Type 2 diabetes mellitus, skin autofluorescence and brain atrophy

Running title: **Diabetes Mellitus, SAF and brain atrophy**

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Abstract

Type 2 Diabetes Mellitus (T2DM) is associated with brain atrophy, but the mechanisms underlying this link are unknown. Advanced glycation end products (AGEs) accumulate in T2DM resulting in inflammation, oxidative stress, and protein cross-linking, which are known contributors to neurodegeneration. We aimed to study whether tissue AGE accumulation is associated with T2DM-related brain atrophy. We performed brain magnetic resonance imaging (MRI), cognitive tests and non-invasive skin autofluorescence (SAF, a measure of tissue AGE levels) on people aged >55 years with and without T2DM. Multivariable linear regression was used to study the relationships between T2DM, SAF and gray matter volume. There were 486 people included in the study. T2DM was associated with greater SAF. Greater SAF, T2DM and cognitive impairment were each associated with lower gray matter volume independently of age, sex and total intracranial volume. SAF partially mediated the association between T2DM and gray matter volume. Longitudinal studies may help confirm whether tissue AGE accumulation is associated with brain atrophy in T2DM.

Type 2 Diabetes Mellitus (T2DM) is associated with an increased risk of incident cognitive impairment and dementia[1]. Brain atrophy may be a key driver of T2DM-related cognitive dysfunction[2]. T2DM is also associated with the excessive accumulation of advanced glycation end products (AGEs) in tissues[3]. AGEs are products of non-enzymatic reactions between reactive carbonyl groups of compounds (such as glucose)with proteins, lipids or nucleic acids[4]. There is a large body of evidence from *in vitro* model research supporting a role for AGEs in neurodegeneration and Alzheimer's disease (AD)[4]. Greater serum levels of AGEs are associated with cognitive decline[5] and lower gray matter volumes in older people[4]. Given these observations, it is possible that they play a mechanistic role in T2DM-related brain atrophy. However, there have been no previous studies examining the role of AGEs in T2DM-related brain atrophy.

Tissue AGEs can be measured reproducibly and non-invasively in the skin by means of a specialised light emitter and detector. Skin autofluorescence (SAF) measured in this manner has been shown to be highly correlated with biopsy-derived skin AGE concentrations[6]. We hypothesized that SAF levels would either mediate or modify the association between T2DM and brain atrophy.

Research Design and Methods

Sampling

We used a cross-sectional study design, and sampling methods have been described previously[2]. Participants were included from 2 studies: the Cognition and Diabetes in Older Tasmanians study(CDOT) and the Tasmanian Study of Cognition and Gait(TASCOG). Participants with T2DM aged ≥55 years were recruited into CDOT between January 2008 and January 2010 using the National Diabetes Service Scheme(NDSS) database as a sampling frame. The TASCOG sample was recruited by posting approach letters to eligible

registrants aged ≥55 years, living in the same Southern Tasmanian postcodes as those in the CDOT study and has been described previously[7]. The phenotype of T2DM was based on self-report and confirmed using a single plasma glucose level according to standard criteria (fasting plasma glucose ≥7.0mmol/L, random plasma glucose ≥11.1mmol/L, and HbA_{1c}>6.5%(48mmol/mol)). People living in a nursing home, those with insufficient English for cognitive testing or contraindication to MRI were excluded. The Southern Tasmanian Health and Medical Human Research Ethics Committee and the Monash University Human Research Ethics Committee approved the study and we obtained written, informed consent.

Measurements

Skin autofluorescence– We used the AGE reader (DiagnOpticsBV,Groningen,the Netherlands)to measure SAF. The spectrometer reader uses a light source to illuminate ~4cm^2 of skin on the volar surface of the right arm 10cm below the elbow fold. SAF is calculated as the ratio between the emission light and reflected excitation light, multiplied by 100 and expressed in arbitrary units (AU). In our laboratory, the test-retest reliability for SAF was high (ICC 0.93, n=11)when individuals were measured 5 days apart.

MRI Scans– MRI scans were obtained using a single 1.5T General Electric scanner with the following sequences: high-resolution T1 weighted spoiled gradient echo (SPGR) (TR35 ms, TE 7ms, flip angle 35°, field of view 24cm, 120 contiguous slices, isotropic voxel size 1mm^3); T2 weighted fast spin echo (TR 4300ms; TE 120ms; NEX 1; turbo factor 48; voxel size 0.90×0.90×3mm); FLAIR (fluid attenuated inversion recovery) (TR = 8802ms, TE = 130ms, TI = 2200ms, voxel size 0.50×0.50×3mm); gradient echo (GRE, TR=0.8ms; TE=0.015; flip angle 30°; voxel size = 0.9x0.9x7mm).

Brain Volumes– 3D-T1 and axial GRE sequences were registered into standard Montreal Neurological Institute (MNI) space using FMRIB's Linear Image Registration Tool (FLIRT).

A multispectral segmentation process was applied using 3D-T1 and gradient echo (GRE) sequences using Statistical Parametric Mapping software version 5 (SPM5)[8] to produce tissue probability maps of gray and white matter. Tissue maps were smoothed using an isotropic 8mm Gaussian kernel. A single expert manually segmented both hippocampi using established methods known to have high test-retest reliability in our laboratory (ICC 0.97)[9]. Tissue volumes of the segmented areas (total gray, white matter, hippocampal) were calculated using standard voxel counting algorithms.

Other Measurements - Standardized questionnaires were used to record demographic and clinical information. Weight, height, waist, hip circumferences, habitual physical activity using a pedometer worn over one week and blood pressure (BP) in a sitting position as an average of 3 recordings from the right arm were measured, and Body Mass Index (BMI) calculated. A standardized cognitive battery was used to test domains of memory, speed, executive and visuospatial function (Supplementary Table 1)as described previously[2]. Diagnosis of cognitive impairment was assigned, blinded to T2DM status, if function in any of the domains was less than 1.5 standard deviations from age, sex and education adjusted norms.

Data analysis

The analyses were conducted on a complete dataset consisting of those in whom both measures of SAF and brain imaging were available.

Logistic regression was used to describe the associations of T2DM and brain atrophy with cognitive impairment. Linear regression was used to estimate the associations of SAF and T2DM with measures of brain atrophy. Covariates for age, sex, total intracranial volume (TICV) and other variables were added to the regression models for brain atrophy if their inclusion produced a statistically significant increase in model fit or changed the coefficient

of the covariate for T2DM by >10%. Putative factors considered were hypertension (defined as mean BP>140/90mmHg or previous diagnosis), ever smoked tobacco, creatinine, means steps per day, history of ischemic heart disease, stroke, hyperlipidemia, BMI and waist-hip ratio, and the use of specific medications that have been shown to influence AGE levels (pravastatin, irbesartan, metformin)[10-12]. We then examined whether SAF mediated the associations estimated between T2DM and brain atrophy. For this, we entered SAF into the model relating T2DM to brain volume outcome measures adjusting for age, sex, smoking, serum creatinine and total intracranial volume. If the introduction of SAF substantially attenuated the regression coefficient of the binary covariate for T2DM and the coefficient of SAF remained largely unchanged from its value without T2DM in the model, it was considered a potential mediator. We also investigated any modifying effect (interaction) of SAF using a test of significance of the coefficient of a covariate formed as the product of the covariates for T2DM and SAF. Statistical analyses were carried out using STATA version 11.1(StatCorp.College Station Tx.).

Results

There were 285 people with T2DM (mean age 67.5 years, SD 6.9) and 201 in the non-T2DM comparison group (mean age 73.4 years, SD 6.9) with SAF measures. A total of 7 participants had inaccurate measures of SAF and were excluded from the analysis. Summary measures of the characteristics of each group are presented in Table 1. Comparisons of the characteristics of people with and those without T2DM are presented in Supplementary Table 2.

Associations of SAF with study factors

Greater SAF was associated with greater age (β=0.014, p<0.001), but not with sex (β=0.07, p=0.26). After adjustment for age and sex, T2DM was associated with greater SAF (β=0.14,

p=0.007). In the whole sample (T2DM and non-T2DM), greater BMI (p<0.001), less habitual physical activity (p=0.009) and greater serum creatinine (p=0.006) were individually associated with greater SAF. Among those with T2DM, greater SAF was associated with greater HbA_{1c}(p<0.001) and longer duration of T2DM (p=0.016). Among those without T2DM, there was no association between SAF and $HbA_{1c.}$

T2DM was associated with lower gray matter volume (GMV) (standardized β= –0.020,p=0.05,), but not with total hippocampal volume (standardized β=0.03, p=0.51) or white matter volume (standardized β=0.002, p=0.90). Lower gray matter volume was significantly associated with the risk of any cognitive impairment (ß =-0.03, CI -0.04 to -0.01, p=0.005). In the whole sample, greater levels of SAF were significantly associated with the risk of any cognitive impairment (ß =0.41, CI 0.01 to 0.82, p=0.05), and with lower GMV (standardized β=–0.036, p<0.001,), but not with hippocampal volume (standardized β=–0.046, p=0.258) or white matter volume (standardized β=0.019, p=0.096) independent of age, sex, smoking serum creatinine and TICV. Addition of SAF attenuated the association between T2DM and GMV by 20% rendering it non-significant (standardized β= –0.016, p=0.12), whereas SAF remained independently associated with GMV in the model(standardized β= –0.034, p<0.001). Additional adjustments for BMI, HbA_{1c} or duration of T2DM did not change these relationships (data not shown). There was no interaction between T2DM and SAF in explaining GMV. Figure 1 shows the scatter plots of the association between SAF and GMV stratified by T2DM status.

Discussion

This is the first study examining the relationship between tissue AGE accumulation, T2DM and GMV. T2DM was associated with greater accumulation of tissue AGEs (as measured by SAF) and with lower GMV. Consistent with our previous study of circulating AGEs[4], we

found that greater SAF was independently and modestly associated with lower GMV, but additionally demonstrate that SAF may partially mediate the association between T2DM and lower GMV. The associations we describe are novel, and provide a solid basis for further studying the relationship between tissue AGE accumulation and brain atrophy.

There is strong evidence from basic science research that tissue AGE accumulation plays a role in the pathogenesis of dementia [13]. In the case of Alzheimer's disease (the most common type of dementia), autopsy studies have shown the process of atrophy is due to the accumulation of extracellular amyloid plaque and intracellular tau neurofibrillary tangles[14]. Greater levels of AGEs have been found to be co-located with amyloid plaques[15, 16]and paired helical filament tau in sporadic AD[17] and may act by stabilising plaques and promoting fibrillation of tau through protein cross-linking[18, 19]. We speculate that SAF may reflect AGE-mediated cross-linking of other cellular proteins, such as in neurons.

AGEs may also be directly cytotoxic to neurons in culture [20] and able to directly induce inflammation and oxidation [13] by binding with RAGE in mitochondria, generating free radicals and reducing clearance of pre-existing reactive oxygen species[18]. Furthermore, RAGE interacts with serum β-amyloid, increasing the transport of β-amyloid across the blood brain barrier, activating pro-inflammatory cytokines and reducing cerebral blood flow[21].

Strengths of our study include the large sample size, reproducible and sensitive measures of tissue AGEs and brain structure, clear definition of T2DM and careful statistical modelling. We carefully adjusted as required for smoking, renal function, BMI, hypertension, and hyperlipidemia that may be related to both AGEs and brain or vascular health. Although medications used to treat these conditions (Pravastatin, Irbesartan)[10, 11] and specific anti-diabetes drugs such as Metformin[12] have been postulated to have a protective effect against the effects of AGEs, adjusting for the use of these medications did not change our findings

(data not shown). Our study has some limitations. Our study is cross-sectional, limiting inferences of causality and needs to be confirmed in longitudinal analyses. The AGE reader does not measure AGEs that do not exhibit autofluorescence (non-fluorophores), and may also measure non-AGE fluorophores[6]. However, the results of a number of earlier studies support the use of SAF as a surrogate marker of fluorescent and non-fluorescent AGE content in the skin [6, 22].

Given the modest strength of beta coefficients for SAF with GMV, it is likely that AGE accumulation is only one of a large number of pathways that contribute to the development of dementia in T2DM, explaining why SAF only partially mediated the T2DM-gray matter volume relationship. The clinical relevance of these results is uncertain, but they support further research to understand the role of AGEs in the pathogenesis of dementia in relation to T2DM and overall. Prospective studies are needed to assess if tissue AGE accumulation is causally related to brain atrophy in T2DM, and subsequently, to study whether limiting AGE accumulation may slow neurodegeneration.

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C.M. drafted and revised the manuscript, performed statistical analysis and data interpretation. G.M. contributed to discussion and reviewed manuscript. J.F. contributed to discussion and reviewed manuscript. R.B. performed image analysis and interpreted data. L.B. contributed to analysis and interpretation of the data and reviewed the manuscript. A.V. contributed to interpretation of data and reviewed the manuscript. T.P. supervised image analysis and contributed to discussion and reviewed manuscript. J.C. performed image analysis and interpreted data. V.S. developed study concept and design, performed analysis and interpretation of data, revised the manuscript, and obtained funding.

V.S. 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.

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Table 1. Sample Characteristics

	Mean (sd) or N(%) Total n=486
Age (years)	69.9 (7.5)
Female sex	208 (43)
Diabetes	285 (59)
Formal education (years)	11.3 (3.7)
Self reported history of hypertension or mean SBP >140 or mean DBP >90 mmHg	374 (77)
Use of blood pressure lowering medications	284 (58)
Statin use	223 (46)
Ischemic Heart Disease	81 (17)
TIA or Stroke	32 (7)
Hyperlipidemia	153 (31)
Ever smoked	253 (52)
BMI (kg/m^2) Normal (BMI 20-25) Overweight (BMI 25-30) Obese (BMI>30)	29.1 (5.0) 86 (18) 216 (44) 177 (36)
Mean steps per day	6337 (3372)
Serum creatinine (μmol/L)	78.5 (24.7)

Any cognitive impairment	146 (30%)
Skin autofluorescence (AU)	2.05 (0.53)

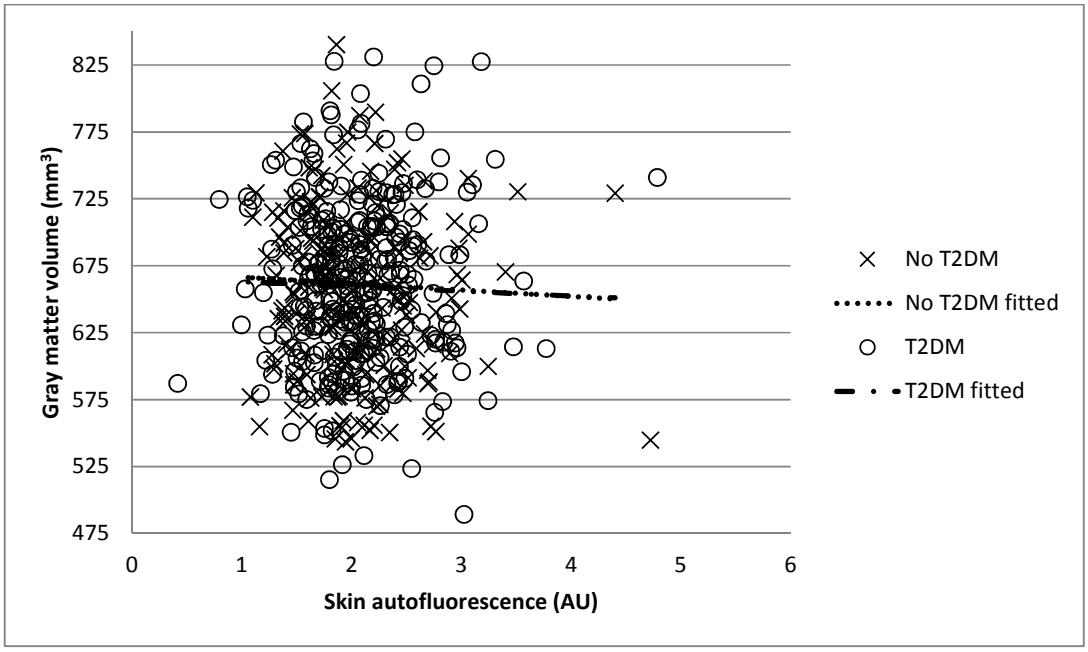

Figure 1.The association of skin autofluorescence with gray matter volume. The raw data points of the association of skin autofluorescence (SAF) and gray matter volume (GMV) stratified by T2DM. The fitted lines show the stratified associations between SAF and GMV adjusted for age, sex, smoking, serum creatinine and total intracranial volume. In those without T2DM: SAF ß= - 4.78, standardized ß= -0.41, p= 0.003, adjusted R^2=0.97; In those with T2DM: SAF ß= - 3.60, standardized ß= -0.03, p= 0.02, adjusted R^2=0.96 .The high R^2 reflect the adjustment for total intracranial volume (head size), which is collinear with gray matter volume. The fitted lines for the two groups also overlap considerably demonstrating a lack of interaction between T2DM and SAF in explaining GMV.

Online Appendix

Supplementary Table 1. Cognitive domains and tests

Cognitive Domain	Tests
Memory factor	Hopkins Immediate Verbal Recall
	Hopkins Delayed Verbal Recall
	Hopkins Recognition
Visuospatial and planning factor	Rey Complex Figure Copy Task
	Rey Complex Figure Recall Task
Speed factor	Digit Symbol Coding
	Digit Symbol Search
Executive function	COWAT Word test
	COWAT Category Test
	Digit Span Recall
	Stroop Dot Time
	Stroop Word Time
	Stroop Color time

Supplementary Table 2. Sample characteristics by diabetes status

	T2DM n=285	No T2DM n=201	P value
Age (years)	67.5 (6.9)	73.4 (6.9)	<0.001
Female sex	41% (117/285)	45% (91/201)	0.35
Formal education (years)	11.5 (3.4)	11.2 (3.9)	0.40
Self reported history of hypertension or mean SBP >140 or mean DBP >90 mmHg	88% (251/285)	72% (144/201)	<0.001
Use of blood pressure lowering medications	70% (199/285)	43% (86/201)	<0.001
Statin use	62% (176/285)	23% (47/201)	<0.001
Ischemic Heart Disease	19% (54/285)	13% (27/201)	0.11
TIA or Stroke	8% (24/285)	4% (8/201)	0.053
Hyperlipidemia	48% (138/285)	7% (15/201)	<0.001
Ever smoked	53% (152/285)	51% (102/201)	0.60
BMI (kg/m^2) Normal (BMI 20-25) Overweight (BMI 25-30) Obese (BMI>30)	30.5 (5.1) 11% (31/285) 41% (117/285) 47% (135/285)	27.2 (4.2) 27% (55/201) 50% (100/201) 21% (42/201)	<0.001 <0.001 0.048 <0.001
Mean steps per day	6377 (3660)	6414 (3014)	0.91
Fasting blood glucose (mmol/l)	7.7 (2.3)	5.3 (0.5)	<0.001
HbA_{1c} (%) (mmol/mol)	7.2 (1.2) 55	5.6 (0.3) 38	<0.001
Serum creatinine (μmol/L)	78.7 (25)	78.4 (24)	0.91
Age at diabetes diagnosis (years)	57.5 (11.3)		
Median duration of T2DM (years)	7 (4-12)		
Use of oral glucose lowering medications	62% (176/285)		
Use of insulin	4% (10/285)		
Any cognitive impairment	107 (38)	39 (19)	<0.001
Skin autofluorescence (AU)	2.03 (0.54)	2.07 (0.51)	0.49