Glycaemic patterns in healthy elderly individuals and in those with impaired glucose metabolism – exploring the relationship with nonglycaemic variables

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PME, BS and CG carried out the analysis of the database and calculated the statistical evaluations; they helped writing the paper. UEN helped write the paper and chose the appropriate references. LR helped write the paper and was instrumental in setting up the figures. MR helped validate the paper and was instrumental in setting up tables and figures. ZS helped write the paper and validated the clinical classification of the included/excluded participants.

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Summary

OBJECTIVE: The SENIORLABOR study data were explored (i) to examine the evolution during senescence of the differences between measured glycated haemoglobin (HbA1c) values and the values predicted by using regression to extrapolate from measured fructosamine levels; (ii) to scrutinise the relationship between the glycation gap and insulin resistance using a homeostasis model assessment, and between the glycation gap and a low-grade inflammation marker (C-reactive protein serum concentration); and (iii) to investigate the glycation gap ranges in relation to triglyceride levels and kidney function.

SUBJECTS AND METHODS: A total of 1432 Swiss individuals aged >60 years and classified as healthy (547), prediabetic (701) or diabetic (184) based on their fasting plasma glucose and HbA1c values were included in the study. The glycation gap was evaluated and assigned to one of four categories: <−0.5; −0.5 to <0.0; 0.0 to ≤0.5; >0.5.

RESULTS: In healthy and prediabetic participants, the homeostasis model assessment for estimation of insulin resistance (p <0.01), high-sensitivity C-reactive protein (p <0.001) and triglyceride (p = 0.02) values tended to increase with increasing glycation gap category and were highest in the glycation gap category >0.5. Homeostasis model assessment for estimation of insulin resistance, high-sensitivity C-reactive protein and triglyceride levels tended to increase with increasing glycation gap category and were highest in the glycation gap category >0.5. Significant differences (p <0.01) between glycation gap categories were seen among different high-sensitivity C-reactive protein concentration groups. Interestingly, in diabetic participants, homeostasis model assessment for estimation of insulin resistance values, triglyceride concentrations and estimation of glomerular filtration values all decreased with decreasing glycation gap category. In the group of participants with a glycation gap >0.5, high-sensitivity C-reactive protein values tended to increase with increasing glycation gap, whereas for participants with type 2 diabetes and in the glycation gap group >0.5, high-sensitivity C-reactive protein levels tended to decrease as the glycation gap increased. The percentage of participants with type 2 diabetes mellitus increased from 2% in the glycation gap category <−0.5 to 76% in the glycation gap category >0.5. In contrast, the percentage of healthy participants fell from 85% to 7%.

CONCLUSION: This is the first time that a direct comparison of healthy, prediabetic and diabetic participants, all assessed under identical conditions and using identical methodology, has clearly demonstrated a different glycation gap pattern. Thus, we contribute evidence that the glycation gap might be of interest in the care of diabetic patients and their prophylaxis, while acknowledging that more studies are needed to confirm our findings. (Trial registration number ISRCTN53778569)

Keywords: glycated haemoglobin, glucose metabolism, glycation gap, prediabetes, type 2 diabetes, senescence

Introduction

The non-enzymatic glycation of proteins through the Maillard reaction is a physiological process which takes place in plants, animals and humans alike. This posttranslational linkage between protein amino and guanidino groups and sugar carbonyl compounds transitions to Amadori and Heynes intermediates before ending up in a stable state, i.e. an advanced glycation end product. Glycation affects the interactions of proteins with specific receptors [1, 2], (auto-)antibodies [3], protein half-lives, senescence [4] and protein folding [5, 6]. Oligosaccharide glycan is covalently attached to polypeptide side chains. This may occur through co- and/or post-translational glycation/glycosylation, now possibly thought to be under control of micro-ribonucleic acid [7]. This glycation serves to aid the recognition of certain cell types. Advanced glycation end products

are responsible for blood vessel malfunction through their reactions with specific receptors [8]. The stimulation of receptors for advanced glycation end products by their agonists activates nuclear factor-kappa B and increases endothelial permeability to macromolecules, causing kidney impairment [9, 10]. Advanced glycation end products block nitric oxide, boosting reactive oxygen species and causing consecutive inflammation [11]. Glucose concentrations are similar in the intra- and extracellular compartments due to the glucose T1 channel homeostatic mechanism, which is markedly expressed in the microvascular system of the kidneys. Hyperglycaemic metabolic state causes an intracellular toxic effect which leads to microvascular complications with time [12].

The glycation extent of haemoglobin (glycated haemoglobin A1c) is an indicator of plasma glucose levels over the ~100 days prior to a venipuncture, and the laboratory assays for its quantification are steadily changing [13, 14]. The remainder of glycoproteins are collectively termed fructosamines [15], and most of these are glycated albumin. Investigations into both HbA1c and fructosamine values are appropriate for clinical use [16, 17]. The difference between the measured HbA1c value and the HbA1c value predicted from fructosamine values is termed the glycation gap. It has a broad distribution in patients with nephropathy (from −3.0% to 5.5%) [18]. Glycation gap values oscillating within >−0.5 and <0.5 were assumed to be in the tolerable range. A drop below −0.5 was considered metabolically fit, whereas a glycation gap >0.5 was considered metabolically unfit [19, 20].

Glycation gap evaluations currently comply with the reference change value criteria for glycaemic control, as documented by their stability in the follow-up of patients with type 2 diabetes [21]. To date, the glycation gap ranges in healthy controls are unknown. It has been suggested that glycation gap is not associated with chronic kidney disease in nondiabetic individuals [22].

Dyslipidaemia, a spectrum of quantitative and qualitative changes in lipids and lipoproteins, is now acknowledged to accompany the metabolic abnormality of hyperglycaemia [23], and a low-grade inflammatory background to insulin insufficiency emerges in the literature [24].

We have recently found laboratory evidence of impaired glucose metabolism among subjectively healthy elderly Swiss individuals [25], and with the current data we hope to improve our understanding by considering the involvement of glycation gap calculations in diabetic organ failure [18]. Therefore, in the present study we will relate the glycation gap to lipid metabolism, low grade inflammation as evidenced by high-sensitivity C-reactive protein, and kidney function as estimated by glomerular filtration rates in primarily healthy seniors, those with prediabetes, and in a small number of participants with treated type 2 diabetes mellitus.

Materials and methods

Study population

We conducted a cross-sectional cohort study. Consecutive subjectively healthy older volunteers aged ≥60 years were recruited between February 2009 and December 2011 as part of the SENIORLABOR study (International Standard

Registered Clinical/Social Study Number 53778569), an ongoing investigation on the Swiss plateau aimed at creating appropriate reference intervals for several analytes in older citizens. The study participants were contacted through newspaper advertisements, clubs and associations where there was a high probability that the membership would include healthy older citizens (e.g., mountaineering clubs, sports clubs) and, for a few participants, through the personal contacts of those involved in organising the study. The personal histories of the participants were collected and anthropometric measurements (body weight, height and body mass index) were taken. We excluded candidates presenting overt comorbidities and excessive alcohol consumption. The food intake of participants was confirmed to be typical of the Swiss midlands and consisted of an approximate energy consumption per person per day of 2661 kcal (11,135 kJ), composed of 14% proteins, 51% carbohydrates and 35% fat [26]. The mean body mass index of the participants was in the upper normal range for both genders, based on the definition issued by the World Health Organization. There were no overweight participants in the cohort. The primary exclusion criteria for the present study were: current steroid use, thyroid dysfunction (free-thyroxine level <9 or >19 pmol/l), high-sensitivity C-reactive protein concentration above 10 mg/l, and being underweight (body mass index <18.5 kg/m^2). The participant inclusion algorithm is illustrated in fig. 1. Out of 1467 study participants, 35 were excluded due to missing glycated haemoglobin A1c values. All remaining participants (n = 1432, 100%) were included in the study. The subjects were subdivided into healthy and diabetic participants. The glycated haemoglobin A1c values and fasting plasma glucose levels in the healthy subjects (n = 1248, 87.2%) remained below the cut-off values for diabetes according to the current reference intervals (glycated haemoglobin A1c ≥6.5%, fasting plasma glucose ≥7 mmol/l). The diabetic subjects (n = 184, 12.8%) were either diagnosed with type 2 diabetes and receiving treatment or were identified as diabetics because their laboratory values exceeded the glycated haemoglobin and/or fasting plasma glucose cut-offs.

Figure 1: Chart representing all the recruited participants, with those retained and those excluded (n = number of participants). See text for primary exclusion criteria. GG = glycation gap; T2DM = type 2 diabetes mellitus
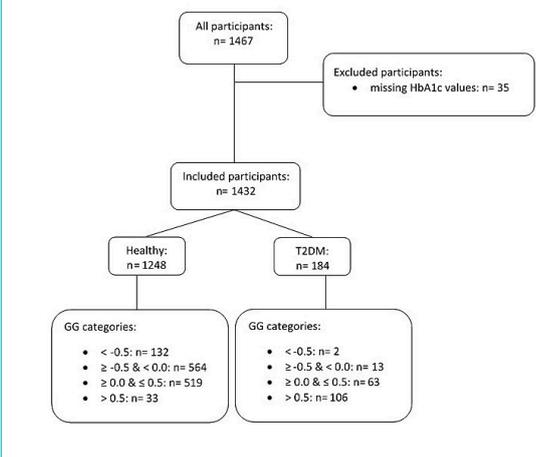
This study was conducted in accordance with the ethical guidelines of the 1957 Declaration of Helsinki and was approved by the cantonal institutional review board (Kantonale Ethikkommission Bern 166/08, Bern, Switzerland). All participants provided written informed consent.

Biochemical measurements

The venous blood samples were drawn by venipuncture into S-Monovette tubes (Sarstedt, Sevelen, Switzerland) after an overnight fasting period. The blood samples were processed (centrifuged, aliquoted and analysed within two hours or frozen at –80°C at our site) immediately to enable standardised preanalytics.

Fasting plasma glucose levels were measured using the enzymatic hexokinase procedure on a Roche Integra 800 (Rotkreuz, Switzerland). Glycated haemoglobin A1c was measured with International Federation of Clinical Chemistry-approved chromatography (high-power liquid chromatography D-10, Biorad, Reinach, Switzerland), a testing system certified by the United States of America National Glycohemoglobin Standardization Program. This system has a coefficient of variation of <2.8%, a range in agreement with currently acknowledged requirements [27]. Fructosamine levels were measured using a spectrophotometric assay based on the reduction of nitroblue tetrazolium to formazan and were assayed on an Abbott ARCHITECT (Abbott Switzerland, Baar). Insulin was measured with an electrochemiluminescence immunoassay (Cobas 6000). Insulin resistance was estimated with the homeostasis model assessment index (serum insulin [µU/ml] x serum glucose [mmol/l] divided by 22.5). The following homeostasis model assessment index values and cut-offs were used: ≤2.0 = no insulin resistance, >2.0 to <2.5 = indication for insulin resistance, ≥2.5 to ≤5.0 = insulin resistance likely; values >5.0 are typically seen in patients with type 2 diabetes mellitus [28]. Triglyceride levels were measured using an enzymatic colorimetric method on a Roche Integra 800 (Roche Diagnostics, Rotkreuz, Switzerland). Hypertriglyceridaemia was defined as triglyceride concentrations >1.7 mmol/l (150 mg/dl), in accordance with the Canadian Cardiovascular Society guidelines [29].

The isotope dilution mass spectrometry-standardised creatinine concentration was determined using a modified Jaffe method on a Cobas Integra 800 instrument. Commercially available materials were used for quality control. Glomerular filtration rates were calculated using cystatin C equations [30]. The inter-day coefficients of variation for creatinine were 4.27% at 42 µmol/l and 1.96% at 556 µmol/l. High-sensitivity C-reactive protein levels were measured using a Siemens ProSpec instrument. Glomerular filtration rates were estimated using the combined chronic kidney disease estimation, which uses creatinine and corrected cystatin C levels (Chronic Kidney Disease Epidemiology Collaboration equation). The arithmetic mean of the estimated glomerular filtration rate$_{cys}$ was obtained from the Caucasian, Asian, Paediatric and Adult cohort equation [31], and the estimated glomerular filtration rate$_{crea.}$ was used for the calculation.

The classification of chronic kidney disease used in our study is based on either measured or estimated glomerular filtration rates. Chronic kidney disease stages 1 and 2 denote normal or slightly reduced kidney function (glomeru-

lar filtration rate ≥90 ml/min/1.73 m^2 or 60–89 ml/min/1.73 m^2). In chronic kidney disease stages 3 and 4, functional impairment is moderate (glomerular filtration rate 30–59 ml/min/1.73 m^2) or severe (glomerular filtration rate 15–29 ml/min/1.73 m^2).

The serum high-sensitivity C-reactive protein concentrations were categorised as low (<1.0 mg/l), average (1.0–3.0 mg/l) or high (>3.0 mg/l), in accordance with the statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association [32]. Participants with high-sensitivity C-reactive protein values >10 mg/l were excluded from the study at the outset.

The glycation gap for each participant was calculated by determining the difference between the measured glycated haemoglobin A1c level and the value predicted from the fructosamine measurement using the regression equation (predicted glycated haemoglobin A1c = 3.2426 + (0.01146 x fructosamine level)). Glycation gap values oscillating within >−0.5 and <0.5 were assumed to be in the tolerable range. A drop below −0.5 was considered metabolically fit, whereas a glycation gap >0.5 was considered metabolically unfit [19]. Therefore, we subclassified participants into four glycation gap categories: <−0.5, −0.5 to <0.0, 0.0 to ≤0.5, and >0.5.

The current American Diabetes Association reference intervals for the diagnosis of type 2 diabetes mellitus (glycated haemoglobin A1c ≥6.5%, fasting plasma glucose ≥7.0 mmol/l) were used for this study [33]. We used the baseline classifications of prediabetes tested by Heianza et al. [34]. These authors used combinations of four glycated haemoglobin intervals, A1c 6.0–6.4%, 5.7–6.4%, <6.0% or <5.7%, and four fasting plasma glucose level intervals, 6.1–6.9 mmol/l, 5.6–6.9 mmol/l, <6.1 mmol/l or <5.6 mmol/l, to create multiple classifications of prediabetes.

Class A referred to a glycated haemoglobin A1c level of 6.0–6.4% combined with a fasting plasma glucose level of 6.1–6.9 mmol/l, and these criteria were predictive of a cumulative risk of developing type 2 diabetes mellitus of as high as 100% after 5.6 years. Class B used the combination of glycated haemoglobin A1c 5.7–6.4% and fasting plasma glucose 6.1–6.9 mmol/l. Class C used the combination of glycated haemoglobin A1c 6.0–6.4% and fasting plasma glucose 5.6–6.9 mmol/l. Classes B and C both resulted in a similar cumulative risk of developing type 2 diabetes mellitus of approximately 80%. Class D was defined by the combination of glycated haemoglobin A1c 5.7–6.4% and fasting plasma glucose 5.6–6.9 mmol/l, and was predictive of a cumulative risk of developing type 2 diabetes mellitus of approximately 50%. Finally, the combinations of glycated haemoglobin A1c <6.0% and fasting plasma glucose <6.1 mmol/l (class E) and of HbA1c <5.7% and fasting plasma glucose <5.6 mmol/l (class F) were predictive of an approximately 0–10% cumulative risk of developing type 2 diabetes mellitus.

The accuracy and precision of our assays complies with external and internal quality control requirements and is certified (international standardisation organisation/IEC 17000,5.5 www.seco.admin.ch) by the Swiss commission for quality assurance in the medical laboratory.

Data analysis

The statistical analyses were performed using MedCalc for Windows, version 15.6.2 (MedCalc Software, Ostend, Belgium). The distributions of the analysed variables were tested using the Shapiro-Wilk test. For a preliminary analysis, the data were logarithmically transformed as appropriate to correct the skewness of the data. Outliers were detected using the Tukey method, in which an outside value was defined as a value smaller than the lower quartile minus 1.5 times the interquartile range or larger than the upper quartile plus 1.5 times the interquartile range. Three such values were identified. Since this is a low number of outliers and since there was no indication of any error, these outliers were not excluded from the statistical analyses.

The preliminary appraisals revealed a non-normal distribution of the data, so most statistical analyses were performed using rank-based methods, which can be expected to perform as intended with skew distributions and certain outliers. Between-group comparisons were performed using Student's t-test and the Mann-Whitney test for independent samples. Proportions were compared using the χ^2 test. To evaluate a possible trend in analyte concentrations among the four glycation gap categories, the rank-based Jonckheere-Terpstra test for trends was used to assess prediabetes involving HbA1c and fasting plasma glucose measurements. Correlations between variables were estimated using Spearman's rank correlation analysis, depending on the distribution of the researched variable. A p-value of <0.05 was considered to indicate statistical significance in all tests.

Results

The clinical laboratory assay intervals of the healthy and the diabetic participants differed with various degrees of significance (table 1). Age, body mass index, homeostasis model assessment index, fasting plasma glucose level, glycation gap, concentrations of high-sensitivity C-reactive protein, triglyceride and total cholesterol, and estimated glomerular filtration rate values were clearly increased,

with various degrees of statistically significance, in participants with type 2 diabetes mellitus. Please note that the glycation gap can be either negative or positive (see also fig. 5 below).

In the cohort of healthy participants, men had significantly higher body mass index values, homeostasis model assessment indices for insulin resistance, and fasting plasma glucose levels than women (p <0.001), and only slightly higher estimated glomerular filtration rate values (p = 0.04), whereas total cholesterol levels were significantly increased in women (p <0.001). In accordance with this, within the type 2 diabetic participants we found slightly higher estimated glomerular filtration rate values in men than in women (p = 0.045), whereas age (p = 0.01), high-sensitivity C-reactive protein concentrations (p = 0.03) and cholesterol levels (p <0.001) were significantly higher in women. Haemoglobin concentrations did not fall below 11.0 g/dl in any of the participants.

We then explored the extent of serum protein glycation. We calculated the median values and 95% confidence intervals (CIs) separately for fasting plasma glucose level, glycated haemoglobin A1c and glycation gap. Healthy participants had a mean fasting plasma glucose level of 5.20 mmol/l (95% CI 5.20 to 5.20), a mean glycated haemoglobin A1c of 5.80% (95% CI 5.70 to 5.80), corresponding to 39.9 mmol/mol as expressed by International Federation of Clinical Chemistry units, and a mean glycation gap of −0.10 (95% CI −0.10 to −0.10). Subjects with laboratory evidence for glucose metabolism impairment, i.e. diabetic participants, had a mean fasting plasma glucose level of 6.60 mmol/l (95% CI 6.40 to 6.78), a mean glycated haemoglobin A1c of 6.70% (95% CI 6.60 to 6.80), and a mean glycation gap of 0.60 (95% CI 0.60 to 0.70).

Detailed results for the homeostasis model assessment used to estimate insulin resistance, the concentrations of high-sensitivity C-reactive protein and triglyceride, and the estimated glomerular filtration rate are presented by glycation gap category in fig. 2. In the healthy participants, homeostasis model assessment for estimation of insulin resistance, high-sensitivity C-reactive protein and triglyceride values tended to increase with glycation gap category

Table 1: Baseline characteristics of healthy and diabetic participants.

	Healthy n = 1248	T2DM n = 184	p-value
Hypertensive/nonhypertensive, %	36/64	53/47	0.02
Smokers/nonsmokers, %	6/94	10/90	0.43
Women/men, %	56/44	33/67	<0.01
Age, years	71.8 (7.78)	74.4 (7.63)	<0.001
Age, range	60–99	60–97	–
Body mass index, kg/m^2	25.24 (3.62)	27.2 (4.84)	<0.001
HOMA-IR	2.11 (1.41)	3.48 (2.70)	<0.001
FPG, mmol/l	5.25 (0.51)	6.97 (2.39)	<0.001
HbA1c, %*	5.76 (0.32)	6.70 (1.01)	<0.001
Glycation gap	−0.11 (0.36)	0.72 (0.92)	<0.001
hsCRP, mg/l	2.30 (3.98)	3.24 (6.79)	<0.01
Triglycerides, mmol/l	1.36 (0.70)	1.81 (1.39)	<0.01
Total cholesterol, mmol/l	5.82 (1.11)	5.17 (1.13)	<0.001
eGFR, ml/min/1.73 m^2	83.4 (16.11)	75.73 (17.80)	<0.001

eGFR = estimated glomerular filtration rate; FPG = fasting plasma glucose; HbA1c = glycated haemoglobin A1c; HOMA-IR = homeostasis model assessment for estimation of insulin resistance; hsCRP = high-sensitivity C-reactive protein; T2DM = type 2 diabetes mellitus Values are mean values. Differences between healthy and diabetic participants were calculated using Student's t-test and the χ^2 test, as appropriate. * International Federation of Clinical Chemistry: 39.9 mmol/mol (38.8, 39.9) for the healthy, and 49.7 (48.6, 50.8) for the diabetic participants.

Figure 2: Variations in homeostasis model assessment for estimation of insulin resistance (HOMA-IR), high-sensitivity C-reactive protein (hsCRP), triglyceride (TG) and glomerular filtration rate levels according to glycation gap category and diabetic status.Statistical box and whiskers diagram around median values. (A) Differences between healthy (H) and diabetic (D) participants were calculated with chi-square and Mann-Whitney tests, as appropriate; (B) hsCRP, high-sensitivity C-reactive protein; (C) TG, triglyceride; (D) GFR, glomerular filtration rate. As can be seen from the figure, in healthy participants, homeostasis model assessment for estimation of insulin resistance (HOMA-IR), high-sensitivity C-reactive protein (hsCRP) and triglyceride (TG) values increased with glycation gap category (p<0.001). In diabetic participants, differences between healthy and diabetic participants were calculated with chi-square and Mann-Whitney tests, as appropriate.HOMA-IR, triglyceride (TG) and estimated glomerular filtration rate (eGFR) values decreased up to glycation gaps ≤0.5, and behaved inversely in the glycation gap category >0.5. High-sensitivity C-reactive protein values increased up to glycation gaps ≤0.5 and behaved inversely in the glycation gap category >0.5. Dots represent outliers of the four analytes as indicated on the ordinate.
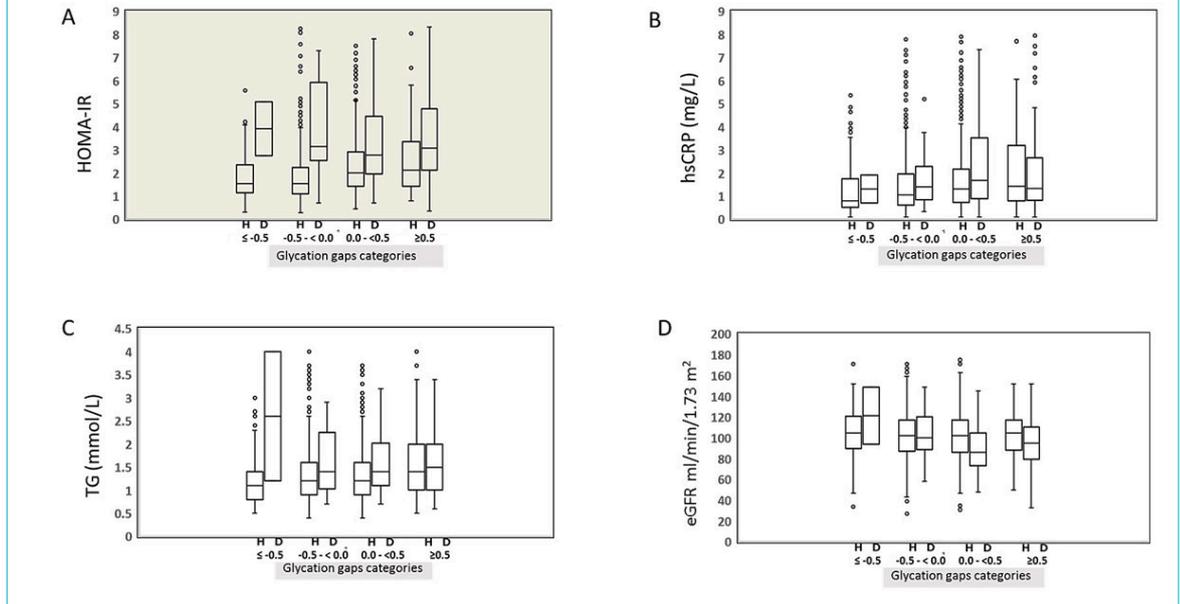
and were highest in the glycation gap category >0.5. Likewise, the Jonckheere-Terpstra test revealed a significant trend towards higher levels in the higher glycation gap categories (p <0.001). With estimated glomerular filtration rate, however, no significant trend was apparent (p = 0.32). In the type 2 diabetic participants, homeostasis model assessment for estimation of insulin resistance, triglyceride and estimated glomerular filtration rate values tended to decrease up to a glycation gap ≤0.5, and behaved inversely in the pathological glycation gap category (glycation gap >0.5). In addition, the high-sensitivity C-reactive protein values tended to increase up to a glycation gap ≤0.5 and behaved inversely when they reached a pathological glycation gap >0.5. When the Jonckheere-Terpstra test was applied, however, no significant trend was identified (p >0.05).

Next, we calculated the percentage of healthy and diabetic participants in each glycation gap category. A χ^2 test for trend analysis revealed a significant trend (p <0.001) towards higher glycation gap levels from the healthy to the type 2 diabetic participants. In fact, the percentage of participants with a glycation gap >0.5 increased sharply, from 2% in healthy subjects to 58% in diabetic subjects, while the percentage of participants with a glycation gap <−0.5 decreased from 11% in healthy to 1% in diabetic subjects (fig. 3). We then investigated the glycation gap values in the healthy participants according to the four baseline classifications of prediabetes (fig. 4) [35]. Using the Jonckheere-Terpstra test, we found a statistically significant trend towards lower glycation gap levels as the cumulative risk of developing diabetes decreased (p <0.001), with the

highest glycation gap level in the 100% cumulative risk group (A).

When we analysed the extent of insulin resistance, we found 41 (3.3%) healthy and 41 (22.3%) diabetic subjects with homeostasis model assessments for estimation of insulin resistance >5.0. Of these participants, 25 (61.0%) healthy and 36 (87.8%) type 2 diabetic subjects had glycation gaps ≥0.0. With regard to hidden inflammation, 221 (17.7%) healthy and 54 (29.5%) diabetic subjects were in the higher high-sensitivity C-reactive protein level category (>3.0 mg/l) (using the Centers for Disease Control and Prevention / American Heart Association recommendations), and 121 (54.8%) healthy and 52 (96.3%) type 2 diabetic subjects were in the two glycation gap categories

Figure 3: Percentage of participants in each glycation gap category. Diabetic participants clearly exhibited a trend towards higher glycation gap categories. The grey shading darkens as the glycation gap changes from below zero to above zero, reflecting the impairment of glycaemic control.
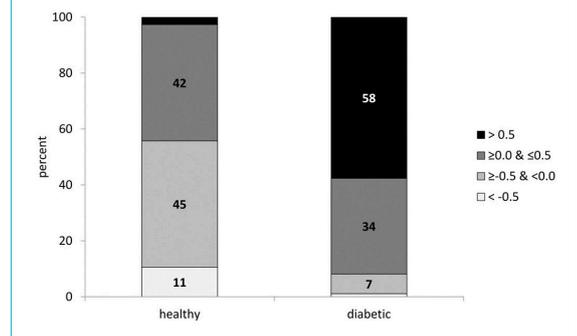
≥0.0. After applying the Clinical Chemistry of Switzerland guidelines for hypertriglyceridaemia, 245 (19.6%) healthy and 66 (35.9%) type 2 diabetic participants had elevated triglyceride levels (>1.7 mmol/l), and among those participants, 128 (52.2%) healthy and 59 (89.4%) type 2 diabetic subjects were in the glycation gap categories ≥0.0. When we analysed the participants according to chronic kidney disease stage, as determined by estimated glomerular filtration rates, we found 124 (9.9%) healthy and 32 (17.4%) diabetic subjects within stage 3 or higher, and 62 (50.0%) apparently healthy and 31 (96.9%) type 2 diabetic participants were found in the glycation gap categories ≥0.0.

We then assessed associations between fasting plasma glucose level, glycated haemoglobin A1c and glycation gap through linked non-glycaemic laboratory assays (table 2), which are important in the diagnosis of type 2 diabetes. Using Spearman rank correlation analysis, significant correlations between homeostasis model assessment for estimation of insulin resistance and fasting plasma glucose level (p <0.001), glycated haemoglobin A1c (p <0.001), and glycation gap (p <0.001) in healthy participants became apparent. The association was strongest for fasting plasma glucose level. However, in the type 2 diabetic participants, only fasting plasma glucose level was significantly associated with homeostasis model assessment for estimation of insulin resistance (p <0.001).

Treatment with metformin may widen the glycation gap [19]. This prompted us to evaluate how many type 2 diabetic participants were treated with metformin. Out of the 184 participants with type 2 diabetes, 54 were taking antidiabetic drugs, including insulin injections, and the participants taking metformin had a mean glycation gap of 0.7%.

The measured fructosamine ranges were used to calculate the corresponding glycated haemoglobin A1c concentrations in each participant (fig. 5). It appears that within the reference interval of fructosamine used [19], glycated haemoglobin A1c values were distributed in a narrow range, whereas outside the reference interval (below or above it), this distribution spreads substantially.

Figure 4: Glycation gap values in healthy participants, according to four baseline classifications of prediabetes defined by FPG and HbA1c (see text, [33] and [34]). Multiple combinations of FPG and HbA1c levels are used to present the glycation gap and the impairment of glycaemic control (abscissa). These combinations are: (a) HbA1c 6.0–6.4% and FPG 6.1–6.9 mmol/l, (b) HbA1c 6.0–6.4% and FPG 5.6–6.9 mmol/l, (c) HbA1c 5.7–6.4% and FPG 6.1–6.9 mmol/l, (d) HbA1c 5.7–6.4% and FPG 5.6–6.9 mmol/l, (e) HbA1c <6.0% and FPG <6.1 mmol/l, (f) HbA1c <5.7% and FPG <5.6 mmol/l. The six alphabetic characters represent, from left to right, increasing impairment of fasting glucose. (e) and (f) are the healthy groups according to Heianza et al. [34]. Please note that the glycation gap changes from below zero to above zero as glycaemic control deteriorates. Impairment of glycaemic regulation increases from left to right.
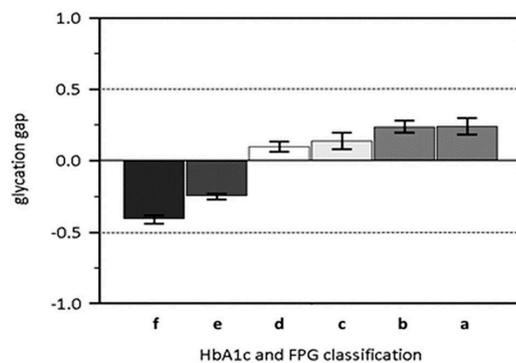
Table 2: Degree of insulin resistance relating to inflammatory, lipid and kidney clinical chemistry criteria in the healthy, non-diabetic cohort (n = 1248).

	HOMA-IR	hsCRP	Triglycerides	Cholesterol	eGFR
FPG	rs = 0.48 p <0.001	rs = 0.07 p = 0.02	rs = 0.15 p <0.001	rs = -0.08 p <0.01	rs = 0.00 p >0.05
Age 60 to 69	rs = 0.57 p <0.001	rs = 0.15 p <0.001	rs = 0.22 p <0.001	rs = -0.09 p = 0.04	rs = 0.00 p >0.05
Age 70 to 79	rs = 0.41 p <0.001	rs = -0.02 p >0.05	rs = 0.09 p = 0.04	rs = -0.10 p = 0.03	rs = 0.04 p >0.05
Age ≥80	r = 0.35 p <0.001	r = 0.00 p >0.05	r = 0.08 p >0.05	r = -0.04 p >0.05	r = 0.05 p >0.05
HbA1c	rs = 0.19 p <0.001	rs = 0.05 p >0.05	rs = 0.12 p <0.001	rs = -0.01 p >0.05	rs = -0.11 p <0.001
Age 60 to 69	rs = 0.16 p <0.001	rs = 0.06 p >0.05	rs = 0.09 p = 0.046	rs = 0.02 p >0.05	rs = -0.03 p >0.05
Age 70 to 79	rs = 0.24 p <0.001	rs = 0.03 p >0.05	rs = 0.13 p <0.01	rs = -0.03 p >0.05	rs = -0.08 p >0.05
Age ≥80	r = 0.12 p >0.05	r = -0.03 p >0.05	r = 0.16 p = 0.01	r = -0.04 p >0.05	r = -0.10 p >0.05
Glycation gap	rs = 0.22 p <0.001	rs = 0.16 p <0.001	rs = 0.14 p <0.001	rs = -0.09 p <0.01	rs = -0.03 p >0.05
Age 60 to 69	rs = 0.17 p <0.001	rs = 0.16 p <0.001	rs = 0.12 p = 0.01	rs = -0.05 p >0.05	rs = 0.04 p >0.05
Age 70 to 79	rs = 0.23 p <0.001	rs = 0.17 p <0.001	rs = 0.14 p <0.01	rs = -0.11 p = 0.02	rs = -0.09 p <0.05
Age ≥80	rs = 0.30 p <0.001	rs = 0.17 p = 0.01	rs = 0.20 p <0.01	rs = -0.14 p = 0.04	rs = -0.08 p >0.05
p <0.001		p <0.05		p >0.05	

eGFR = estimated glomerular filtration rate; FPG = fasting plasma glucose; HbA1c = glycated haemoglobin A1c; HOMA-IR = homeostasis model assessment for estimation of insulin resistance; hsCRP = high-sensitivity C-reactive protein HOMA-IR is a measure of insulin resistance, hsCRP reflects systemic inflammation, triglycerides and free cholesterol reflect lipid homoeostasis, and estimated glomerular filtration rate represents existing kidney function in the healthy cohort. The shading intensity goes from clear (highly significant) to darker (not significant).

In the healthy cohort, fasting plasma glucose level was significantly correlated with high-sensitivity C-reactive protein (p = 0.02), triglyceride (p <0.001) and total cholesterol (p <0.01) levels. Furthermore, glycated haemoglobin A1c was significantly correlated with triglyceride concentration (p <0.001) and estimated glomerular filtration rate (p <0.001). When we analysed the glycation gap among the healthy participants, we found additional associations with high-sensitivity C-reactive protein (p <0.001), triglyceride (p <0.001) and total cholesterol (p <0.01) concentrations (table **2**). The correlation analyses for homeostasis model assessment for estimation of insulin resistance and the high-sensitivity C-reactive protein, triglyceride and total cholesterol concentrations were performed separately for three arbitrarily defined age groups: 60–69 years, 70–79 years and ≥80 years. As can be seen from table 2, triglyceride levels were most strongly associated with fasting plasma glucose levels, whereas estimated glomerular filtration rate was most strongly associated with glycated haemoglobin A1c in the age group >80 years.

Discussion

In a quest to establish reference intervals for the selected clinical chemistry parameters, including those relevant to the diagnosis of type 2 diabetes, we recruited healthy residents with presumably uniform lifestyles and dietary patterns [36]. Swiss plateau nutritional habits typically include the consumption of dairy products and predictable proportions of protein, fat and carbohydrates. This means the participants have comparable glycation exposure of tissues and proteins, including the blood plasma components accessible for laboratory assays.

The estimation of the glycation gap and fructosamine levels [37] has been used in longitudinal studies to predict complications of diabetes [38], but it also has the potential for use in patient care and for the monitoring of therapeutic regimens. In other evaluations of SENIORLABOR data, we have excluded participants treated for type 2 diabetes mellitus or with laboratory evidence of metabolic disease. In this study, however, they are included, and their laboratory values are reported and also analysed in relationship to the healthy cohort, representing the SENIORLABOR confidence intervals. It is possible that biological variation has

had an influence on the interpretation of our report, but its effect is minimised by the large number of subjects recruited and the resulting statistical power. Variation exists within consecutive measurements of many laboratory parameters in the same individual (www.eflm.eu/) [39–41]. It comes as little surprise that the conventional separators between healthy and diabetic individuals, i.e., fasting plasma glucose, glycated haemoglobin A1c and homoeostasis model assessment for insulin resistance values, were highly significantly different between the two groups. Our data further separate the two groups by triglyceride and total cholesterol concentrations and estimated glomerular filtration rates. As expected, we observed that neither glycation gap nor HbA1c were associated with chronic kidney disease in nondiabetic individuals [22]. We also now report for the first time the robust physiological importance of the glycation gap in this context. Our results indicate that healthy elderly individuals display a significant direct relationship between glycation gap and homeostasis model assessment-measured insulin resistance, high-sensitivity C-reactive protein levels, and triglyceride concentrations, and that this relationship is much stronger than that between these parameters and glycated haemoglobin A1c or fasting plasma glucose levels. This fact strongly challenges the idea that the glycation gap is a spurious empirical measure of the disagreement between the two indirect estimates of glycaemic control.

Such an understanding of glycation gap requires an appreciation of the fact that carbohydrate metabolism, even when monitored by continuous glucose monitoring, involves additional players, i.e. deglycation enzymes, erythrocytic processes such as the mean red blood cell age, and glucokinase mutations [12, 42–44].

Expert associations, such as the American Diabetes Association or the International Diabetes Federation, currently focus on precision medicine based on a large selection of laboratory assays, the ratios among them, and are integral part of such big data sets. Metabolic syndrome encompasses stages of diabetes mellitus, hypertriglyceridaemia and hypertension, and it also might involve HNF1 homeobox B, which controls transcription factors such as tumour necrosis factor-1α in the context of immunological changes in type 2 diabetes mellitus [45]. The cohort of SENIORLABOR is homogenous by race, allowing us to ex-

Figure 5: Relationship between fructosamine and HbA1c. This 4-panel figure compares glycated haemoglobin A1c and fructosamine levels in three different fructosamine concentration ranges (data from 1431 participants). A all participants. B subjects with fructosamine concentrations <205 μmol/l. The glycated haemoglobin A1c values range from 4.5% to 7.0%. C Participants exhibiting fructosamine values within the reference interval. Glycated haemoglobin A1c values are within a narrow window. D individuals with high fructosamine levels have glycated haemoglobin A1c values between 5.8% and 11%.
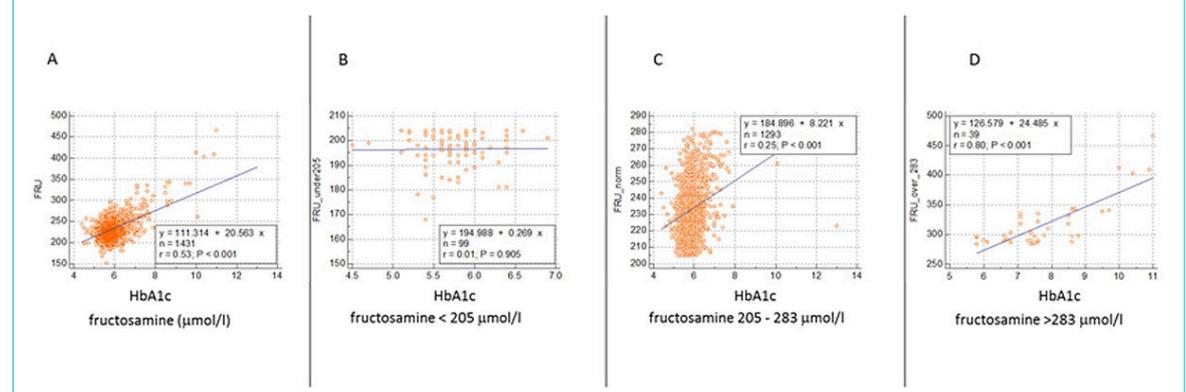
clude the impact of ethnic/major genetic diversity, which is known to play a role in maturity onset diabetes of the young (www.diabetesgenes.org), as this won't have a substantial influence on fasting glucose changes over time in non-diabetic individuals of European descent [46]. The performance of the pancreatic glucose sensor might influence the pattern of our results. We used homeostasis model assessment to assess insulin resistance, which is known to increase with aging. The effect of age was previously analysed separately in individuals with and without type 2 diabetes [25, 47]. This analysis also revealed gender differences in homeostasis model assessment for estimation of insulin resistance cut-off values in non-diabetic women of 19% when using the Youden Index. We here describe these gender differences without offering an explanation. Whereas disciplined nutritional habits can be assumed in the examined study population, the limited increments in HbA1c values observed here might be caused by anti-diabetic drug overdosage or one of the following enhancers/depressors of HbA1c results: haemoglobin gene variations, even when clinically silent [48], mild haemolytic anaemia, or folic acid deficiency. Thus, the pilot Spanish epidemiology of chronic renal disease cross-sectional study [47] is consistent with the present findings of a clear distinction between female and male metabolisms. The Adult Treatment Panel III criteria for metabolic syndrome include: abdominal obesity (given as waist circumference >102 cm for men and >88 cm for women), high-density lipoprotein-associated cholesterol (men <1.0 mmol/l, women <1.3 mmol/l), blood pressure ≥130/≥85 mm Hg, fasting plasma glucose ≥6.1 mmol/l, and triglyceride concentration ≥1.7 mmol/l. Being overweight or obese is also associated with insulin resistance and the diagnosis of metabolic syndrome [49].

Our separate consideration of four categories of glycation gap, i.e., two below and two above zero, was borrowed from earlier studies. We choose this method in order to draw an error grid [38] and made it clear that the incremental values from negative to positive indicated progression from a healthy to a diabetic metabolism. This finding presents evidence that the glycation gap is affected by the glycation of both short-lived and long-lived proteins and reflects current and past glycation history.

One limitation to our study is the current uncertainty about the best method for measuring glycation, e.g., by high-power liquid chromatography, capillary electrophoresis or immunological and enzymatic assays controlled by reference laboratorys [50]. More recently, the constitution of the glycated haemoglobin has been scrutinised as a previously unknown influence on glycated haemoglobin A1c results. Racial differences also affect this variable [48]. Efforts to comply with International Federation of Clinical Chemistry reference systems and to improve biosensing methods are maintained though [14]. Indeed, the nitroblue tetrazolium reduction we used consists of reducing analytes commonly named 'fructosamine', but nitroblue tetrazolium is also an active oxygen species that produces a formazan dye which any unbound sugar molecule in the sample would convert. Some researchers prefer the measurement of glycated albumin, which is largely in agreement with fructosamine [51]. The need to improve the management of diabetes makes advances in measurement

technology for glycated proteins necessary. It has been suggested that variations in the level of the deglycating enzyme fructosamine-3-kinase, with its genetic locus, play a role in glycation gap variations [52].

The uneven distribution of the non-diabetic and type 2 diabetes mellitus patients and the low number of type 2 diabetes patients in the negative glycation gap categories may have led to underestimates of the below zero categories in such patients. It also allows us to assume that HbA1c [53] remains a more reliable risk predictor than glycation gap alone [53, 54].

Alcohol consumption above one drink (10 g alcohol) daily was an exclusion criterion for this study. Hence, the effects of downstream sequelae of hepatic insulin resistance-implicating lipid species such as triglyceride, inflammatory signalling or other modifications [55] on the homeostasis model assessment for insulin resistance here observed cannot be excluded.

In conclusion, appreciation of the uneven chemical protein-based glycoconjugation of N-terminal glycation during senescence and along different degrees of glucose metabolism impairment remains in focus thanks to improved laboratory technology. It remains inappropriate, from the data presented here, to propose glycation gap measurements for clinical care, although glycation gap and HbA1c measurements point to a difference between HbA1c and other measures of mean glycaemia [20]. This confers on the glycation gap and its calculation a meaning regarding the appreciation of the homoeostasis of glucose metabolism. New markers for metabolic abnormalities now include the glycation gap, and studying the extent of glycation, up to advanced glycation end products, might help to distinguish biological from chronological aging [56–58].

Potential competing interests
None of the co-authors have any conflicts of interest to declare.

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