Amyloid-related protein changes associated with dementia differ according to severity of hypoglycemia

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ABSTRACT

Introduction Hypoglycemia in type 2 diabetes (T2D) may increase risk for Alzheimer's disease (AD), but no data on changes in AD-related proteins with differing degrees of hypoglycemia exist. We hypothesized that milder prolonged hypoglycemia would cause greater AD-related protein changes versus severe transient hypoglycemia.

Research design and methods Two prospective case-control induced hypoglycemia studies were compared: study 1, hypoglycemic clamp to 2.8 mmol/L (50 mg/dL) for 1 hour in 17 subjects (T2D (n=10), controls (n=7)); study 2, hypoglycemic clamp to 2.0 mmol/L (36 mg/dL) undertaken transiently and reversed in 46 subjects (T2D (n=23), controls (n=23)). Blood sampling at baseline, hypoglycemia and 24-hour post-hypoglycemia, with proteomic analysis of amyloid-related proteins performed.

Results In control subjects, the percentage change from baseline to hypoglycemia differed between study 1 and study 2 for 5 of 11 proteins in the AD-related panel: serum amyloid A1 (SAA1) (p=0.009), pappalysin (PAPPA) (p=0.002), apolipoprotein E2 (p=0.02), apolipoprotein E3 (p=0.03) and apolipoprotein E4 (p=0.02). In controls, the percentage change from baseline to 24 hours differed between studies for two proteins: SAA1 (p=0.003) and PAPPA (p=0.004); however, after Bonferroni correction only SAA1 and PAPPA remain significant. In T2D, there were no differential protein changes between the studies.

Conclusions The differential changes in AD-related proteins were seen only in control subjects in response to iatrogenic induction of hypoglycemic insults of differing length and severity and may reflect a protective response that was absent in subjects with T2D. Milder prolonged hypoglycemia caused greater AD-related protein changes than severe acute hypoglycemia in control subjects.

Trial registration numbers NCT02205996, NCT03102801.

Significance of this study

What is already known about this subject?
► We recently reported that severe, transient iatrogenic induction of hypoglycemia has a detrimental effect on Alzheimer's disease (AD)-associated proteins and that the increased risk subjects with type 2 diabetes (T2D) have for development of AD may be exacerbated by hypoglycemia.
► Prior to this report, there had been no previous studies looking at AD-related proteins in plasma in response to hypoglycemia.

What are the new findings?
► Milder but prolonged hypoglycemia leads to greater changes in AD-related proteins when compared with severe transient hypoglycemia.
► Interestingly, however, the differential changes were seen only in control subjects, suggesting that control subjects are able to mount a protective robust anti-inflammatory response to counteract the enhanced stress of prolonged hypoglycemia, but that subjects with T2D cannot.

How might these results change the focus of research or clinical practice?
► AD protein responses to hypoglycemia at differing glycemic control points may advise on the optimum diabetes control to address AD risks, with further work focused on glucose variability and AD protein modulation.
► Optimal diabetes control using therapeutic agents that do not cause hypoglycemia would appear to be the best treatment strategy to address AD.

INTRODUCTION

Along with the well-documented macrovascular and microvascular complications of diabetes, a marked increase in dementia in patients with type 2 diabetes (T2D) is also well recognized.[1–4] Alzheimer's disease (AD), the most common form of dementia, comprises 60%–80% of all dementia cases.[5] Epidemiological evidence indicates that patients with T2D are at increased risk for developing AD.[6–8] Elevation in key circulating AD-related proteins, such as plasma amyloid precursor protein (APP), have been reported in association with AD, with levels of APP showing a positive trend with increasing cognitive impairment.[9 10]

Optimal management of T2D involving tighter glucose control increases the risk for, and frequency of, hypoglycemic episodes. Hypoglycemia has been directly linked to

cognitive dysfunction, potentially increasing the risk of dementia.[11]

We previously undertook a clinical interventional study, specifically designed to mimic the physiological responses to hypoglycemia seen clinically in patients with diabetes[12] and reported that a severe hypoglycemic episode had detrimental effects on circulating AD-related proteins in patients with T2D,[13] thus providing a mechanistic link between T2D-associated hypoglycemia and AD. It is, however, not known whether a differing length and degree of hypoglycemic insult would cause similar perturbations in plasma AD-related protein levels.

We hypothesized that milder prolonged hypoglycemia would lead to greater changes in AD-related proteins when compared with a severe transient hypoglycemic episode. To this end, we compared amyloid-related proteins levels following a milder prolonged iatrogenic-induced hypoglycemic insult (study 1) with an acute severe iatrogenic-induced hypoglycemia (study 2) in subjects with and without T2D.

RESEARCH DESIGN AND METHODS
Study design
Study 1 design
A prospective case-control study in adult subjects with T2D (n=10) and control subjects (n=7); all but one of the subjects were Caucasian (one subject with T2D was South Asian), aged 40–53 years. As previously described,[14] a hypoglycemic clamp to 2.8 mmol/L (50 mg/dL) was undertaken for 1 hour.

Blood sampling was performed at baseline, at hypoglycemia after 1 hour and 24 hours post-hypoglycemia.

Study 2 design
A prospective case-control study was performed in 46 subjects, adult subjects with T2D (n=23) and control subjects (n=23); all subjects were Caucasian, aged 40–70 years. As previously described,[12] a hypoglycemic clamp to 2.0 mmol/L (36 mg/dL) was undertaken transiently and then immediately reversed. Blood sampling was performed at baseline, at the point of hypoglycemia and at 24 hours posthypoglycemia.

Study subjects
Both studies were undertaken at the Diabetes Centre at Hull Royal Infirmary. Inclusion criteria for the T2D cohort in both studies included a duration of diabetes <10 years and maintenance on a stable dose of medication (metformin, statin and/or ACE inhibitor/angiotensin receptor blocker) over the prior 3 months; no glycemic control medications other than metformin were allowed; hemoglobin A1c (HbA1c) levels <10% (86 mmol/mol)); no history of either hypoglycemic unawareness or hypoglycemia within a 3-month period. In the control group, diabetes was excluded with an oral glucose tolerance test and subjects were not on any medication nor had any known medical condition. All subjects had a body mass index (BMI) between 18 and 49 kg/m^2, and all had

normal renal and hepatic biochemical indices and no history of cancer nor any contraindication to insulin infusion to achieve hypoglycemia (ischemic heart disease, epilepsy, seizure history, drop attacks, history of adrenal insufficiency and treated hypothyroidism). All subjects had a medical history, clinical examination, routine blood tests and an ECG performed.

Biochemical markers
As previously described,[12–14] 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 and triglycerides were measured enzymatically using a Beckman AU 5800 analyser (Beckman-Coulter, High Wycombe, UK).

SOMA-scan assay
As previously described,[13] the SOMAscan assay used to quantify proteins was performed on an in-house Tecan Freedom EVO liquid handling system (Tecan Group, Maennedorf, Switzerland) using buffers and SOMAmers from the SOMAscan HTS Assay 1.3K plasma kit (SomaLogic, Boulder, Colorado, USA) according to manufacturer's instructions and as described previously.[15 16]

Data processing and analysis
Initial relative fluorescent units (RFUs) were obtained from microarray intensity images using the Agilent Feature Extraction Software (Agilent, Santa Clara, California, USA). 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 a high degree of hemolysis (Haptoglobin log RFU <10) were excluded from the analysis.

Statistical analyses were performed on $\log_2$ RFU values using R V.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[17] packages. For differential protein analysis, we applied limma models containing contrasts between timepoints, as well as contrasts between healthy patients 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.[18]

Statistical analysis
Based on our previous study to detect a significant rise in APP in the control population, a sample size of seven patients was calculated giving 80% power to detect a mean increase of 18 000 RFU of APP, with a alpha error of 0.05.[13] Data trends were visually evaluated for each

Table 1 Demographic and biochemical parameters of control subjects and subjects with T2D included in study 1 and study 2

	Study 1 controls (n=7)	Study 2 controls (n=23)	P value	Study 1 T2D (n=10)	Study 2 T2D (n=23)	P value
Age (years)	47±6	60±10	0.003	46±6	64±8	<0.0001
Sex (M/F)	4 M/3 F	11 M/12 F		7 M/3 F	12 M/11 F	
BMI (kg/m²)	29±4	28±3	0.640	36±7	32±4	0.03
Systolic BP (mm Hg)	126±15	122±8	0.280	127±20	132±8	0.31
Diastolic BP (mm Hg)	75±13	75±6	1.000	75±11	81±7	0.08
Duration of diabetes (years)	N/A	N/A		3.3±2.3	4.5±2.2	0.14
HbA1c (mmol/mol)	33.6±2.9	37.2±2.2	0.004	49±12	51±11	0.62
HbA1c (%)	5.2±0.3	5.6±0.2	0.006	6.6±1.0	6.8±1.0	0.48
Total cholesterol (mmol/L)	5.1±0.8	4.8±0.77	0.230	5.3±0.7	4.2±1.0	0.36
Triglyceride (mmol/L)	1.2±0.5	1.3±0.6	0.540	1.7±0.8	1.7±0.7	0.96
CRP (mg/L)	0.8±0.0	5.1±10.3	0.26	2.8±1.8	3.1±2.9	0.94

Data are presented as mean±1 SD.
BMI, body mass index; BP, blood pressure; CRP, C reactive protein; F, female; HbA1c, hemoglobin A1c; M, male; T2D, type 2 diabetes.

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. Within-group comparisons of changes between timepoints were compared using Student's t-test. To account for multiple comparisons, the significance level cut-off was estimated using the Bonferroni method and a value of 0.0045 was considered statistically significant. The sample size was too small to adjust for baseline covariates. To rule out underlying association of age, BMI and HbA1c with the AD proteins, one-to-one correlation was estimated for age, BMI and HbA1c with all the AD proteins for each study using Pearson's correlation. Statistical analysis was performed using GraphPad Prism (San Diego, California, USA) and STATA V.16 (StataCorp, College Station, Texas, USA).

RESULTS

Study 1 included 17 subjects (T2D (n=10) and control (n=7)). Study 2 included 46 subjects (T2D (n=23) and control (n=23)). Demographic and biochemical data for the control subjects and subjects with T2D included in study 1 and study 2 are shown in table 1. Subjects did not differ in their medication between studies.

Both control subjects (p=0.003) and subjects with T2D (p<0.0001) in study 2 were older than their study 1 counterparts. BMI did not differ between studies for controls. For subjects with T2D, BMI was greater in study 1 (p<0.03). HbA1c was higher in study 2 versus study 1 control subjects (p=0.006), in keeping with the reported association of increasing HbA1c with age,[19] although was still well within the normal non-diabetic range in both cohorts. Association between age and BMI and each of the AD-related proteins for each study were estimated and no correlations were found, suggesting that neither age

nor BMI were confounding factors between the studies. HbA1c did not correlate with any of the AD-related proteins for either study (online supplemental tables 1 and 2).

Total cholesterol, triglycerides, blood pressure and CRP, as a marker for inflammation, did not differ between control groups or between T2D groups (table 1).

Eleven AD-related proteins were included in the analysis: amyloid precursor protein (APP), amyloid P component (APCS), serum amyloid A1 (SAA1), pappalysin (PAPPA), microtubule-associated protein tau (MAPT), apolipoprotein A1 (APOA1), noggin, apolipoprotein E (APOE), apolipoprotein E2 (APOE2), apolipoprotein E3 (APOE3) and apolipoprotein E4 (APOE4).

Differences between T2D and controls at baseline in each study

At baseline, levels of all 11 AD-related proteins were comparable between T2D and controls in study 1 (p=ns).

At baseline, only APP differed between T2D and controls, being elevated in T2D in study 2 (p=0.02) as previously reported.[13]

Because study 1 and study 2 were performed at different times and analysed separately, and not as part of a single study, it is not possible to directly compare protein levels between studies. Therefore, relative change, rather than absolute change, in proteins was undertaken to account for assay differences. For the 10 AD-related proteins where there was no difference between T2D and controls at baseline in their respective studies, baseline protein levels were normalized to 1 to compare the per cent change from baseline to subsequent timepoints in both studies.
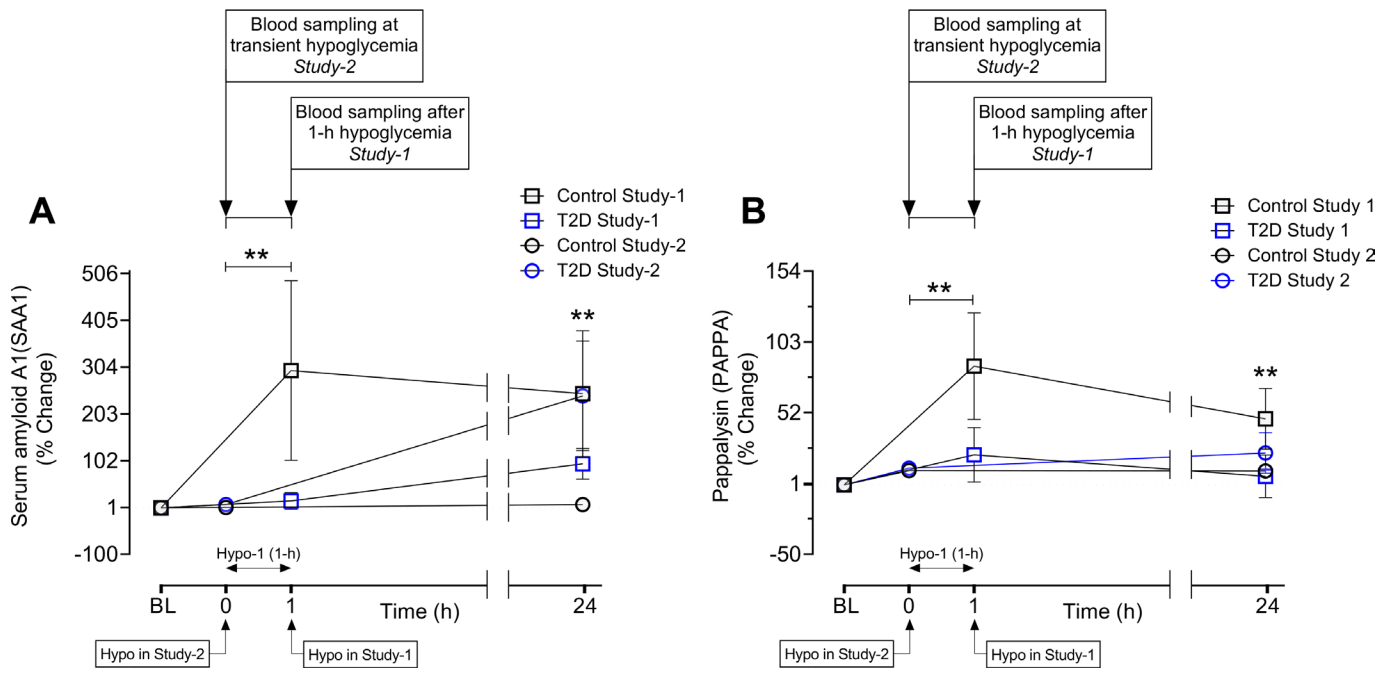
Figure 1 Per cent (%) changes of Alzheimer's disease (AD)-related proteins in response to hypoglycemia in two different prospective studies in control subjects and subjects with type 2 diabetes (T2D). Line graphs showing changes as percentage of two AD-related proteins, serum amyloid A1 (SAA1) (A) and pappalysin (PAPPA) (B) from baseline to hypoglycemia and to 24 hours posthypoglycemia in study 1 (open white square, control and open blue square, T2D) and study 2 (open white circle, control and open blue circle, T2D). Baseline protein levels were normalized to 1 to show the % change from baseline to subsequent timepoints. SAA1 and PAPPA showed a significant differential percentage change from baseline to hypoglycemia and from baseline to 24 hours in controls. There were no differential percentage changes in T2D for SAA1 or PAPPA in any of the studies. Two-way arrows in the graphs indicate the duration of hypoglycemia for study 1. Data were present here as mean % change of proteins±SEM. **p<0.01. BL, baseline; Hypo, hypoglycemia.

Differences between studies in per cent change from baseline
Baseline to hypoglycemia

In control subjects, the percentage change from baseline to hypoglycemia differed between study 1 and study 2 for 6 of the 11 proteins included in the panel: SAA1 (p=0.017),

PAPPA (p=0.004) (figure 1A,B); APOE2 (p=0.015), APOE3 (p=0.03) and APOE4 (p=0.01) (figure 2A–C and table 2) and APOE (p=0.04) (figure 3B). To account for multiple comparisons, significance cut-off was adjusted using Bonferroni correction, which yielded a p value
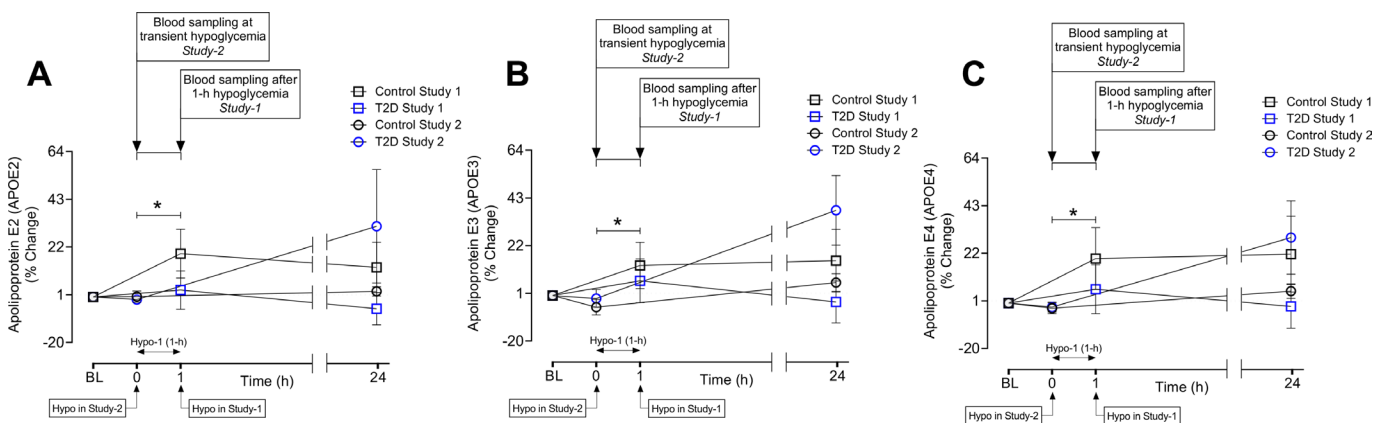

Figure 2 Per cent (%) changes of apolipoprotein Es (APOEs) in response to hypoglycemia in two different prospective studies in control subjects and subjects with type 2 diabetes (T2D). Line graphs showing changes as percentage of three Alzheimer's disease (AD)-related apolipoproteins, APOE2 (A), APOE3 (B) and APOE4 (C) from baseline to hypoglycemia and to 24 hours posthypoglycemia in study 1 (open white square, control and open blue square, T2D) and study 2 (open white circle, control and open blue circle, T2D). Baseline protein levels were normalized to 1 to show the % change from baseline to subsequent timepoints. APOE2, APOE3 and APOE4 showed a significant differential percentage change from baseline to hypoglycemia in control but not in T2D. There were no differential percentage changes of the apolipoproteins 24 hours posthypoglycemia compared with baseline both in control and in T2D. Two-way arrows in the graphs indicate the duration of hypoglycemia for study 1. Data were present here as mean % change of proteins±SEM. *P<0.05. BL, baseline; Hypo, hypoglycemia.

Table 2 A comparison of the change in plasma protein levels of Alzheimer's disease (AD)-related proteins following a milder prolonged iatrogenic-induced hypoglycemic insult (study 1) with an acute severe iatrogenic-induced hypoglycemia (study 2) in subjects with and without T2D

Proteins (% change)	Control (baseline to hypoglycemia)		Control (baseline to 24 hours)		T2D (baseline to hypoglycemia)		T2D (baseline to 24 hours)	
	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2
APP	−26.1±21.2 p=0.07	124.0±42.0	11.0±31.0 p=0.53	−9.4±15.2	41.4±36.2 p=0.36	83.7±25.7	15.2±20.8 p=0.9	23.9±43.2
APCS	1.9±4.5 p=0.06	−6.5±1.9	−3.7±5.6 p=0.84	−4.5±1.6	1.8±9.3 p=0.9	0.9±2.6	−3.2±7.5 p=0.5	19.3±21.5
SAA1	289.0±229.2 p=0.017	−0.8±3.8	246.5±136.0 **p=0.003**	7.2±5.9	15.2±18.0 p=0.56	7.2±4.2	95.3±32.8 p=0.42	241.8±118.5
PAPPA	83.3±45.4 p=0.004	10.3±3.3	47.6±21.8 **p=0.004**	9.9±2.2	21.6±19.5 p=0.48	12.0±2.4	6.2±15.4 p=0.49	23.0±14.6
MAPT	10.4±19.4 p=0.63	17.7±6.1	8.0±23.1 p=0.72	3.4±2.5	33.5±16.5 p=0.22	17.4±4.7	13.3±8.8 p=0.97	14.2±14.0
APOA1	12.5±8.6 p=0.28	2.3±4.2	−0.4±5.0 p=0.87	0.6±3.1	6.0±7.5 p=0.97	6.4±5.2	−10.2±6.7 p=0.45	15.3±22.2
NOG	36.0±45.2 p=0.93	39.0±13.6	14.5±26.4 p=0.25	−4.9±4.9	61.8±31.8 p=0.13	24.0±8.5	24.7±26.5 p=0.37	2.2±11.7
APOE	26.0±14.4 p=0.04	−4.8±6.7	26.0±13.1 p=0.18	5.1±7.4	11.2±17.1 p=0.68	4.4±8.0	1.2±8.4 p=0.25	40.7±22.2
APOE2	20.6±12.6 p=0.01	0.2±2.5	13.0±11.0 p=0.14	2.5±2.0	3.1±8.5 p=0.5	−1.1±1.7	−5.1±7.1 p=0.34	31.1±25.0
APOE3	14.6±12.0 p=0.03	−5.1±3.4	15.4±13.8 p=0.35	5.7±3.9	6.5±9.7 p=0.38	−1.3±4.0	−2.8±9.2 p=0.1	37.6±15.3
APOE4	22.2±16.0 p=0.01	−2.3±2.3	21.7±16.7 p=0.13	5.3±3.2	6.1±10.7 p=0.32	−1.7±2.2	−1.4±9.6 p=0.24	29.0±16.2

Per cent change from baseline to hypoglycemia and from baseline to 24 hours are shown for study 1 and study 2, as well as Students' t-tests of the comparison of percentage change between studies.
P values in bold/italics indicate p<0.01.
APCS, amyloid P component; APOA1, apolipoprotein A1; APOE2, apolipoprotein E2; APOE3, apolipoprotein E3; APOE, apolipoprotein E; APOE4, apolipoprotein E4; APP, amyloid precursor protein; BL, baseline; MAPT, microtubule-associated protein tau; NOG, noggin; PAPPA, pappalysin; SAA1, serum amyloid A1; T2D, type 2 diabetes.

cut-off of 0.0045. On application of this cut-off, only PAPPA remained significant.

For SAA1, the percentage change increase in controls in milder prolonged hypoglycemia (study 1) was marked, whereas there was minimal percentage decrease in response to severe acute hypoglycemia (study 2) (SAA1: 289.0±229.2 vs −0.8±3.8 % change of SAA1 from baseline, controls study 1 vs study 2, p=0.017) (figure 1A).

Likewise, while PAPPA and APOE2 increased in response to hypoglycemia in both studies, the percentage change increase in controls was marked in response to milder prolonged hypoglycemia (study 1) and much less so in response to acute severe hypoglycemia (study 2) (PAPPA 83.3±45.4 vs 10.3±3.3 % change of PAPPA from baseline in control, controls study 1 vs study 2, p=0.004 (figure 1B); APOE2: 20.6±12.6 vs 0.2±2.5 % change of

APOE2 from baseline, controls study 1 vs study 2, p=0.01) (figure 2A).

For APOE, APOE3 and APOE4, there was a percentage change increase in response to milder prolonged hypoglycemia, but a percentage change decrease in response to acute severe hypoglycemia (APOE: 26.0±14.4 vs −4.8±6.7 % change of APOE from baseline, controls study 1 vs study 2, p=0.04; APOE3: 14.6±12.0 vs −5.1±3.4 % change of APOE3 from baseline, controls study 1 vs study 2, p=0.03; APOE4: 22.2±16.0 vs −2.3±2.3 % change of APOE4 from baseline, controls study 1 vs study 2, p=0.01) (figures 2B,C and 3B).

For study 1, MAPT and NOG correlated (r=0.8), APOE correlated with APOE3 and APOE4 (r=0.8 and 0.8, respectively), APOE3 correlated with APOE2 and APOE4 (r=0.97 and 0.8, respectively). For study 2, MAPT and
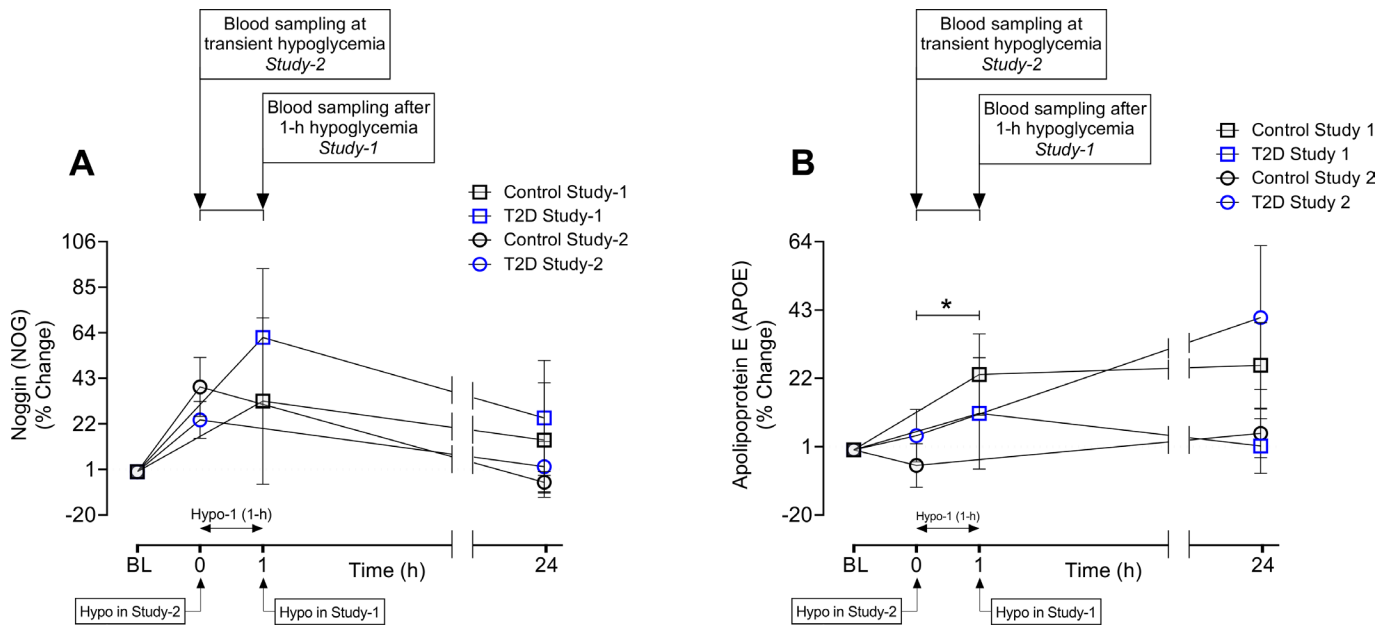
Figure 3 Line graphs showing changes as percentage of Alzheimer's disease (AD)-related protein, noggin (NOG) (A) and APOE (B) in control and type 2 diabetes (T2D) in two different prospective studies. Two-way arrows in the graphs indicate the duration of hypoglycemia for study 1. Data were present here as mean % change of proteins±SEM. BL, baseline; Hypo, hypoglycemia. *p<0.05.

APP (r=0.5) and NOG and APP correlated (r=0.6): APOE correlated with APOE2, APOE3 and APOE4 (r=0.7, 0.8 and 0.9, respectively), APOE3 correlated with APOE2 and APOE4 (r=0.8 and 0.9, respectively). Correlations were considered significant at p=0.0035 to adjust for multiple comparisons.

For subjects with T2D, there were no significant percentage change differences from baseline to hypoglycemia for any of the above proteins between the two studies.

Interestingly, APP showed opposite effects in control subjects in response to hypoglycemia. While transient hypoglycemia (study 2) caused 124% increase (124.0%±42.0%) of APP, less-severe prolonged hypoglycemia (study 1) resulted in ~26% decrease (−26.1%±21.2%) of APP in controls (figure 4A); this differential trend between the two studies was close to significance (p=0.07). These data suggest a rapid clearance of APP levels within 1 hour of hypoglycemia in controls. In T2D, the % change of APP was increased in both studies (~41% in study 1 and ~84% in study 2) in response to hypoglycemia relative to baseline; however, unlike in controls, less severe prolonged hypoglycemia did not result in a decreased percentage change of APP below baseline in T2D, suggesting a defect in APP clearance from blood in T2D (41.4±36.2 vs −26.1±21.2 % change of APP in prolonged hypoglycemia in study 1, T2D vs control, p=ns) (figure 4A). Other AD-related proteins, for example, APOA1 (figure 4B), APCS (figure 4C), MAPT (figure 4D) and Noggin (figure 3) did not differ in % change from baseline to hypoglycemia for study 1 vs study 2 in either control or T2D cohorts.

Baseline to 24 hours

In control subjects, the percentage change from baseline to 24 hours differed between study 1 and study 2 for only two proteins: SAA1 (p=0.003) and PAPPA (p=0.004) (figure 1 and table 2).

While SAA1 and PAPPA increased in both studies at 24 hours posthypoglycemia, the percentage change increase was marked in response to milder prolonged hypoglycemia (study 1) and much less so in response to acute severe hypoglycemia (study 2) (SAA1: 246.5±136.0 vs 7.2%±5.9% baseline to 24 hours, controls study 1 vs study 2, p=0.003; PAPPA: 47.6±21.8 vs 9.9%±2.2% baseline to 24 hours, controls study 1 vs study 2, p=0.004) (figure 1A,B). Both SAA1 and PAPPA were significant after Bonferroni correction.

Again, for subjects with T2D, there were no percentage change differences from baseline to 24 hours for any of the proteins between the two studies (figures 1–4).

DISCUSSION

This is the first study comparing the response of plasma AD-related proteins with differing lengths and severities of hypoglycemia. Here, we show that there are differential changes in AD-related proteins according to the nature of the hypoglycemic insult. The percentage change from baseline to hypoglycemia differed between study 1 (milder prolonged hypoglycemia for a 1-hour period) and study 2 (acute severe hypoglycemia) for 6 of the 11 proteins (SAA1, PAPPA, APOE, APOE2, APOE3 and APOE4) but only in control subjects. The percentage change from baseline to 24 hours differed between study
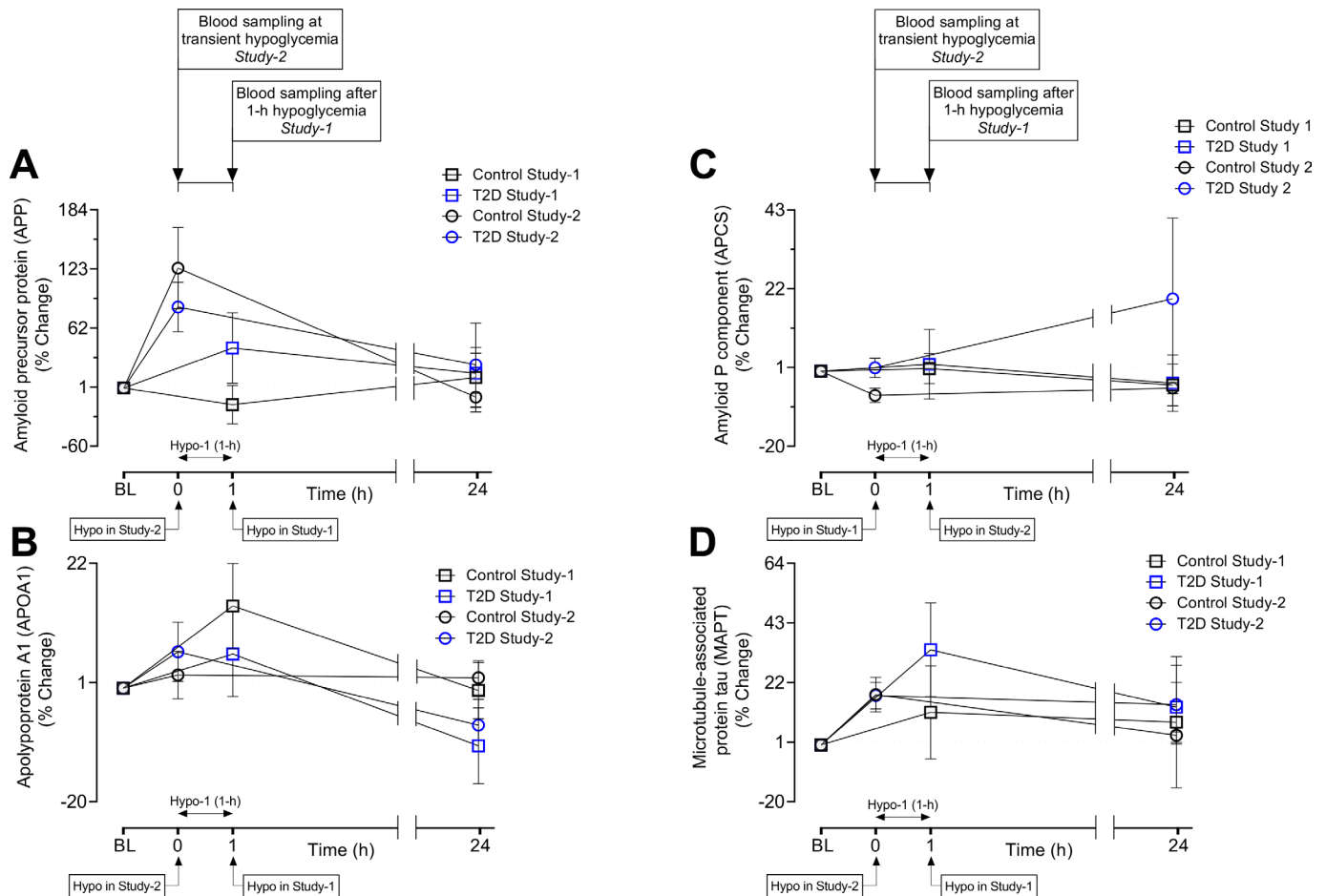
Figure 4 Alzheimer's disease (AD)-related proteins that did not differ as percentage changes in response to hypoglycemia in two different prospective studies in control subjects and subjects with type 2 diabetes (T2D). Line graphs showing changes as percentage of four AD-related proteins, amyloid precursor protein (APP) (A), apolipoprotein A1 (APOA1) (B), amyloid P component (APCS) (C), microtubule-associated protein tau (MAPT) (D) from baseline to hypoglycemia and to 24 hours posthypoglycemia in study 1 (open white square, control and open blue square, T2D) and study 2 (open white circle, control and open blue circle, T2D). Baseline protein levels were normalized to 1 to show the % change from baseline to subsequent timepoints. There were no differential percentage changes for APP, MAPT, APCS or APOA1 either in control or in T2D from baseline to hypoglycemia or from baseline to 24 hours. Two-way arrows in the graphs indicate the duration of hypoglycemia for study 1. Data were present here as mean % change of proteins±SEM. BL, baseline; Hypo, hypoglycemia.

1 and study 2 for only two proteins (SAA1 and PAPPA) and, again, only in control subjects.

It must be emphasized that the absolute changes in protein levels we reported previously in severe acute hypoglycemia (study 2)[13] are not contradictory to the differential relative changes we report here. Specifically, in absolute values at baseline in study 2, APP was elevated in T2D; at hypoglycemia, APCS was elevated in T2D while APP and noggin were elevated in controls; and at 24-hours, noggin had normalized in both cohorts, APP and MAPT normalized in controls but showed a below-baseline decrease in T2D. Therefore, the changes that occurred in response to hypoglycemia in T2D were similar in both study 1 and study 2 and were therefore not altered by the length or severity of the hypoglycemic insult. This is in marked contrast to the differential changes seen in controls between study 1 and study 2. Differences in APP trends between the severity of the hypoglycemic insult (study 1 vs study 2) and between

controls and T2D, may be due to differential clearance of the serum protein. APP is a membrane protein widely expressed, especially in the synapses of neurons, which is key in the development of AD pathogenesis.[20] APP consists of a single membrane-spanning domain that is cleaved by either alpha or beta secretase that may then be internalized[20]; however, the kinetics of its clearance have not been determined nor whether these differ in T2D.

SAA1 is an acute-phase protein important in maintaining homeostasis in healthy tissues; however, increased expression in the brain has been reported in AD.[21] While most reports in the literature have considered SAA1 to be a proinflammatory mediator, more recent publications have questioned this paradigm, conversely purporting it to be an anti-inflammatory mediator.[22]

PAPPAs cleave IGF-binding proteins, and PAPPA-2 overexpression promotes Aβ peptide accumulation in AD.[23] Increased circulatory PAPPA-1 has been reported in subjects with T2D,[24] perhaps reflective of the common

underlying protein misfolding pathophysiology.[25] IGF proteins, once cleaved, negatively modulate the acute phase response,[26 27] and thus PAPPA also serves as an anti-inflammatory mediator.

Studies are inconclusive as regards circulating APOE levels in AD,[28] with some reporting an increase,[29] some a decrease[30] and some no change.[31] APOE is a well-recognized modulator of inflammation, suppressing the production of proinflammatory cytokines and converting proinflammatory M1 macrophages to an anti-inflammatory M2 phenotype.[32]

Taken together, these data suggest that control subjects are potentially able to mount a robust anti-inflammatory response to counter the presumably heightened inflammation that accompanies a prolonged, as opposed to an acute, hypoglycemic insult, but that the ability for subjects with T2D to mount such a response is blunted. Therefore, subjects with T2D have less capacity to regain homeostasis after such an insult, making them more susceptible to the negative sequelae of homeostatic perturbations. While T2D is recognized as an inflammatory pathology, in large measure there were few baseline differences compared with controls, but this may reflect the relatively short duration of disease in this cohort of patients with T2D.

The strengths of this study include inclusion of subjects with T2D having a short disease duration and being relatively treatment naïve and not on polypharmacy, characteristics that did not differ between studies and that the same study design with the hyperinsulinemic clamp protocol to hypoglycemia development was employed. As these were two separate studies, it was not possible to have the subjects undergo the two different hypoglycemic levels in random order in a randomized cross-over fashion. The age difference between studies may have contributed to the differences seen, with a blunting of the responses seen in study 2. The relatively small subject numbers in each study cohort, for both subjects with T2D and control subjects, is the major limitation of this study as larger numbers may have revealed greater changes in plasma levels of amyloid-related proteins. However, in both studies, the subjects were subjected to significant, though differing, iatrogenic-induced hypoglycemic episodes, study 1 inducing prolonged though less severe hypoglycemia while study 2 induced severe and acute hypoglycemia; in both cases, it is highly likely that protein-level changes would have become evident, especially as the posthypoglycemia follow-up in both studies was to 24 hours. The panels used in the two studies were very similar, although not identical, and therefore some proteins present in the second panel were not available in the first. In addition, the protein panel analysis for each study was performed separately and not as part of a single study; therefore, relative change in protein level was undertaken here rather than absolute change to account for assay differences. A further limitation is that only circulatory protein levels were measured that may not be reflective of tissue levels. As subjects enrolled in

these studies were, with a single exception, Caucasian, these results may not be generalizable to other ethnic populations. It should be noted that when correction for multiple testing using Bonferroni was used that only PAPPA and SAA1 remained significant, likely due to the small sample size leading to increased SD and the inherent conservatism of Bonferroni testing that may lead to false negative results.

In conclusion, these data support our hypothesis that milder prolonged hypoglycemia would lead to greater changes in AD-related proteins when compared with severe transient hypoglycemia. Interestingly, however, the differential changes were seen only in control subjects, suggesting that control subjects are able to mount a robust anti-inflammatory response to counteract the enhanced stress of prolonged hypoglycemia, but that subjects with T2D cannot.

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Supplementary table 1: Correlation between Age, BMI, HbA1c and AD proteins for Study 1

Correlations[a]

		Age	BMI	HbA1c	APP	APCS	SAA1	PAPPA	MAPT	APOA1	NOG	APOE	APOE3	APOE4	APOE2
Age	Pearson Correlation	1	-.331	-.312	.304	.331	-.062	-.067	-.161	.630**	-.364	.190	.202	.314	.496*
	Sig. (2-tailed)		.211	.257	.235	.194	.812	.798	.537	.007	.151	.465	.437	.220	.043
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
BMI	Pearson Correlation	-.331	1	.351	-.189	-.283	-.096	.422	-.061	-.440	-.160	.186	.203	.112	-.185
	Sig. (2-tailed)	.211		.219	.484	.288	.723	.104	.823	.088	.554	.492	.451	.680	.493
	N	16	16	14	16	16	16	16	16	16	16	16	16	16	16
HbA1c	Pearson Correlation	-.312	.351	1	-.425	.036	-.286	.135	.244	-.053	.594*	.117	-.072	-.090	-.175
	Sig. (2-tailed)	.257	.219		.114	.899	.301	.632	.381	.852	.019	.679	.798	.749	.532
	N	15	14	15	15	15	15	15	15	15	15	15	15	15	15
APP	Pearson Correlation	.304	-.189	-.425	1	-.229	.510*	-.381	-.104	-.064	-.241	-.423	-.467	-.455	-.371
	Sig. (2-tailed)	.235	.484	.114		.376	.037	.132	.691	.808	.351	.091	.059	.067	.143
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
APCS	Pearson Correlation	.331	-.283	.036	-.229	1	.072	.233	.378	.319	.187	.542*	.424	.456	.458
	Sig. (2-tailed)	.194	.288	.899	.376		.784	.369	.135	.212	.472	.025	.090	.066	.065
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
SAA1	Pearson Correlation	-.062	-.096	-.286	.510*	.072	1	-.125	.555*	-.129	.329	-.235	-.172	-.272	-.331
	Sig. (2-tailed)	.812	.723	.301	.037	.784		.634	.021	.621	.197	.365	.509	.291	.195
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
PAPPA	Pearson Correlation	-.067	.422	.135	-.381	.233	-.125	1	-.032	-.317	-.151	.181	.467	.351	.307
	Sig. (2-tailed)	.798	.104	.632	.132	.369	.634		.903	.215	.562	.488	.059	.167	.231
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
MAPT	Pearson Correlation	-.161	-.061	.244	-.104	.378	.555*	-.032	1	-.110	.801**	.009	.115	.032	-.080
	Sig. (2-tailed)	.537	.823	.381	.691	.135	.021	.903		.673	.000	.973	.661	.903	.761
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
APOA1	Pearson Correlation	.630**	-.440	-.053	-.064	.319	-.129	-.317	-.110	1	-.112	.299	.169	.292	.410
	Sig. (2-tailed)	.007	.088	.852	.808	.212	.621	.215	.673		.667	.243	.516	.256	.102
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
NOG	Pearson Correlation	-.364	-.160	.594*	-.241	.187	.329	-.151	.801**	-.112	1	-.035	-.015	-.075	-.137
	Sig. (2-tailed)	.151	.554	.019	.351	.472	.197	.562	.000	.667		.895	.954	.775	.600
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
APOE	Pearson Correlation	.190	.186	.117	-.423	.542*	-.235	.181	.009	.299	-.035	1	.818**	.812**	.642**
	Sig. (2-tailed)	.465	.492	.679	.091	.025	.365	.488	.973	.243	.895		.000	.000	.005
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
APOE3	Pearson Correlation	.202	.203	-.072	-.467	.424	-.172	.467	.115	.169	-.015	.818**	1	.968**	.841**
	Sig. (2-tailed)	.437	.451	.798	.059	.090	.509	.059	.661	.516	.954	.000		.000	.000
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
APOE4	Pearson Correlation	.314	.112	-.090	-.455	.456	-.272	.351	.032	.292	-.075	.812**	.968**	1	.901**
	Sig. (2-tailed)	.220	.680	.749	.067	.066	.291	.167	.903	.256	.775	.000	.000		.000
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17
APOE2	Pearson Correlation	.496*	-.185	-.175	-.371	.458	-.331	.307	-.080	.410	-.137	.642**	.841**	.901**	1
	Sig. (2-tailed)	.043	.493	.532	.143	.065	.195	.231	.761	.102	.600	.005	.000	.000	
	N	17	16	15	17	17	17	17	17	17	17	17	17	17	17

Supplementary table 2: Correlation between Age, BMI, HbA1c and AD proteins for Study 2

Correlations[a]

		Age	BMI	HbA1c	APP	APCS	SAA1	PAPPA	MAPT	APOA1	NOG	APOE	APOE3	APOE4	APOE2
Age	Pearson Correlation	1	.237	.244	.380**	.061	-.054	.075	-.065	-.046	.222	-.293*	-.309*	-.275	-.311*
	Sig. (2-tailed)		.112	.102	.009	.689	.720	.622	.668	.761	.139	.048	.037	.064	.035
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
BMI	Pearson Correlation	.237	1	.451**	.250	.167	-.068	-.138	-.059	-.144	.282	-.160	-.056	.005	-.089
	Sig. (2-tailed)	.112		.002	.094	.268	.654	.360	.696	.340	.057	.288	.713	.971	.557
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
HbA1c	Pearson Correlation	.244	.451**	1	.283	-.052	-.084	.088	.019	-.280	.151	-.078	-.167	-.144	-.185
	Sig. (2-tailed)	.102	.002		.057	.732	.579	.559	.899	.059	.315	.607	.268	.339	.218
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APP	Pearson Correlation	.380**	.250	.283	1	.085	-.050	.102	.541**	-.140	.579**	-.246	-.387**	-.201	-.257
	Sig. (2-tailed)	.009	.094	.057		.573	.740	.500	.000	.353	.000	.100	.008	.181	.085
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APCS	Pearson Correlation	.061	.167	-.052	.085	1	.247	-.082	.091	.244	.012	.025	-.033	.127	.014
	Sig. (2-tailed)	.689	.268	.732	.573		.098	.589	.548	.102	.935	.871	.825	.402	.924
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
SAA1	Pearson Correlation	-.054	-.068	-.084	-.050	.247	1	.298*	-.058	.130	.015	.101	.009	.073	.158
	Sig. (2-tailed)	.720	.654	.579	.740	.098		.044	.701	.388	.920	.503	.955	.628	.295
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
PAPPA	Pearson Correlation	.075	-.138	.088	.102	-.082	.298*	1	.043	-.229	.037	-.061	-.133	-.054	.017
	Sig. (2-tailed)	.622	.360	.559	.500	.589	.044		.777	.125	.810	.685	.378	.722	.913
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
MAPT	Pearson Correlation	-.065	-.059	.019	.541**	.091	-.058	.043	1	.138	.394**	.196	.003	.061	.020
	Sig. (2-tailed)	.668	.696	.899	.000	.548	.701	.777		.361	.007	.192	.987	.687	.893
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APOA1	Pearson Correlation	-.046	-.144	-.280	-.140	.244	.130	-.229	.138	1	.031	.423**	.442**	.402**	.374*
	Sig. (2-tailed)	.761	.340	.059	.353	.102	.388	.125	.361		.838	.003	.002	.006	.010
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
NOG	Pearson Correlation	.222	.282	.151	.579**	.012	.015	.037	.394**	.031	1	-.132	-.242	-.193	-.224
	Sig. (2-tailed)	.139	.057	.315	.000	.935	.920	.810	.007	.838		.383	.105	.198	.135
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APOE	Pearson Correlation	-.293*	-.160	-.078	-.246	.025	.101	-.061	.196	.423**	-.132	1	.860**	.773**	.680**
	Sig. (2-tailed)	.048	.288	.607	.100	.871	.503	.685	.192	.003	.383		.000	.000	.000
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APOE3	Pearson Correlation	-.309*	-.056	-.167	-.387**	-.033	.009	-.133	.003	.442**	-.242	.860**	1	.929**	.839**
	Sig. (2-tailed)	.037	.713	.268	.008	.825	.955	.378	.987	.002	.105	.000		.000	.000
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APOE4	Pearson Correlation	-.275	.005	-.144	-.201	.127	.073	-.054	.061	.402**	-.193	.773**	.929**	1	.893**
	Sig. (2-tailed)	.064	.971	.339	.181	.402	.628	.722	.687	.006	.198	.000	.000		.000
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
APOE2	Pearson Correlation	-.311*	-.089	-.185	-.257	.014	.158	.017	.020	.374*	-.224	.680**	.839**	.893**	1
	Sig. (2-tailed)	.035	.557	.218	.085	.924	.295	.913	.893	.010	.135	.000	.000	.000	
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46