronounced with increasing age; hypoglycaemia causes their enhanced expression that may lead to the accelerated development of AD in susceptible patients or perhaps those particularly prone to severe and recurrent hypoglycaemia, as hypoglycaemia has been associated with AD.[15] What is of potential concern is that the patients with T2D in this study had a relatively short duration of disease (<5 years) and they were all on a single therapy (metformin) that was not associated with hypoglycaemia and, yet, they showed exaggerated changes in the AD-related proteins. Repetition of this study in patients of longer diabetes duration or on hypoglycaemic agents, including insulin, may show an even greater differential in AD-related protein expression. Further, it would be of interest to determine whether a similar profile of protein changes is observed when study subjects are subjected to a less severe but more prolonged hypoglycaemic episode, and for blood samples to be taken beyond 24 h and perhaps up to 1 week. Given the changes in APP, further work on the protective effects of APP metabolites needs to be undertaken.[61] Finally, that hypoglycaemia is having these effects is being assumed, rather than a response to counter-regulatory parameters.

The strengths of this study were inclusion of a group of T2D subjects of short disease duration who were relatively treatment naïve and not on polypharmacy. The main study limitation is the small study numbers and, with a larger population, even greater differences in plasma levels of amyloid-related proteins may have been discerned. However, it is important to note that these patients were subjected to

a severe hypoglycaemic episode, during which it would be anticipated that changes in protein levels would have become apparent. While subjects with T2D were older and more obese, this should not have altered the expression of these proteins. A further limitation is that peripheral blood samples were only collected up to the 24-h time-point, and it would be important in future studies to extend this period, perhaps for up to 1 week, to profile the time course of post-hypoglycaemia protein changes better.

## 5 | CONCLUSION

In conclusion, these data are in accord with the hypothesis that hypoglycaemia has a detrimental effect on AD-associated proteins. At baseline, subjects with T2D had elevated APP and reduced SNCA levels in plasma, reflecting changes previously reported in AD. These baseline changes were exacerbated in response to induced hypoglycaemia with increased APP and decreased SNCA in both T2D and controls. The other measured proteins did not differ at baseline between the cohorts. In the post-hypoglycaemia period, APCS, MAPT and APOA1 decreased, and APOE3 and PAPPA increased in T2D only, while noggin levels decreased in both cohorts, and SAA levels remained unchanged. Taken together, the circulating protein levels reported here suggest that subjects with T2D have an increased risk of the development of AD, and this risk may be exacerbated by hypoglycaemia.

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### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

### AUTHOR CONTRIBUTIONS

Abu Saleh Md Moin analysed the data and wrote the manuscript; Ahmed Al-Qaissi performed the clinical studies and edited the manuscript; Thozhukat Sathyapalan supervised clinical studies and data collection and contributed to the writing of the manuscript; Stephen L. Atkin contributed to study design, data interpretation and the writing of the manuscript. Alexandra E. Butler analysed the data and wrote the manuscript. All authors approved the final version of the manuscript. Stephen L. Atkin is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

### CONSENT FOR PUBLICATION

All authors gave their consent for publication.

### PEER REVIEW

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### DATA AVAILABILITY STATEMENT

Availability of data and materials: All the data for this study will be made available upon reasonable request to the corresponding author.

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## SUPPORTING INFORMATION

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

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