Hypoglycemia in Type 2 Diabetes exacerbates Amyloid-related proteins associated with dementia

Abu Saleh Md Moin, PhD[1], Ahmed Al-Qaissi MD[2], Thozhukat Sathyapalan MD[2], Johannes Graumann, PhD[3], Stephen L Atkin MD[4]*, Alexandra E. Butler MD[1]*

1. Diabetes Research Center (DRC), Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation (QF), PO Box 34110, Doha, Qatar

2. Academic Endocrinology, Diabetes and Metabolism, Hull York Medical School, Hull, UK.

3. Scientific Service Group Biomolecular Mass Spectrometry, Max Planck Institute for Heart and Lung Research, Ludwigstr. 43, 61231 Bad Nauheim Germany

4. Royal College of Surgeons of Ireland, Bahrain

*joint senior authors

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Corresponding author. Alexandra E Butler, Diabetes Research Center (DRC), Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation (QF), PO

Box 34110, Doha, Qatar. Email: aeb91011@gmail.com; abutler@hbku.edu.qa Phone: +97466876499.

Abstract

Objective: Hypoglycemia is a common complication of tighter glucose control regimens in type 2 diabetes (T2D) and may increase risk of Alzheimer's disease (AD). We hypothesized that changes in amyloid-related protein levels during induced hypoglycemia would be exacerbated during/following hypoglycemia in type 2 diabetes (T2D).

Methods: A prospective, parallel study in T2D (n=23) and controls (n=23). Hypoglycemia (<2.2mmol/l; <40mg/dl) was achieved by intravenous insulin infusion. Blood sampling was performed at baseline, hypoglycemia and post-hypoglycemia; proteomic (Somalogic) analysis of amyloid-related proteins performed: Amyloid-precursor protein [APP], Amyloid P-component [APCS], Noggin, Alpha-synuclein [SNCA], Microtubule-associated protein tau [MAPT], Pappalysin [PAPPA], Serum amyloid-A1 [SAA1], Apolipoprotein-A1 [ApoA1] and Apolipoprotein-E3 [ApoE3].

Results: APP was elevated ($p<0.01$) at baseline in T2D while SNCA was reduced ($p<0.01$). At hypoglycemia, APCS ($p<0.01$) was elevated and SNCA ($p<0.05$) reduced in T2D, whilst APP ($p<0.01$) and Noggin ($p<0.05$) were elevated and SNCA ($p<0.01$) reduced in controls. Following hypoglycemia, APP and MAPT normalized in controls but decreased below baseline in T2D; Noggin normalized in both cohorts; SNCA normalized in T2D but decreased below baseline in controls. PAPPA, SAA1, APCS, ApoA1 and ApoE3 changes were less marked.

Conclusions: Baseline parameters showed an increase in APP and a reduction in SNCA in T2D, reflecting the baseline levels previously reported in AD; these baseline changes were exaggerated, with APP increase and SNCA decrease, in response to hypoglycemia seen in both T2D and controls. Hypoglycemia was associated with changes in AD-associated proteins of APCS, MAPT, ApoA1, ApoE3 and PAPPA in T2D only, and Noggin in both cohorts.

Key words: Type 2 diabetes, hypoglycemia, dementia, Alzheimer's disease, amyloid-related proteins.

Introduction

Type 2 diabetes (T2D) prevalence has reached pandemic proportions. The International Diabetes Federation (IDF) currently estimates that 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 (3-6). Predictions suggest that the worldwide prevalence of patients suffering from dementia will increase from 35.6 million in 2010 to 115.4 million in 2050 (7). Given the association between T2D and dementia, and the dramatic predicted increases in each disease individually, the combined effect may exceed these predictions and have an even more devastating effect on the global population.

Alzheimer's disease (AD) comprises 60-80% of all dementia cases (8). Epidemiological studies have shown an increased risk for subjects with T2D developing AD (9-11). Elevation in plasma amyloid precursor protein (APP) has been reported in association with AD, levels tending to

increase with increasing cognitive impairment (12, 13). Whilst no difference in plasma levels of alpha-synuclein (SNCA) had previously been reported (14), a recent study showed lower concentrations of SNCA in red blood cells in the circulation of AD subjects (15).

Improved management of T2D involves stricter glucose control, increasing the risk and frequency of hypoglycemic episodes. Hypoglycemia itself is directly linked to cognitive dysfunction, complicating diabetic management and perhaps increasing the risk of dementia (16).

We hypothesized that changes in amyloid-related protein levels during induced hypoglycemia would be exacerbated during/following hypoglycemia in type 2 diabetes (T2D), thus providing a potential mechanistic link between T2D-related hypoglycemia and AD. This study was specifically designed to mimic the physiological responses to hypoglycemia as would be seen in diabetic patients in clinical practice (17). To this end, we analyzed the levels of amyloid-related proteins following acute iatrogenic induction of hypoglycemia in subjects with and without T2D.

Materials and Methods

A prospective parallel study was performed in the Diabetes Research Centre at Hull Royal Infirmary in adult T2D (n=23) and control (n=23) subjects between November 2011 and May 2013. All participants provided written informed consent. The trial was approved by the North West-Greater Manchester East Research Ethics Committee (REC number:16/NW/0518), registered at www.clinicaltrials.gov (NCT03102801) and conducted according to the Declaration of Helsinki.

All participants were Caucasian, aged 40-70 years. For T2D, diabetes duration was <10 years; all were on a stable dose of medication (metformin, statin and/or angiotensin converting enzyme inhibitor/angiotensin receptor blocker) over the prior 3 months. Exclusion criteria for T2D

cohort: any medications for glycemic control except metformin; poor glycemic control [HbA1c levels ≥10% (86mmol/mol)]; hypoglycemic unawareness or history of severe hypoglycemia in previous 3 months. Exclusion criteria for control group: diagnosis of type 1 or 2 diabetes or HbA1c >6% (42mmol/mol). Exclusion criteria for both groups: body mass index (BMI)<18 or >50kg/m^2; excessive alcohol consumption; renal or liver disease; history or presence of malignant neoplasms within prior 5 years; psychiatric illness; history of acute or chronic pancreatitis or gastrointestinal tract surgery; steroid treatment; pregnant or breastfeeding women; any contraindication to insulin infusion to achieve hypoglycemia (ischemic heart disease, epilepsy, history of seizures, drop attacks, history of adrenal insufficiency and treated hypothyroidism).

Participants were screened by medical history, clinical examination, routine blood tests and an electrocardiogram. On the day of the study, participants avoided habitual exercise (defined as brisk walking >20min) for >24h and individuals with T2D on medication withheld their oral hypoglycemic agents on the morning of the visit. Participants were weighed (Marsden Weighing Machine Group Ltd, UK) and height was taken barefoot using a wall-mounted stadiometer. Blood pressure was measured using a sphygmomanometer (Datascope Duo Masimo Set, Mindray Ltd, UK) and a fasting blood sample was collected before insulin infusion (baseline). A continuous insulin infusion was performed to induce hypoglycemia. Blood samples were taken at 0, 30, 60, 120 and 240 minutes after hypoglycemia. After 240 minutes, participants were provided lunch and were given their (morning) diabetes medications. Patients took their evening medication as prescribed that day. Subjects reattended 24-hours following the induction of hypoglycemia; patients withheld their medications until they completed the blood tests in the fasted state, after which breakfast was provided. Prior to discharge, blood glucose was checked

using a glucose analyser (HemoCue® glucose 201+) to ensure normal levels, together with other vital signs.

Insulin Infusion

Following an overnight fast, bilateral ante-cubital fossa indwelling cannulas were inserted 30-60 minutes prior to the commencement of the test (0830h). To induce hypoglycemia, soluble intravenous insulin (Humulin S, Lilly, UK) was given in a pump starting at a dose of 2.5mU/kg body weight/min with an increment of 2.5mU/kg body weight/min every 15min by hypoglycemic clamp (18), until two readings of capillary blood glucose measured by a glucose analyser (HemoCue® glucose 201+) ≤2.2 mmol/L (<40mg/dl) or a reading of ≤2.0 mmol/L (36mg/dl) (18). The blood sample schedule was timed subsequently in respect to the time point that hypoglycemia occurred. Following the identification of hypoglycemia, intravenous glucose was given in the form of 150 ml of 10% dextrose and a repeat blood glucose check was performed after 5 minutes if blood glucose was still <4.0 mmol/L.

Biochemical markers:

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

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 plama kit (SomaLogic, Boulder, CO)

according to manufacturer's instructions and as described previously (19, 20). The assay was performed in 96-well plates containing up to 85 plasma samples, 3 quality control and 5 calibrator plasma 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 a 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).

Data processing and analysis

Initial Relative Fluorescent Units (RFUs) were obtained from microarray intensity images using the Agilent Feature Extraction Software (Agilent, Santa Clara, CA). Raw RFUs were normalized and calibrated using the software pipeline provided by SomaLogic. This included (a) microarray hybridization normalization based on spiked-in hybridization controls, (b) plate-specific intensity normalization, (c) median signal normalization, and (d) median calibrator scaling of single RFU intensities according to calibrator reference values. Samples with high degree of hemolysis (Haptoglobin log RFU < 10) were excluded from 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 analyzed using the autonomics and limma (21) packages. For

differential protein analysis we applied limma models containing contrasts between timepoints, as well as contrasts between healthy and diabetic patients 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 covariate to the model. Limma obtained P values were corrected using the Benjamini-Hochberg method (22).

Statistical analysis:

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. A p-value of <0.05 was considered statistically significant. Within group comparisons are as follows: changes from baseline, and from hypoglycemia, 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 subgroup (i.e. condition:timepoint), while taking the patient ID as a random effect using the R package limma. Subsequently, we computed the p value for two contrasts: baseline to hypoglycemia for both T2D and controls, and false discovery rate (FDR) corrected at a value of <0.05 as the cutoff for significance.

Results:

46 subjects were recruited (23 people with T2D, 23 controls)(17). Demographic and clinical characteristics of the subjects are presented in **Table 1.**

Nine amyloid-related proteins were included in the analysis: Amyloid precursor protein [APP], Amyloid P component [APCS], Noggin, Alpha-synuclein [SNCA], Microtubule-associated protein tau [MAPT], Pappalysin [PAPPA] and Serum amyloid A1 [SAA1], Apolipoprotein A1 [ApoA1] and Apolipoprotein E3 [ApoE3].

Differences between T2D and controls

In the T2D cohort, baseline levels of APP were elevated (35594±6559 vs 18535±1837 RFU, T2D vs control, $p<0.01$) (Figure 1A) and SNCA decreased (5836±272 vs 7175±420 RFU, T2D vs control, $p<0.01$) (Figure 1B). Baseline levels of the other proteins did not differ between cohorts (Figure 2A – 2D) and (Figure 3A – 3D).

At hypoglycemia, APCS was elevated in T2D (35128±1307 vs 31594±1473 RFU, T2D vs control, $p<0.05$) (Figure 2B).

Within cohort changes at the point of hypoglycemia

Significant changes occurred in the control group in response to hypoglycemia with APP and Noggin being elevated (Control, hypoglycemia vs baseline: APP, 37549±7046 vs 18535±1837 RFU, $p<0.01$) (Figure 1A); Noggin, 2899±344 vs 2084±123 RFU, $p<0.05$) (Figure 2C) and SNCA levels decreasing (Control, hypoglycemia vs baseline: 5558±310 vs 7175±420 RFU, $p<0.01$) (Figure 1B).

In the T2D cohort, APP levels were already elevated as compared to controls, so whilst there was a further increase in response to hypoglycemia, this did not reach significance. Whilst SNCA levels were already relatively decreased in T2D, as in controls, SNCA levels were further

depressed in T2D in response to hypoglycemia (T2D, hypoglycemia vs baseline: alpha-synuclein, 4931±261 vs 5836±272 RFU, $p<0.05$) (Figure 1B).

Changes in protein levels in the 24-hour follow-up period

In the post-hypoglycemia period, the elevated hypoglycemia-induced APP levels gradually decreased to baseline during the 24-hour follow-up period in controls (Controls: 30-minutes vs hypoglycemia, 21714±4539 vs 37549±7046 RFU, $p<0.05$; 1-hour vs hypoglycemia, 18994±3077 vs 37549±7046 RFU, $p<0.01$; 2-hour vs hypoglycemia, 17905±4890 vs 37549±7046 RFU, $p<0.05$; 4-hour vs hypoglycemia, 17475±3219 vs 37549±7046 RFU, $p<0.01$; 24-hour vs hypoglycemia, 16237±2788 vs 37549±7046 RFU, $p<0.01$). In T2D, APP levels were depressed below baseline levels in the follow-up period (T2D: 30-minutes vs hypoglycemia, 30455±5064 vs 46877±6340 RFU, $p<0.05$; 1-hour vs hypoglycemia, 30507±5665 vs 46877±6340 RFU, $p<0.05$; 2-hour vs hypoglycemia, 26690±6211 vs 46877±6340 RFU, $p<0.05$; 4-hour vs hypoglycemia, 20684±3780 vs 46877±6340 RFU, $p<0.001$; 24-hour vs hypoglycemia, 21728±3955 vs 46877±6340 RFU, $p<0.001$) (Figure 1A).

Following a hypoglycemia-induced decrease in SNCA, levels normalized to baseline in the T2D cohort. In the control group, however, levels continued to decrease further during the follow-up period relative to baseline (Control: 30-minutes vs baseline, 4944±223 vs 7175±420 RFU, $p<0.0001$; 1-hour vs hypoglycemia, 5170±284 vs 7175±420 RFU, $p=0.0001$; 2-hour vs hypoglycemia, 5196±340 vs 7175±420 RFU, $p<0.001$; 4-hour vs hypoglycemia, 5089±460 vs 7175±420 RFU, $p<0.001$; 24-hour vs hypoglycemia, 5232±392 vs 7175±420 RFU, $p<0.001$) (Figure 1B).

In both cohorts, MAPT (tau) levels showed a non-significant increasing trend in response to hypoglycemia 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-minutes vs hypoglycemia, 147±7 vs 166±10 RFU, p=ns; 1-hour vs hypoglycemia, 143±7 vs 166±10 RFU, $p<0.05$; 2-hour vs hypoglycemia, 130±9 vs 166±10 RFU, $p<0.01$; 4-hour vs hypoglycemia, 132±5 vs 166±10 RFU, $p<0.01$; 24-hour vs hypoglycemia, 136±6 vs 166±10 RFU, $p<0.01$) (Figure 2A).

APCS levels were unchanged in the control group and largely unchanged in the T2D cohort, with only the 2-hour timepoint being decreased relative to hypoglycemia (T2D: 2-hour vs hypoglycemia, 31589±1394 vs 35128±1307, $p<0.05$) (Figure 2B).

Hypoglycemia-induced elevations in Noggin also returned to baseline in both the control and T2D cohorts during the follow-up period (Control: 30-minutes vs hypoglycemia, 2121±171 vs 2899±344 RFU, $p<0.05$; 1-hour vs hypoglycemia, 2162±203 vs 2899±344 RFU, $p<0.05$; 2-hour vs hypoglycemia, 1896±198 vs 2899±344 RFU, $p<0.01$; 4-hour vs hypoglycemia, 1809±104 vs 2899±344 RFU, $p<0.01$; 24-hour vs hypoglycemia, 2017±143 vs 2899±344 RFU, $p<0.05$) (T2D: 30-minutes vs hypoglycemia, 2383±243 vs 2788±228 RFU, p=ns; 1-hour vs hypoglycemia, 2236±221 vs 2788±228 RFU, $p<0.05$; 2-hour vs hypoglycemia, 2058±283 vs 2788±228 RFU, $p<0.05$; 4-hour vs hypoglycemia, 1974±188 vs 2788±228 RFU, $p<0.001$; 24-hour vs hypoglycemia, 2048±157 vs 2788±228 RFU, $p<0.001$) **(**Figure 2C).

PAPPA was unchanged during the study in the control subjects; in the T2D subjects, PAPPA tended to increase in the follow-up period relative to baseline, specifically at the 30-minute and 4-hour timepoints (T2D: 30-minutes vs baseline, 18299±1129 vs 15260±973 RFU, $p<0.05$; 1-hour vs hypoglycemia, 17367±1236 vs 15260±973 RFU, p=ns; 2-hour vs hypoglycemia,

14907±1000 vs 15260±973 RFU, p=ns; 4-hour vs hypoglycemia, 18246±1301 vs 15260±973 RFU, p<0.05; 24-hour vs hypoglycemia, 16498±1000 vs 15260±973 RFU, 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 hypoglycemia or at any time-point in the 24-hour follow-up period in control subjects; in the T2D subjects, the level of ApoA1 trended downwards in the follow-up period, reaching significance at 24-hours (T2D: 24-hours vs hypoglycemia, 11426±417 vs 12780±397 RFU, p<0.05) (Figure 3B).

ApoE3 levels were raised at 4-hours post-hypoglycemia in the T2D cohort only (T2D: 4-hours vs baseline, 173319±12097 vs 143355±10655 RFU, p<0.05; 4-hours vs hypoglycemia, 173319±12097 vs 142301±11440 RFU, p<0.05) (Figure 3C).

ApoE4 levels were no different between control and T2D subjects and remained unchanged throughout the study (Figure 3D).

The string network interaction (STRING version 11.0) revealed that plasma AD-related proteins (APCS, APP, SAA1 and APOA1, MAPT and APP and PAPPA) that showed differences in response to hypoglycemia are interconnected, suggesting linkage between hypoglycemia and induction of AD in T2D (Figure 4A–C).

Moreover, Ingenuity Pathway Analysis of the AD-related proteins also revealed the possible cellular pathways and mechanistic linkage of 4 proteins (APCS, APP, PAPPA, SAA1) in hypoglycemia-mediated development of AD (Figure 5).

Discussion

There have been no previous studies looking at Alzheimers-related proteins in plasma in response to hypoglycemia. Here, we show changes in plasma Alzheimer-related proteins in response to iatrogenic-induced hypoglycemia 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 (12, 13, 15). These changes were also observed in the post-hypoglycemia period for T2D and control subjects, with an elevation of APP that subsequently and significantly reduced in both controls and T2D, whilst SNCA significantly reduced in both controls and T2D following hypoglycemia, indicating that hypoglycemia induced further changes of these AD related proteins. Similarly, Noggin appeared to be increased by hypoglycemia, then fell significantly afterwards to baseline levels. The AD related proteins MAPT, PAPPA, ApoA1 and ApoE3 remained unchanged in controls whilst, in T2D, MAPT and ApoA1 were reduced following hypoglycemia but returned to baseline levels whilst PAPPA and ApoE3 increased in response to hypoglycemia over the following 4-hour time course but returned to baseline at 24 hours. Some proteins, such as SAA1, remained unchanged throughout the study period in both cohorts.

AD and T2D are both diseases characterized by increased prevalence with aging, genetic predisposition and comparable pathological features of amyloid deposition in brain (23-26) and pancreatic islets (27, 28), respectively. The pathogenesis of amyloid diseases involving, for example, hippocampal cells in Alzheimer's disease, dopaminergic neurons in Parkinson's disease and beta-cells in type 2 diabetes, involves abnormal interactions of amyloidogenic proteins with cellular machinery and membranes (29). Thus, these diverse conditions are all considered to be diseases of protein misfolding (30-32). Much evidence points to the prefibrillar oligomers as being the primary cytotoxic form of these proteins (33-36).

AD is characterized by accumulation of β-amyloid (Aβ) and tau proteins in the brain. Evidence points to the generation of Aβ from amyloid precursor protein (APP) as the critical step in development of AD (37) and elevated serum levels have been reported (12, 13), as are reported here.

Amyloid P component (APCS) has been found in the senile plaques and neurofibrillary tangles in the brain of subjects with Alzheimer's disease (38) and may hinder proteolysis of Aβ deposits, thereby promoting plaque formation (39); serum levels have been reported to be decreased (38), in accord with our findings in the current study.

Alpha-synuclein induces the fibrillization of MAPT (tau); while mostly associated with the pathophysiology of synucleinopathies such as Parkinson's disease and Lewy body-associated dementia, it is also involved in Alzheimer's disease-related brain pathology through its interactions with Aβ (40).

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 (41) and increases in plasma tau have been associated with AD (42). In this study, there was a transient increase in MAPT (tau) levels in response to hypoglycemia, with levels in the follow-up period returning to baseline.

Pappalysins (PAPPAs) are known to cleave IGF-binding proteins (IGFBPs), and overexpression of PAPPA-2 has been shown to play a role in Aβ peptide accumulation in Alzheimer's disease (43). Increased serum levels of PAPPA-1 have been reported in T2D subjects (44) and this is in accord with their suggested shared pathophysiology (45). Elevated levels of PAPPA were only seen in patients with T2D following the hypoglycemic event and were increased for to up to 4 hours, as levels returned to baseline at 24 hours.

Studies are inconclusive as regards circulating ApoE levels in AD (46), with some reporting an increase (47), some a decrease (48) and some no change (49). In this study, we found an increase in ApoE3 levels in T2D following hypoglycemia and lasting until the 4-hour 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 (50) and dementia (51). In this study, ApoA1 showed an overall decrease, still present at 24-hours, following hypoglycemia in T2D but not in controls.

Serum amyloid A1 (SAA1) is an acute phase protein that may play a housekeeping role in normal tissue, but increased expression has been shown in the brain in Alzheimer's disease (52). There were no changes in SAA in either the controls or T2D in response to hypoglycemia.

That so many of the proteins changed in response to induced hypoglycemia is not surprising given the interrelated protein interactions shown clearly in the string analysis that were in accord with other reports (12, 13, 38, 42, 44, 50); however, the differential effect of the protein response in T2D compared to controls was marked.

Taken together, these results suggest that an AD pattern of proteins may be seen at baseline for patients with T2D; hypoglycemia causes their enhanced expression that may lead to an accelerated development of AD in susceptible patients or perhaps those particularly prone to severe and recurrent hypoglycemia, as hypoglycemia has been associated with AD (16). What is of potential concern is that the patients with T2D in this study had a relatively short duration of disease (less than 5 years) and they were all on single therapy (metformin) that is not associated with hypoglycemia and, yet, they showed exaggerated changes in the AD-related proteins.

Repetition of this study in patients of longer diabetes duration or on hypoglycemic agents including insulin may show an even greater differential in AD protein expression.

The strengths of this study include the 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 serum levels of amyloid-related proteins may have been discerned. However, it is important to note that these patients were subject to a severe hypoglycemic episode, during which it would be anticipated that changes in protein levels would have become apparent. Whilst subjects with T2D were older and more obese, this should not have altered the expression of these proteins.

In conclusion, at baseline, T2D subjects had elevated APP and reduced SNCA levels in plasma, reflecting the changes previously reported in AD. These baseline changes were exacerbated in response to induced hypoglycemia with an increase in APP and a decrease in SNCA seen in both T2D and controls. The other measured proteins did not differ at baseline between the T2D and control cohorts. In the post-hypoglycemia period, APCS, MAPT and ApoA1 decreased, and ApoE3 and PAPPA increased in T2D only, whilst Noggin levels decreased in both cohorts, and SAA levels remained unchanged. Taken together, the circulating protein levels reported here suggest that T2D subjects have an increased risk for development of AD, and this risk may be exacerbated by hypoglycemia.

DECLARATIONS

Ethics approval and consent to participate: The trial was approved by the North West-Greater Manchester East Research Ethics Committee (REC number:16/NW/0518), registered at www.clinicaltrials.gov (NCT03102801) and conducted according to the Declaration of Helsinki.

All participants provided written informed consent.

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

Conflict of interest: None of the authors have any conflict of interest to declare.

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Author contributions: ASMM - analyzed the data and wrote the manuscript. JG– performed experiments and contributed to the writing of manuscript; TS– supervised clinical studies and data collection and contributed to the writing of manuscript; SLA– contributed to study design, data interpretation and the writing of the manuscript. AEB- analyzed the data and wrote the manuscript. All authors approved the final version of the manuscript.

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References

1. DF Diabetes Atlas Belgium: International Diabetes Federation; 2017 [8th:[Available from: ttp://www.diabetesatlas.org.
2. Strachan MW, Reynolds RM, Marioni RE, Price JF. Cognitive function, dementia and type 2 diabetes mellitus in the elderly. Nat Rev Endocrinol. 2011;7(2):108-14.
3. Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. Lancet Neurol. 2006;5(1):64-74.
4. Cukierman T, Gerstein HC, Williamson JD. Cognitive decline and dementia in diabetes--systematic overview of prospective observational studies. Diabetologia. 2005;48(12):2460-9.
5. Strachan MW, Deary IJ, Ewing FM, Frier BM. Is type II diabetes associated with an increased risk of cognitive dysfunction? A critical review of published studies. Diabetes Care. 1997;20(3):438-45.
6. Gudala K, Bansal D, Schifano F, Bhansali A. Diabetes mellitus and risk of dementia: A meta-analysis of prospective observational studies. J Diabetes Investig. 2013;4(6):640-50.
7. Prince M, Bryce R, Albanese E, Wimo A, Ribeiro W, Ferri CP. The global prevalence of dementia: a systematic review and metaanalysis. Alzheimers Dement. 2013;9(1):63-75 e2.
8. Alzheimer's A. 2014 Alzheimer's disease facts and figures. Alzheimers Dement. 2014;10(2):e47-92.
9. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology. 1999;53(9):1937-42.
10. Sims-Robinson C, Kim B, Rosko A, Feldman EL. How does diabetes accelerate Alzheimer disease pathology? Nat Rev Neurol. 2010;6(10):551-9.
11. Zilkens RR, Davis WA, Spilsbury K, Semmens JB, Bruce DG. Earlier age of dementia onset and shorter survival times in dementia patients with diabetes. Am J Epidemiol. 2013;177(11):1246-54.
12. Yun SM, Cho SJ, Jo C, Park MH, Han C, Koh YH. Elevation of plasma soluble amyloid precursor protein beta in Alzheimer's disease. Arch Gerontol Geriatr. 2020;87:103995.
13. Bush AI, Beyreuther K, Masters CL. Beta A4 amyloid protein and its precursor in Alzheimer's disease. Pharmacol Ther. 1992;56(1):97-117.
14. Shi M, Zabetian CP, Hancock AM, Ginghina C, Hong Z, Yearout D, et al. Significance and confounders of peripheral DJ-1 and alpha-synuclein in Parkinson's disease. Neurosci Lett. 2010;480(1):78-82.
15. Baldacci F, Daniele S, Piccarducci R, Giampietri L, Pietrobono D, Giorgi FS, et al. Potential Diagnostic Value of Red Blood Cells alpha-Synuclein Heteroaggregates in Alzheimer's Disease. Mol Neurobiol. 2019;56(9):6451-9.
16. Chin SO, Rhee SY, Chon S, Baik SH, Park Y, Nam MS, et al. Hypoglycemia is associated with dementia in elderly patients with type 2 diabetes mellitus: An analysis based on the Korea National Diabetes Program Cohort. Diabetes Res Clin Pract. 2016;122:54-61.
17. Al-Qaissi A, Papageorgiou M, Deshmukh H, Madden LA, Rigby A, Kilpatrick ES, et al. Effects of acute insulin-induced hypoglycaemia on endothelial microparticles in adults with and without type 2 diabetes. Diabetes, obesity & metabolism. 2019;21(3):533-40.

18. Hepburn DA, Frier BM. Hypoglycemia Unawareness in Patients with Insulin-Treated Diabetes-Mellitus. Saudi Medical Journal. 1991;12(3):182-90.
19. Kraemer S, Vaught JD, Bock C, Gold L, Katilius E, Keeney TR, et al. From SOMAmer-based biomarker discovery to diagnostic and clinical applications: a SOMAmer-based, streamlined multiplex proteomic assay. PLoS One. 2011;6(10):e26332.
20. Suhre K, Arnold M, Bhagwat AM, Cotton RJ, Engelke R, Raffler J, et al. Connecting genetic risk to disease end points through the human blood plasma proteome. Nat Commun. 2017;8:14357.
21. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.
22. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001;125(1-2):279-84.
23. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A. 1985;82(12):4245-9.
24. Masters CL, Multhaup G, Simms G, Pottgiesser J, Martins RN, Beyreuther K. Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. EMBO J. 1985;4(11):2757-63.
25. Bahmanyar S, Higgins GA, Goldgaber D, Lewis DA, Morrison JH, Wilson MC, et al. Localization of amyloid beta protein messenger RNA in brains from patients with Alzheimer's disease. Science. 1987;237(4810):77-80.
26. Goedert M. Neuronal localization of amyloid beta protein precursor mRNA in normal human brain and in Alzheimer's disease. EMBO J. 1987;6(12):3627-32.
27. Westermark P, Wilander E. The influence of amyloid deposits on the islet volume in maturity onset diabetes mellitus. Diabetologia. 1978;15(5):417-21.
28. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes. 2003;52(1):102-10.
29. Jayaraman S, Gantz DL, Haupt C, Gursky O. Serum amyloid A forms stable oligomers that disrupt vesicles at lysosomal pH and contribute to the pathogenesis of reactive amyloidosis. Proc Natl Acad Sci U S A. 2017;114(32):E6507-E15.
30. Campioni S, Mannini B, Zampagni M, Pensalfini A, Parrini C, Evangelisti E, et al. A causative link between the structure of aberrant protein oligomers and their toxicity. Nat Chem Biol. 2010;6(2):140-7.
31. Stefani M. Structural features and cytotoxicity of amyloid oligomers: implications in Alzheimer's disease and other diseases with amyloid deposits. Prog Neurobiol. 2012;99(3):226-45.
32. Guerrero-Munoz MJ, Castillo-Carranza DL, Kayed R. Therapeutic approaches against common structural features of toxic oligomers shared by multiple amyloidogenic proteins. Biochem Pharmacol. 2014;88(4):468-78.
33. Hirakura Y, Carreras I, Sipe JD, Kagan BL. Channel formation by serum amyloid A: a potential mechanism for amyloid pathogenesis and host defense. Amyloid. 2002;9(1):13-23.

34. Janson J, Ashley RH, Harrison D, McIntyre S, Butler PC. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. Diabetes. 1999;48(3):491-8.
35. Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, et al. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science. 2003;300(5618):486-9.
36. Gurlo T, Ryazantsev S, Huang CJ, Yeh MW, Reber HA, Hines OJ, et al. Evidence for proteotoxicity in beta cells in type 2 diabetes: toxic islet amyloid polypeptide oligomers form intracellularly in the secretory pathway. Am J Pathol. 2010;176(2):861-9.
37. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci. 2011;34:185-204.
38. Nishiyama E, Iwamoto N, Kimura M, Arai H. Serum amyloid P component level in Alzheimer's disease. Dementia. 1996;7(5):256-9.
39. Verwey NA, Schuitemaker A, van der Flier WM, Mulder SD, Mulder C, Hack CE, et al. Serum amyloid p component as a biomarker in mild cognitive impairment and Alzheimer's disease. Dement Geriatr Cogn Disord. 2008;26(6):522-7.
40. Twohig D, Nielsen HM. alpha-synuclein in the pathophysiology of Alzheimer's disease. Mol Neurodegener. 2019;14(1):23.
41. Guo T, Noble W, Hanger DP. Roles of tau protein in health and disease. Acta Neuropathol. 2017;133(5):665-704.
42. Mattsson N, Zetterberg H, Janelidze S, Insel PS, Andreasson U, Stomrud E, et al. Plasma tau in Alzheimer disease. Neurology. 2016;87(17):1827-35.
43. Mihelcic M, Simic G, Babic Leko M, Lavrac N, Dzeroski S, Smuc T, et al. Using redescription mining to relate clinical and biological characteristics of cognitively impaired and Alzheimer's disease patients. PLoS One. 2017;12(10):e0187364.
44. Heidari B, Fotouhi A, Sharifi F, Mohammad K, Pajouhi M, Paydary K, et al. Elevated serum levels of pregnancy-associated plasma protein-A in type 2 diabetics compared to healthy controls: associations with subclinical atherosclerosis parameters. Acta Med Iran. 2015;53(7):395-402.
45. Akter K, Lanza EA, Martin SA, Myronyuk N, Rua M, Raffa RB. Diabetes mellitus and Alzheimer's disease: shared pathology and treatment? Br J Clin Pharmacol. 2011;71(3):365-76.
46. Irizarry MC. Biomarkers of Alzheimer disease in plasma. NeuroRx. 2004;1(2):226-34.
47. Taddei K, Clarnette R, Gandy SE, Martins RN. Increased plasma apolipoprotein E (apoE) levels in Alzheimer's disease. Neurosci Lett. 1997;223(1):29-32.
48. Siest G, Bertrand P, Qin B, Herbeth B, Serot JM, Masana L, et al. Apolipoprotein E polymorphism and serum concentration in Alzheimer's disease in nine European centres: the ApoEurope study. ApoEurope group. Clin Chem Lab Med. 2000;38(8):721-30.
49. Scacchi R, Gambina G, Ruggeri M, Martini MC, Ferrari G, Silvestri M, et al. Plasma levels of apolipoprotein E and genetic markers in elderly patients with Alzheimer's disease. Neurosci Lett. 1999;259(1):33-6.
50. Button EB, Robert J, Caffrey TM, Fan J, Zhao W, Wellington CL. HDL from an Alzheimer's disease perspective. Curr Opin Lipidol. 2019;30(3):224-34.

51. Saczynski JS, White L, Peila RL, Rodriguez BL, Launer LJ. The relation between apolipoprotein A-I and dementia: the Honolulu-Asia aging study. Am J Epidemiol. 2007;165(9):985-92.
52. Urieli-Shoval S, Linke RP, Matzner Y. Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. Curr Opin Hematol. 2000;7(1):64-9.

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	<0.0001
Sex (M/F)	12/11	11/12	0.77
Weight (kg)	90.9±11.1	79.5±8.8	<0.0001
Height (cm)	167±14	169±5	0.64
BMI (kg/m^2)	32±4	28±3	<0.0001
Systolic BP (mmHg)	132±8	122±8	0.001
Diastolic BP (mmHg)	81±7	75±6	0.003
Duration of diabetes (years)	4.5±2.2	N/A	
HbA1c (mmol/mol)	51.2±11.4	37.2±2.2	<0.0001
HbA1c (%)	6.8±1.0	5.6±0.2	<0.0001
Total cholesterol (mmol/l)	4.2±1.01.0	4.8±0.77	0.014
Triglyceride (mmol/l)	1.7±0.7	1.34±0.6	0.055
HDL-cholesterol (mmol/l)	1.1±0.3	1.5±0.4	0.001
LDL-cholesterol (mmol/l)	2.23±0.8	2.7±0.87	0.051
CRP (mg/l)	3.10±2.87	5.30±1110.03	0.66

BMI: Body mass index, BP: Blood pressure, HDL-cholesterol: High density lipoprotein cholesterol, LDL-cholesterol: Low density lipoprotein cholesterol, CRP: C-reactive protein. HbA1c: Hemoglobin A1c

Figure legends.

Figure 1. Comparison of Alzheimer-related proteins, Amyloid precursor protein (APP) and α-synuclein (SNCA) in plasma before, during and after iatrogenic induction of hypoglycemia. Blood sampling was performed at baseline, at hypoglycemia and post-hypoglycemia (30 minutes, 1-hour, 2-hours, 4-hours and 24-hours) for controls (white circles) and for T2D (black squares). At baseline, blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 (for control, C). At point of hypoglycemia, blood sugar (BS) was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 (for control). Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for Amyloid-precursor protein [APP] (A), Alpha-synuclein [SNCA] (B). Statistics: [(* $p< 0.05$ or ** $p< 0.01$, control vs T2D); (# $p<0.01$, control baseline vs control hypoglycemia); ($ $p<0.05$ T2D baseline vs T2D hypoglycemia);(#$ $p<0.05$ control hypoglycemia vs control post-hypoglycemia timepoints); (^ $p<0.05$ T2D hypoglycemia vs T2D post-hypoglycemia timepoints); (#% control baseline vs control post-hypoglycemia timepoints). RFU, relative fluorescent units.

Figure 2. Comparison of Alzheimer-related proteins in plasma of before, during and after iatrogenic induction of hypoglycemia. Blood sampling was performed at baseline, at hypoglycemia and post-hypoglycemia (30 minutes, 1-hour, 2-hours, 4-hours and 24-hours) for controls (white circles) and for T2D (black squares). At baseline, blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 (for control, C). At point of hypoglycemia, blood sugar (BS) was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 (for control). Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for Microtubule-associated protein tau [MAPT] (A), Amyloid P component [APCS] (B), Noggin (C) and Pappalysin [PAPPA] (D). Statistics: [(* p<

0.05 control vs T2D); (# $p<0.01$, control baseline vs control hypoglycemia); ($ $p<0.05$ T2D baseline vs T2D hypoglycemia); (#$, $p<0.05$ control hypoglycemia vs control post-hypoglycemia timepoints); (^ $p<0.05$ T2D hypoglycemia vs T2D post-hypoglycemia timepoints). RFU, relative fluorescent units.

Figure 3. Comparison of Alzheimer-related proteins in plasma of before, during and after iatrogenic induction of hypoglycemia. Blood sampling was performed at baseline, at hypoglycemia and post-hypoglycemia (30 minutes, 1-hour, 2-hours, 4-hours and 24-hours) for controls (white circles) and for T2D (black squares). At baseline, blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 (for control, C). At point of hypoglycemia, blood sugar (BS) was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 (for control). Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for Serum amyloid A1 [SAA1]) (A), Apolipoprotein A1 (APOA1) (B), Apolipoprotein E3 (APOE3) (C) and Apolipoprotein E4 (APOE4) (D). Statistics: * $p<0.05$ control vs T2D). RFU, relative fluorescent units.

Figure 4. STRING interaction network. STRING version 11.0 interaction network showing the interactions of APCS, APP, SAA1 and APOA1 (A), MAPT and APP (B) and PAPPA (C).

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/). 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 colored lines between the proteins represent the various types of interaction evidence in STRING (databases, experiments, neighborhood, gene fusion, co-occurrence, text mining, co-expression, and homology).

Figure 5. Ingenuity pathway analysis. Ingenuity pathway analysis of four amyloid-related protein genes (Amyloid P component [APCS], Amyloid precursor protein [APP], Pappalysin [PAPPA] and Serum amyloid A1 [SAA1].