Research: Complications
Incretin hormones, insulin, glucagon and advanced glycation end products in relation to cognitive function in older people with and without diabetes, a population-based study

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Accepted 3 February 2020

Abstract

Aim The aim of this observational study was to investigate relationships between physiological levels of glucometabolic biomarkers and cognitive test results in a population-based setting.

Methods Cross-sectional data were obtained from the Swedish population-based Malmö Diet and Cancer Study Re-examination 2007–2012 comprising 3001 older people (mean age 72 years). Through oral glucose tolerance testing (OGTT), fasting and post-load levels of serum insulin, plasma glucagon, serum glucose-dependent insulinotropic peptide (GIP) and plasma glucagon-like peptide-1 (GLP-1) were measured. Insulin resistance and insulin sensitivity levels were calculated. In 454 participants, advanced glycation end products (AGEs) were estimated through skin autofluorescence. Associations between biomarkers and two cognitive tests, the Mini-Mental State Examination (MMSE) and A Quick Test of Cognitive Speed (AQT) respectively, were explored in multiple regression analyses.

Results Positive associations following adjustments for known prognostic factors were found between MMSE scores and insulin sensitivity ($B = 0.822, P = 0.004$), 2-h plasma glucagon ($B = 0.596, P = 0.026$), 2-h serum GIP ($B = 0.581, P = 0.040$) and 2-h plasma GLP-1 ($B = 0.585, P = 0.038$), whereas negative associations were found between MMSE scores and insulin resistance ($B = -0.734, P = 0.006$), fasting plasma GLP-1 ($B = -0.544, P = 0.033$) and AGEs ($B = -1.459, P = 0.030$) were found.

Conclusions Higher levels of insulin sensitivity, GIP and GLP-1 were associated with better cognitive outcomes, but AGEs were associated with worse outcomes, supporting evidence from preclinical studies. Glucagon was linked to better outcomes, which could possibly reflect neuroprotective properties similar to the related biomarker GLP-1 which has similar intracellular properties. Longitudinal and interventional studies are needed to further evaluate neuromodulating effects of these biomarkers.

Abstract presented at the European Association for the Study of Diabetes (EASD) 2019, Barcelona, Spain

Introduction

Diabetes and impaired glucose metabolism enhance the risk of mild cognitive decrements during the life course and dementia in old age. Contributing factors may be genetic predisposition, vascular risk factors, neurodegeneration, vascular disease and lifestyle factors [1]. Interest has recently been directed towards different biomarkers associated with diabetes that may modulate cognitive function. Preclinical studies have shown that insulin can stimulate neuronal growth and inhibit apoptosis in nerve cells [2], and that brain insulin resistance may disturb the neuroprotective effects of insulin signalling [3]. Such disturbances have been noted in
What’s new?

• Preclinical studies suggest that insulin and incretin hormones exert neuroprotective effects in the brain and that advanced glycation end products (AGEs) are linked to Alzheimer’s disease. Glucagon has not been studied in relation to cognition, but has similar intracellular properties to the incretin hormone glucagon-like peptide-1.

• This cross-sectional population-based study supports previous evidence that insulin and incretin hormones may be neuroprotective and that AGEs may have negative effects on cognition. Furthermore, glucagon was positively associated with cognitive test results, which is a novel finding.

• These results contribute with new information to the important research field of diabetes-related cognitive impairment.

the brain tissue of patients with Alzheimer’s disease [4], possibly due to regulation of amyloid beta (Aß) plaque and neurofibrillary tangle formation [5]. Peripheral insulin levels and insulin resistance have been negatively correlated with cognitive test results, but a recent genetic study could not prove causality [6].

The relationship between glucagon and cognitive function has not been studied. The central nervous system contains only small amounts of the peptide [7] and it is unclear whether it crosses the blood–brain barrier. Glucagon receptors have been found in the brain [8] but their functional significance remains unknown. GCG, the gene that encodes glucagon as well as GLP-1 [9], is expressed in the brainstem but no prohormone-convertase enabling synthesis of glucagon has so far been found in the central nervous system [7].

Incretin hormones such as glucose-dependent insulino-tropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) have been shown in experimental studies to exert pleiotropic effects in regions of the brain associated with learning and memory [10]. Both hormones cross the blood–brain barrier [11], raising the possibility of a gut–brain axis. Animal studies have provided increasing hope that GLP-1 analogue treatment may prevent cognitive decline [12]. There are now also some studies on humans that support this hypothesis, in which subjection to the treatment has been associated with less risk of dementia [12] and Aß load in Alzheimer’s disease [13]. However, the large randomized CAROLINA Cognition sub-study recently reported that the dipeptidyl peptidase-4 inhibitor linagliptin could not prevent cognitive decline in people with type 2 diabetes better than glimepiride [14].

The effect of GIP levels on cognition is less studied, but one study recently showed that administration of synthetic long-lasting GIP analogue molecules in mice could improve cognitive function [15].

Advanced glycation end products (AGEs) are non-enzymatically glycated lipids or proteins that accumulate in different organs due to long-standing hyperglycaemia and inflammation. Elevated levels have been associated with Alzheimer’s disease, possibly by increasing glycation of Aß and phosphorylation of the tau protein [16]. There are few studies on AGEs in relation to cognition, but one cross-sectional population-based study showed that skin autofluorescence, a non-invasive measure of AGEs, was negatively associated with cognitive function [17]. One prospective cohort study also showed that higher AGE levels at baseline were associated with worse cognitive test results at follow-up [18].

Here, we investigate cross-sectional relationships between physiological levels of biomarkers related to glucose metabolism and cognitive function in a population-based cohort of older participants with a view to determining the interplay between these biomarkers and cognitive function.

Participants and methods

Participants

The baseline examination of the prospective population-based Malmö Diet and Cancer Study (MDCS) from southern Sweden was carried out in 1991–1994. It comprised 28 449 participants (participation rate 40%). The follow-up examination focusing on cardiovascular factors (the MDCS-CV) was held during 2007–2012, in which 3734 people (76% of the surviving participants) were examined. Recruitment details, reasons of loss to follow-up and information on potential health selection bias have been described [19,20]. This study is based on cross-sectional data from the follow-up examination. Participants with essential missing data (n = 419), i.e. lacking both cognitive tests (n = 405) or all biomarkers (n = 46) were excluded (n = 14 lacking all these data). As language fluency can affect cognitive test results, 402 people that were born outside Sweden, Norway or Denmark (n = 289) or lacked data for nationality (n = 113) were also excluded. Some met both exclusion criteria for missing data and nationality (n = 88). The final study population comprised 3001 participants (733 excluded).

Clinical examination

The examination took place at the Clinical Research Unit at Skåne University Hospital in Malmö, Sweden. Blood samples were drawn for lipid measurements and an oral glucose tolerance test (OGTT). Blood pressure (BP) and waist circumference were measured.

A questionnaire including questions on sociodemographic, health aspects and drug treatment was conducted. Educational level was categorized as ‘up to 10 years’, ‘11–12 years’ and ‘more than 12 years of schooling’; smoking
habs as ‘never-smoker’, ‘former smoker’ or ‘current smoker’; physical activity as ‘sedentary spare time’, ‘moderate exercise’ and ‘regular exercise’; and alcohol consumption as ‘no consumption’, ‘consumption below risk level’, and ‘consumption above risk level’, defined as > 9 standard alcohol units per week for women or > 14 for men. Diabetes was defined as having fasting glucose ≥ 7 mmol/l, 2-h glucose ≥ 11.1 mmol/l, a self-reported diagnosis or treatment for diabetes. Type of diabetes was not specified.

**Glucometabolic biomarkers**

Laboratory analyses were held at the Department of Clinical Chemistry, Malmö University Hospital, Malmö, Sweden. For participants with diabetes diagnosed before the re-examination, only blood samples at fasting were collected. The OGTT was administered for all other participants, during which blood samples were collected after overnight fasting and 2 h after intake of 75 g glucose.

Plasma glucose, serum insulin, plasma glucagon, serum GIP and plasma GLP-1 were analysed from blood samples at 0 and 120 min. The Hemocue Glucose System (HemoCue AB, Angelholm, Sweden) was used to analyse plasma glucose and the Dako ELISA kit (Glostrup, Denmark) to analyse serum insulin (minimum detection level 3 pmol/l, intra- and interassay coefficients of variation 3.6% and 7.5% respectively). Plasma glucagon was assayed with RIA GL-32K (Merck Millipore, Darmstadt, Germany, minimum detection level 18.5 pg/ml, intra- and interassay coefficients of variation 3.6–6.2% and 8.7–14.7% respectively) and serum GIP with the Millipore Human GIP Total ELISA #EZHGIP-54K (Merck Millipore, minimum detection level 3 pmol/l, intra- and interassay coefficients of variation 3.8–8.8%, and 1.8–6.1% respectively). Plasma GLP-1 concentrations (intact GLP-1 and the metabolite GLP-1 9-36 amide) were determined radio-immunologically (minimum detection level 1 pmol/l, intra- and interassay coefficients of variation 6.0% and 1.5%, respectively), using an N-terminally specific guinea pig anti-GLP-1 antiserum (Linco Research, St Charles, MO, USA) for intact GLP-1 and a C-terminally directed antiserum (code no. 89390) for total GLP-1. Severely haemolytic blood samples were excluded (serum insulin: n = 190 at fasting level and n = 205 at 120 min; serum GIP: n = 194 at fasting level and n = 188 at 120 min).

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) [21] and the Insulin Sensitivity Index (IS [0–120 min]), which has been proven to more accurately reflect euglycemic hyperinsulinaemic clamp measurements [22], were calculated.

In 454 participants, levels of AGEs were estimated using skin autofluorescence [23]. Skin reflectance, i.e. per cent of reflected fluorescent light, was also measured to be adjusted for as it affects skin autofluorescence.

**Cognitive tests**

Participants were interviewed using two cognitive tests administered by research nurses: The Mini-Mental State Examination (MMSE) and A Quick Test of Cognitive Speed (AQT). For logistic reasons there was a time delay between physical examinations and subsequent cognitive testing sessions (mean 264 days). The MMSE is a widely used global cognitive screening test that has been validated in Swedish populations [24]. The AQT test has been validated as a reliable screening test to detect early dementia [25]. It involves describing the colour and shape of geometrical figures on time, and covers the domains processing speed, attention and executive function.

**Statistical analyses**

Analyses were performed using IBM SPSS version 22.0 for Mac OS X. A P-value of < 0.05 was considered significant. Because of the exploratory nature of the study, no alpha-level adjustment was made for multiple testing. Missing data in covariates were imputed with five consecutive imputations. In analyses including all participants, 5.8% in Model 1 and 6.2% in Model 2 (described below) had any imputed data. The variables with most imputed data were alcohol consumption (n = 121) and smoking habits (n = 74). In all other variables, fewer than 13 values were imputed.

To minimize ceiling effects of the MMSE we used a normalizing transformation method that has been validated, creating a scale of 0–100 instead of 0–30 as used in the test [26]. Continuous variables were recalculated into natural logarithmic values to achieve normal distribution when needed.

Independent-samples t-tests for continuous variables and χ² tests for categorical variables were carried out to compare the group with diabetes and the group without diabetes.

Linear associations between biomarkers (z-scores of natural logarithmic values) as exposure variables and cognitive tests as outcome variables were explored in multiple regression analyses. These were then stratified for diabetes status and, in a supplementary analysis, for four levels of glucometabolic status: normal glucose tolerance (NGT) at baseline and follow-up; prediabetes at follow-up (defined as impaired fasting glucose, i.e. 6.1 mmol/l ≤ fasting glucose < 7.0 mmol/l or impaired glucose tolerance, i.e. 7.8 mmol/l ≤ 2-h glucose < 11.1 mmol/l); diabetes acquired after the baseline examination; diabetes diagnosed at baseline or before.

Two adjustment models were used. Model 1 was adjusted for age, sex, education, smoking consumption, alcohol consumption and physical activity (as these lifestyle factors may contribute to both impaired glucose metabolism and cognitive impairment). Model 2 was adjusted for the factors in Model 1 and systolic BP, waist circumference, total cholesterol and drug treatment for hypertension, hyperlipidaemia and diabetes, as evidence suggests that cardiovascular factors largely mediate the relationship between diabetes and cognitive function [1]. These variables were chosen over other measurements of obesity, lipids and BP due to their
closer correlation with fasting glucose in this data set (data not shown).

Unadjusted mean cognitive test results for quartiles of each biomarker were visualized in box-plots. Interactions between diabetes and the biomarkers in relation to cognition were also tested in multiple regression analyses. Partial correlations between biomarkers in the cohort (adjusted for age and sex) were also explored. Finally, mediation analyses were performed using Process version 3.4 for SPSS to investigate whether biomarkers that were significantly correlated to cognitive outcomes could possibly mediate associations between diabetes and cognitive function.

**Table 1** Characteristics of the Malmö Diet and Cancer Study Cardiovascular Re-examination Cohort and comparisons between participants with and without diabetes

| Biomarkers                      | N (valid in original data) | Total study sample (n = 3001)* | Participants without diabetes* (n = 2453) | Participants with diabetes* (n = 548) | P for difference between groups† |
|---------------------------------|----------------------------|--------------------------------|-------------------------------------------|--------------------------------------|---------------------------------|
| P-Glucose 0 min (mmol/l)        | 2997                       | 6.10 (52.2)                    | 5.72 (0.586)                              | 7.78 (2.02)                          | < 0.001                         |
| P-Glucose 120 min (mmol/l)      | 2677                       | 7.11 (2.67)                    | 6.64 (1.73)                              | 11.7 (4.98)                          | < 0.001                         |
| S-Insulin 0 min (pmol/l)        | 2843                       | 8.97 (5.29)                    | 8.39 (4.71)                              | 11.5 (6.77)                          | < 0.001                         |
| S-Insulin 120 min (pmol/l)      | 2525                       | 53.3 (52.2)                    | 50.9 (50.2)                              | 77.4 (64.3)                          | < 0.001                         |
| HOMA-IR                         | 2841                       | 2.51 (1.84)                    | 2.17 (1.31)                              | 4.05 (2.80)                          | < 0.001                         |
| ISI(0–120 min)                  | 2396                       | 4.28 (1.58)                    | 4.46 (1.54)                              | 2.52 (0.70)                          | < 0.001                         |
| P-Glucagon 0 min (pg/ml)        | 2991                       | 79.8 (26.4)                    | 77.9 (26.5)                              | 88.7 (24.3)                          | < 0.001                         |
| P-Glucagon 120 min (pg/ml)      | 2668                       | 72.3 (20.2)                    | 72.0 (20.0)                              | 74.6 (22.4)                          | 0.06                           |
| S-GIP 0 min (pmol/l)            | 2840                       | 47.1 (27.9)                    | 44.8 (25.8)                              | 57.3 (33.6)                          | < 0.001                         |
| S-GIP 120 min (pmol/l)          | 2525                       | 238.7 (109.8)                  | 237.8 (105.9)                            | 247.8 (142.3)                        | 0.19                           |
| P-GLP-1 0 min (pmol/l)          | 2956                       | 8.13 (3.65)                    | 7.92 (3.41)                              | 9.05 (4.44)                          | < 0.001                         |
| P-GLP-1 120 min (pmol/l)        | 2633                       | 17.7 (9.83)                    | 17.9 (9.70)                              | 16.1 (10.9)                          | 0.006                          |
| Skin autofluorescence (AU)      | 454                        | 2.41 (0.498)                   | 2.38 (0.50)                              | 2.53 (0.48)                          | 0.02                           |
| Skin reflectance (% reflected fluorescent light) | 454 | 0.16 (0.052) | 0.16 (0.053) | 0.16 (0.049) | 0.99 |
| Cognitive tests                 |                            |                                |                                           |                                      |                                |
| MMSE score (0 of 30p)           | 3001                       | 28.2 (1.79)                    | 28.2 (1.74)                              | 27.9 (1.99)                          | 0.001                          |
| AQT score (time, s)             | 2985                       | 135.8 (32.3)                   | 134.5 (30.5)                             | 141.8 (38.6)                         | < 0.001                         |
| COVARIATES                      |                            |                                |                                           |                                      |                                |
| Age (years)                     | 3001                       | 72 (5.6)                       | 72 (5.7)                                 | 73 (5.3)                             | 0.008                          |
| Sex (men : women, %)            | 3001                       | 40 : 60                        | 39 : 61                                  | 48 : 52                              | < 0.001                         |
| Educational level (%)           | 2999                       |                                |                                           |                                      | 0.58                           |
| Low (≤ 10 years)                | 69                         | 68                             | 70                                       |                                      |                                |
| Medium (11–12 years)            | 9.5                        | 9.5                            | 9.5                                      |                                      |                                |
| High (>12 years)                | 22                         | 22                             | 20                                       |                                      |                                |
| Physical activity (%)           | 2988                       |                                |                                           |                                      | < 0.001                         |
| Sedentary spare time            | 7                          | 6                              | 12                                       |                                      |                                |
| Moderate exercise               | 76                         | 76                             | 76                                       |                                      |                                |
| Regular exercise                | 17                         | 18                             | 12                                       |                                      |                                |
| Smoking status (%)              | 2927                       |                                |                                           |                                      | 0.24                           |
| Never-smokers                   | 46                         | 46                             | 43                                       |                                      |                                |
| Past smokers                    | 45                         | 44                             | 48                                       |                                      |                                |
| Present smokers                 | 9                          | 9.2                            | 8.2                                      |                                      |                                |
| Alcohol consumption (%)         | 2880                       |                                |                                           |                                      | 0.003                          |
| None                            | 23                         | 21                             | 28                                       |                                      |                                |
| Moderate (< 9 (women) or <11 (men) units/week) | 65 | 67 | 60 |
| High                            | 12                         | 12                             | 13                                       |                                      |                                |
| Anti-hypertensive treatment (%) | 3001                       | 57                             | 52                                       | 81                                    | < 0.001                         |
| Lipid-lowering treatment (%)    | 3001                       | 31                             | 26                                       | 54                                    | < 0.001                         |
| Diabetes drug treatment (%)     | 3001                       | 8.0                            | 0                                        | 43.8                                  | < 0.001                         |
| Systolic BP (mmHg)              | 2997                       | 143 (18.8)                     | 142 (18.8)                               | 147 (18.3)                           | < 0.001                         |
| Waist circumference (cm)        | 2995                       | 92 (13)                        | 91 (12)                                  | 98 (13)                               | < 0.001                         |
| BMI (kg/m²)                     | 2996                       | 26.7 (4.39)                    | 26.3 (4.11)                              | 28.7 (5.04)                           | < 0.001                         |
| Total cholesterol (mmol/l)      | 2995                       | 5.21 (1.07)                    | 5.33 (1.04)                              | 4.64 (1.05)                           | < 0.001                         |

AQT, A Quick Test of Cognitive Speed; AU, arbitrary units; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; ISI, Insulin Sensitivity Index; MMSE, Mini-Mental State Examination.

*Means and so of continuous variables and proportions of groups of categorical variables are presented, analysed through independent-samples t-tests for continuous variables and χ² tests for categorical variables. †Significant P-values are shown in bold.
Ethics

The study was approved by the Ethical Committee of Lund University, Sweden (MDC baseline examination LU-51-90, MDC Re-examination Dnr.532/2006). Written informed consent was obtained from all participants.

Results

Characteristics of the study sample are presented in Table 1. The mean age was 72.4 years (range 61–85 years). Participants with diabetes (n = 548) were on average 0.7 years older than the control group (n = 2453). The proportion of men and the prevalence of cardiovascular risk factors and unhealthy lifestyle habits were also greater in the group with diabetes. Participants with diabetes scored on average 0.3 normalized MMSE score points less and were 7.3 s slower at the AQT test than the control group. Biomarkers and related indices had higher mean values among participants with diabetes, apart from 2-h plasma GLP-1 and ISI, for which the control group had higher mean values.

Changes in mean cognitive test results per 1 SD increment of each biomarker are shown in Table 2 (entire cohort), Table 3 (participants without diabetes) and Table 4 (participants with diabetes). In Fig. 1 unadjusted mean cognitive test results for quartiles of each biomarker are visualized.

No associations were found between insulin and cognition, apart from a weak correlation between 2-h insulin and worse AQT results in people without diabetes. HOMA-IR levels were associated with worse MMSE results after adjustment for Model 1 in the entire cohort (B = −0.73, P = 0.006) and in the subgroup without diabetes. ISI$_{(0, 120 \text{ min})}$ was associated with better MMSE scores (B = 0.82, P = 0.010) and shorter AQT time, i.e. better results (B = −0.010, P = 0.025) after full adjustment (Model 2) in the entire cohort and in the subgroup without diabetes.

Fasting glucagon levels were not correlated to cognitive outcomes, but 2-h plasma glucagon was positively associated with MMSE results after full adjustment (B = 0.64, P = 0.017) in the entire cohort and in people without diabetes (B = 0.68, P = 0.017).

Fasting plasma GLP-1 was negatively associated with MMSE results (B = −0.54, P = 0.033) but 2-h serum GIP (B = 0.60, P = 0.034) and 2-h plasma GLP-1 (B = 0.61, P = 0.033) were positively associated with MMSE results in the study population after full adjustment (Model 2). Results were similar for the subgroup without diabetes. The subgroup with diabetes was much smaller – 18% of the total population.

### Table 2 Multiple regression analyses of changes in mean cognitive test results per 1 SD increment of each biomarker or index (n = 3001)

|                   | Model 1 | Model 2 |
|-------------------|---------|---------|
|                   | N       | Means per 1 SD increment | P-value* | N       | Means per 1 SD increment | P-value* |
| **A. MMSE (points/100, normalized)** |         |         |        |         |         |        |
| S-Insulin 0 min (pmol/l) | 2843    | −0.475  | 0.07  | −0.251 | 0.42    |
| S-Insulin 120 min (pmol/l) | 2525    | −0.461  | 0.10  | −0.437 | 0.16    |
| HOMA-IR | 2841    | −0.734  | 0.006 | −0.427 | 0.19    |
| ISI$_{(0, 120 \text{ min})}$ | 2396    | 0.822   | 0.004 | 0.820 | 0.010   |
| P-Glucagon 0 min (pg/ml) | 2991    | 0.289   | 0.28  | 0.534 | 0.05    |
| P-Glucagon 120 min (pg/ml) | 2668    | 0.596   | 0.026 | 0.640 | 0.017   |
| S-GIP 0 min (pmol/l) | 2840    | 0.050   | 0.85  | 0.299 | 0.27    |
| S-GIP 120 min (pmol/l) | 2525    | 0.581   | 0.040 | 0.603 | 0.034   |
| P-GLP-1 0 min (pmol/l) | 2956    | −0.544  | 0.033 | −0.469 | 0.07    |
| P-GLP-1 120 min (pmol/l) | 2525    | 0.585   | 0.038 | 0.606 | 0.033   |
| Skin autofluorescence (AU)$^9$ | 454     | −1.459  | 0.030 | −1.235 | 0.07    |
| **B. AQT (s)** |         |         |        |         |         |        |
| S-Insulin 0 min (pmol/l) | 2828    | 0.003   | 0.47  | −0.001 | 0.80    |
| S-Insulin 120 min (pmol/l) | 2510    | 0.007   | 0.07  | 0.008 | 0.07    |
| HOMA-IR | 2826    | 0.007   | 0.08  | 0.001 | 0.87    |
| ISI$_{(0, 120 \text{ min})}$ | 2382    | −0.010  | 0.018 | −0.010 | 0.225   |
| P-Glucagon 0 min (pg/ml) | 2975    | 0.006   | 0.13  | 0.002 | 0.56    |
| P-Glucagon 120 min (pg/ml) | 2653    | 0.002   | 0.52  | 0.001 | 0.70    |
| S-GIP 0 min (pmol/l) | 2826    | 0.002   | 0.57  | −0.001 | 0.73    |
| S-GIP 120 min (pmol/l) | 2510    | −0.001  | 0.80  | −0.001 | 0.89    |
| P-GLP-1 0 min (pmol/l) | 2940    | 0.008   | 0.022 | 0.001 | 0.037   |
| P-GLP-1 120 min (pmol/l) | 2510    | −0.001  | 0.81  | −0.001 | 0.90    |
| Skin autofluorescence (AU)$^9$ | 454     | 0.012   | 0.18  | 0.010 | 0.29    |

AQT, A Quick Test of Cognitive Speed; AU, arbitrary units; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; ISI, Insulin Sensitivity Index; MMSE, Mini-Mental State Examination.

*Significant P-values are shown in bold.

†Model 1: adjusted for age, sex, education, smoking, alcohol, physical activity.

‡Model 2: adjusted for age, sex, education, smoking, alcohol, physical activity, systolic BP, waist circumference, total cholesterol, antihypertensive treatment, lipid-lowering treatment, diabetes treatment.

§Analyses with skin autofluorescence are also adjusted for skin reflectance.
sample, hence the regression analyses had less statistical power to detect statistically significant differences. In this group there was a small but significant relationship between fasting GLP-1 (Model 1) and worse MMSE results and between 2-h serum GIP and worse AQT results (Model 2, B = 0.034, P = 0.011).

Skin autofluorescence was associated with worse MMSE results in Model 1 in the whole population (B = −1.459, P = 0.030) and in people without diabetes (B = −1.851, P = 0.012), but not in people with diabetes.

Of the strongest significant associations that were found, one SD increment of each biomarker corresponded to ~ 2 normalized MMSE score points difference or a difference in AQT time of 1 s. The effect sizes corresponded to 4 years of age difference as regards MMSE results (B = −0.50, P < 0.001) and 10 years as regards AQT results (B = 0.010, P < 0.001), adjusted for Model 1.

There was a statistically significant interaction between 2-h serum GIP and diabetes status with MMSE as outcome after full adjustment in the total cohort (B = −0.014, P = 0.038). No interactions were found between the other biomarkers and diabetes status (data not shown) in relation to cognitive test results.

In Table S1, correlations between biomarkers are shown, adjusted for age and sex.

In Table S2, multiple regression analyses of associations between biomarkers and cognitive outcomes stratified for four categories of glucometabolic status are presented. The analyses had less statistical power than the previous ones due to smaller groups, but most notably, associations were positive between 2-h glucagon and MMSE, and negative between AGEs and MMSE within the subgroup with prediabetes but not within the group with NGT. However, post-load incretin levels were positively correlated with MMSE within the group with NGT, but not within the group with prediabetes.

In a mediation analysis (Table S3), the indirect effect of HOMA-IR as a mediator between diabetes and MMSE was significant (34% of the total effect of diabetes on MMSE results, P = 0.0074). No other biomarkers significantly mediated this relationship in the analyses.

**Discussion**

In this cross-sectional, population-based study of older participants, insulin sensitivity and post-load levels of GIP...
and GLP-1 were associated with better cognitive outcomes, and AGEs and insulin resistance with worse outcomes, supporting evidence from previous studies. These data also suggest peripheral insulin resistance as a mediator between diabetes and poor cognition, which could be due to a close correlation with intracerebral insulin resistance. Furthermore, higher glucagon levels were associated with better cognitive test results, which to our knowledge is a novel finding. The effect sizes were small, but the change of cognitive function per SD increment of each biomarker was equivalent to several years of age difference in these participants. Most associations remained significant after adjustment for cardiovascular factors, which may indicate that these factors do not predominantly mediate the associations.

There were few significant associations between biomarkers and cognitive test results in the subgroup with diabetes. This could possibly be due to lack of statistical power. Of the results that were significant, however, it is somewhat surprising that post-load incretin levels correlated with worse cognitive test results (although the beta coefficients were very low).

There are some possible explanations for the association between glucagon and cognitive function, although it is not possible to determine causality in a cross-sectional study. High glucagon may, via systemic hyperglycaemia, increase glucose availability in the brain. Alternatively, glucagon may directly affect neuronal function/synaptic transmission. GLP-1 has been reported to enhance synaptic transmission by a cyclic adenosine monophosphate (cAMP)/protein kinase A-dependent mechanism [27]. Like GLP-1, glucagon receptor activation will increase intracellular CAMP and it is therefore possible that it affects neurotransmission similarly. To date, only trace amounts of glucagon have been found in the central nervous system [7] but it is possible that glucagon crosses the blood–brain barrier under certain conditions, e.g. when the permeability is increased due to hyperglycaemia or neurodegenerative disease [28]. Interestingly, the associations were statistically significant in the group with prediabetes but not NGT.

Our findings within both the whole study population and the subgroup without diabetes support the hypothesis that incretins may have neuroprotective properties, but further longitudinal and interventional studies are needed to

### Table 4 Multiple regression analyses of changes in mean cognitive test results per 1 SD increment of each biomarker among participants with diabetes (n = 548)

|                                      | Model 1† |          | Model 2‡ |          |
|--------------------------------------|----------|----------|----------|----------|
|                                      | N        | Means per 1 SD increment | P-value* | Means per 1 SD increment | P-value* |
| A. MMSE (points/100, normalized)     |          |          |          |          |
| S-Insulin 0 min (pmol/l)             | 525      | 0.246    | 0.66     | 0.375    | 0.52     |
| S-Insulin 120 min (pmol/l)           | 232      | 0.376    | 0.72     | −0.174   | 0.88     |
| HOME-IR                              | 525      | 0.047    | 0.93     | 0.349    | 0.57     |
| ISI(0–120 min)                       | 219      | 0.393    | 0.32     | 1.109    | 0.40     |
| P-Glucagon 0 min (pmol/l)            | 546      | −0.119   | 0.86     | 0.146    | 0.84     |
| P-Glucagon 120 min (pmol/l)          | 245      | 0.545    | 0.53     | 0.326    | 0.71     |
| S-GIP 0 min (pmol/l)                 | 528      | −0.228   | 0.70     | 0.169    | 0.78     |
| S-GIP 120 min (pmol/l)               | 232      | −1.455   | 0.10     | −1.416   | 0.12     |
| P-GLP-1 0 min (pmol/l)               | 541      | −1.310   | 0.015    | −1.157   | 0.033    |
| P-GLP-1 120 min (pmol/l)             | 232      | −1.462   | 0.11     | −1.421   | 0.13     |
| Skin autofluorescence (AU)§          | 83       | 1.087    | 0.55     | 2.116    | 0.28     |

A. QPT (s)

|                                      |          |          |          |          |
| S-Insulin 0 min (pmol/l)             | 522      | −0.009   | 0.30     | −0.009   | 0.31     |
| S-Insulin 120 min (pmol/l)           | 229      | −0.017   | 0.29     | −0.005   | 0.75     |
| HOME-IR                              | 522      | −0.003   | 0.69     | −0.006   | 0.52     |
| ISI(0–120 min)                       | 217      | 0.007    | 0.72     | 0.003    | 0.88     |
| P-Glucagon 0 min (pmol/l)            | 542      | 0.012    | 0.27     | −0.007   | 0.51     |
| P-Glucagon 120 min (pmol/l)          | 242      | < 0.001  | 0.98     | < 0.001  | 0.99     |
| S-GIP 0 min (pmol/l)                 | 525      | −0.001   | 0.92     | −0.008   | 0.42     |
| S-GIP 120 min (pmol/l)               | 229      | 0.032    | 0.017    | 0.034    | 0.011    |
| P-GLP-1 0 min (pmol/l)               | 537      | 0.007    | 0.38     | 0.006    | 0.48     |
| P-GLP-1 120 min (pmol/l)             | 229      | 0.035    | 0.013    | 0.037    | 0.008    |
| Skin autofluorescence (AU)§          | 83       | 0.011    | 0.67     | 0.011    | 0.71     |

AQT, A Quick Test of Cognitive Speed; AU, arbitrary units; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; HOME-IR, Homeostatic Model Assessment of Insulin Resistance; ISI, Insulin Sensitivity Index; MMSE, Mini-Mental State Examination.

*Significant P-values are shown in bold.
†Model 1: adjusted for age, sex, education, smoking, alcohol, physical activity.
‡Model 2: adjusted for age, sex, education, smoking, alcohol, physical activity, systolic BP, waist circumference, total cholesterol, anti-hypertensive treatment, lipid-lowering treatment, diabetes treatment.
§Analyses with skin autofluorescence are also adjusted for skin reflectance.
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Quartiles of HOMA-IR

Quartiles of ISI 0–120 min

Quartiles of 2-h Glucagon (pg/mL)

Quartiles of 2-h GIP (pmol/L)

Quartiles of fasting GLP-1 (pmol/L)

Quartiles of 2-h GLP-1 (pmol/L)

Quartiles of SAF (AU)

Quartiles of ISI 0–120 min

Quartiles of fasting GLP-1 (pmol/L)
investigate causality. The results of the CAROLINA Cognition study do not support this hypothesis (for GLP-1 and in people with diabetes) [14]; however, the study was not placebo-controlled, which is why the lack of effect differences according to cognitive function between treatment arms cannot lead us to any conclusion regarding incretin-related mechanisms in relation to sulfonylurea-related mechanisms for this outcome.

By contrast, there were negative associations between fasting levels of GLP-1 and cognitive function. We hypothesize that the difference between this finding and the positive associations for 2-h GLP-1 could be due to the fact that fasting levels of incretins are often chronically elevated in the context of impaired glucose metabolism and insulin resistance, whereas postprandial incretin levels are reduced [29].

The interaction between 2-h GIP and diabetes status in relation to cognition could imply that the effects of GIP in the brain are more dependent on diabetes status than the effects of GLP-1, but more studies are needed to determine this. It is also possible that inter-regulatory effects between glucagon, GIP and GLP-1 could have affected the results in this study (both cognition and AGE levels) as, for instance, GIP stimulates glucagon secretion whereas GLP-1 inhibits it.

Higher levels of skin autofluorescence were associated with worse cognitive test results, supporting findings from the few previous studies on AGES and cognition. In contrast to the Maastricht study [17], our results were significant also after adjustment for lifestyle factors, but not cardiovascular risk factors, possibly reflecting the close link between AGES and cardiovascular disease [30]. These correlations among people without diabetes were significant for the prediabetes group, raising the possibility that AGE-related pathophysiological mechanisms could be present already at subclinical stages of diabetes.

The strengths of this study include the large sample size with OGTT, the population-based setting and the possibility to adjust for many possible confounding factors. The limitations include the cross-sectional study design, the time latency between measurement of biomarkers and cognitive examinations, and the inability to adjust for depression, dietary intake and APOE-ε4 genotype, which may affect the risk of cognitive impairment.

In conclusion, we have shown that increased insulin sensitivity, 2-h plasma glucagon, 2-h serum GIP and 2-h plasma GLP-1 were positively associated with cognitive test results, whereas insulin resistance (HOMA-IR), fasting plasma GLP-1 and AGES were negatively associated with cognitive test results. Longitudinal and interventional studies are needed to confirm these findings and identify possible neuroprotective mechanisms of these biomarkers.

Funding sources
The study was funded by the Research Council of Sweden (Grant K2011-65X-20752–04-6), the Anders Pålsson Foundation, the Ernhold Lundström Foundation, the Regional Agreement on Medical Training and Clinical Research (ALF) between Skåne County Council and Lund University, the Swedish Alzheimer’s Foundation (Alzheimerfonden) and research grants from Region Skåne.

Competing interests
None declared.

Acknowledgements
The authors wish to thank Erik Nilsson, Kalina Rajaobelina, Kate McCullough, Hugh McCullough, Geert-Jan Biessels and Christophe Tzourio for their contributions to the study and its interpretation. The study was funded by the Research Council of Sweden (Grant K2011-65X-20752–04-6), the Anders Pålsson Foundation, the Ernhold Lundström Foundation, the Regional Agreement on Medical Training and Clinical Research (ALF) between Skåne County Council and Lund University, the Swedish Alzheimer’s Foundation (Alzheimerfonden) and research grants from Region Skåne.

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**Supporting Information**

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

**Table S1.** Partial correlations between biomarkers and related indices adjusted for age and sex.

**Table S2.** Multiple regression analyses of changes in mean cognitive test results per 1 SD increment of each biomarker, comparing participants with normal glucose tolerance (NGT), prediabetes and short or long duration of diabetes. The analyses were adjusted for age, sex, education, smoking, alcohol and physical activity.

**Table S3.** Direct and indirect effects of diabetes and biomarkers on cognitive function using the mediation analysis method process by Andrew F. Hayes for SPSS.