Serum levels of advanced glycation end-products (AGEs) and the decoy soluble receptor for AGEs (sRAGE) can discriminate non-alcoholic fatty liver disease in age-, sex- and BMI-matched normo-glycemic adults

Short running title: glycation products, sRAGE and NAFLD pathogenesis

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19 Abbreviations

AGEs	advance glycation end products
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
BMI	body mass index
CEL	N^{ε}-carboxyethyl-L-lysine
CML	N^{ε}-carboxymethyl-L-lysine
CML-d_2	N^{ε}-carboxy[2H2]methyl-L-lysine
CV	coefficient of variation
CVD	Cardiovascular disease
DMF	1-deoxy-1-morpholinofructose
ELISA	enzyme-linked immunosorbent assay
esRAGE	endogenous secretory receptors of advance glycation end products
FFA	free fatty acid
HOMA-IR	Homeostasis Model of Assessment-Insulin Resistance
HPLC-MS	high-performance liquid chromatography
hsCRP	high sensitive C reactive protein
IL-6	interleukin 6
IL-8	interleukin 8
IR	Insulin resistance
LC-MS	liquid chromatography-mass spectometry
LSM	Liver stiffness measurements
m30	caspase-cleaved cytokeratin 18 fragment
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBT	nitroblue tetrazolium
NFPA	Nanofluoropentanoic acid
OR	odds ratio
PBS	phosphate buffer saline
PDA	photodiode array
RAGE	receptors of advance glycation end products
ROC	receiver operating characteristic
ROS	reactive oxygen species
sRAGE	soluble receptors of advance glycation end products
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor alfa
γGT	glutamyl transpeptidase

Abstract

Background

Non-alcoholic fatty liver disease (NAFLD) is a serious health problem affecting ~25% of the global population. While NAFLD pathogenesis is still unclear, multiple NAFLD parameters, including reduced insulin sensitivity, impaired glucose metabolism and increased oxidative stress are hypothesised to foster the formation of advance glycation end-products (AGEs). Given the link of AGEs with end organ damage, there is scope to examine the role of the AGE/RAGE axis activation in liver injury and NAFLD.

Methods

Age, sex and body mass index matched normo-glycemic NAFLD adults (n=58) and healthy controls (n=58) were enrolled in the study. AGEs were analysed by liquid chromatography-mass spectrometry (CML, CEL), fluorescence (pentosidine, AGE fluorescence), colorimetry (fructosamine) and ELISA (sRAGE). Their association with liver function, inflammation, fibrosis and stage of NAFLD was examined.

Results

Early and advanced glycation end-products, except N^{ε}-carboxymethyl-L-lysine (CML), were 10-30% higher, sRAGE levels 1.7-fold lower, and glycation/sRAGE ratios 4-fold higher in the NAFLD cases compared to controls. While AGEs presented weak to moderate correlations with indices of liver function and damage (AST/ALT, HOMA-IR, TNF-α and TGF-β1), including sRAGE to characterize the AGEs/sRAGE axis strengthened the associations observed. High glycation/sRAGE ratios were associated with 1.3 to 14-fold likelihood of lower AST/ALT ratios. The sum of AGEs/sRAGE ratios accurately distinguished between healthy controls and NAFLD patients (area under the curve of 0.85). Elevated AGEs/sRAGE (>7.8mmol/pmol) was associated with a 12-fold likelihood of the presence of NAFLD.

Conclusion

These findings strengthen the involvement of AGEs-RAGE axis in liver injury and the pathogenesis of NAFLD.

Keywords

NAFLD, glycation, biomarker, liver, sRAGE, CML

1.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) results from fat accumulation in the liver (fat>5% of liver weight), for reasons other than excess alcohol consumption [1]. NAFLD, the most common cause of chronic liver disease in Western countries, covers a spectrum of liver damage from simple fatty liver to non-alcoholic steatohepatitis [2] and cirrhosis [3]. Affecting ~25% of the general population, NAFLD is expected to become the next global epidemic as its pathogenesis is closely linked to obesity and metabolic syndrome [4, 5].

Insulin resistance (IR) is a prevailing factor in the complex and still-unclear disease pathogenesis [6]. Lipid accumulation in the liver, followed by increased oxidative stress and cytokine levels is proposed to lead to necro-inflammation, fibrosis and cirrhosis (two-hit model). Alternatively, multiple insults, such as insulin resistance, free fatty acid (FFA) flux, oxidative and cytokine-induced stress, adipocytokine imbalance and bacteria toxins could act in parallel to induce progressive damage leading to steatosis (multiple-hit model) [7, 8].

Reduced insulin sensitivity and impaired glucose and lipid metabolism [9] favour advance glycation end-products (AGEs) formation, contributing to liver damage [10]. AGEs are formed by the non-enzymatic reaction of a reducing sugar or oxidized lipid with an amino acid, resulting in alteration in the structure and function of proteins [11]. N^{ε}-carboxyethyl-L-lysine (CEL), N^{ε}-carboxymethyl-L-lysine (CML), and pentosidine are the most common and best characterized AGEs used as biomarkers of disease progression [12, 13]. AGEs exert their effect by binding to the AGEs receptors (RAGE) [11]. The AGE/RAGE interaction induces the activation of several intracellular pathways and further synthesis of cytokines [13, 14]. Upregulation of these biological processes lead to further inflammation, reactive oxygen species (ROS) and IR, promoting further AGEs production and in the case of liver fibrosis [15, 16]. While in circulation, AGEs may also bind with the soluble variants of the receptor (sRAGE and esRAGE) which act as scavengers leading to AGEs elimination and prevention of the AGE/RAGE axis activation [11].

In vitro and *in vivo* studies have strengthened the hypothesis that high AGEs levels (endogenous formation and exogenous intake from meat, fat and highly processed food [17, 18]) and AGEs/RAGE axis activation lead to oxidative stress and hepatic inflammation [10]. Lower levels of sRAGE levels are also present in hyperglycemic [19] and hypertensive [20] subjects with IR and components of the metabolic syndrome [21] compared to non-diabetic normotensive subjects. On the contrary, Type1/2 Diabetes Mellitus subjects with renal disease have higher sRAGE levels compare to healthy controls [22]. AGEs and/or sRAGE levels (in isolation) and AGEs/sRAGE ratios have been proposed as potential novel biomarkers for end

organ damage [22-24], but the relationship between sRAGE levels and glycaemic control / insulin resistance remains unclear [25].

To-date, there is a lack of human studies investigating the role of AGEs and (e)sRAGE in the pathogenesis of NAFLD. The aim of the present study was to simultaneously examine, for the first time, early (fructosamine) and advanced glycation products (CML, CEL and pentosidine), as well as sRAGE levels and their respective ratios as proxies for liver injury/damage in a NAFLD case-control study of normo-glycemic adults.

1.2 Material and methods

1.2.1 Study population

Briefly, 58 adults with recent NAFLD diagnosis (based on elevated liver enzymes levels, ultrasound hepatic steatosis evidence and exclusion of any other liver injury) and 58 healthy adults matched for age, sex and BMI with the aforementioned cases were recruited [26, 27]. Patients were excluded if following a weight-loss diet, had changed dietary habits after diagnosis, had type 1 or 2 diabetes mellitus or any malignancy. The study was approved by the Ethics Committees of the Hippokration General Hospital of Athens and Harokopio University and was executed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

Medical records and anthropometric measurements, along with other variables previously described [26-28], were recorded for each participant. Liver stiffness measurements (LSM) by transient elastography (FibroScan®, Echosens, France) were available in 45 of the 58 NAFLD patients, and liver biopsies were available for 22 of the NAFLD subjects. Patients were classified as having simple liver steatosis or NASH, based on the liver injury pattern and the criteria of Brunt et al.[29], modified by Kleiner et al [30].

1.2.2 Biochemical markers

12-h fasting blood was collected from all subjects. Glucose was measured colorimetrically (Cobas 8000, Roche), insulin by chemiluminescence (Centaur analyzer, Siemens) and HOMA-IR (Homeostasis Model of Assessment-Insulin Resistance) was calculated [31]. Inflammatory biomarkers (TNF-α, Il-6, IL-8, adiponectin) were measured by enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA) and High sensitive CRP (hsCRP) using a nephelometric assay (BN II® nephelometer, Siemens). Markers of apoptosis, fas ligand and

caspase-cleaved cytokeratin 18 fragment (m30) were included as non-invasive biomarkers for NAFLD diagnosis. Liver enzymes; aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (γGT), were obtained from patients' medical records or analyzed using routine commercial assays [32].

1.2.3 Chemicals and reagents

CML, CEL, pentosidine and CML-d_2 (HPLC-grade) were from PolyPeptide Laboratories France. Nanofluoropentanoic acid (NFPA), nitroblue tetrazolium, 1-deoxy-1-morpholinofructose (DMF), sodium borohydride, sodium tetraborate, boric acid, sodium carbonate, trichloroacetic acid, hydrochloric acid and PBS were purchased from Sigma Aldrich. Acetonitrile and water were HPLC-grade from VWR International.

1.2.4 Glycation biomarkers

1.2.4.1 Fructosamine (NBT Assay)

Fructosamine was analyzed using the modified NBT assay by Vlassopoulos et al. [33]. Serum (12µL, duplicates) was added to sodium carbonate buffer (120µL, 0.375M) and nitroblue tetrazolium (120µL, 1.2mM). After incubation (10 and 15min, 37^{o}C), absorbencies were measured at 550nm (Multiskan Spectrum v1.2, Thermo Scientific). DMF was used as a standard (0–2mM). The method presented a limit of quantification of 0.04mM, with 2.45 and 0.74% coefficient of variation (CV) for repeatability and reproducibility.

1.2.4.2 AGE fluorescence

Serum (25µL, duplicates) was diluted in PBS (100µL) and measured fluorometrically ($\lambda_{emission}$=370 nm, $\lambda_{excitation}$=440nm, SpectraMax M2e, SoftMax®Pro software). The repeatability and reproducibility of the method were CV 3.7% and CV 1.2%, respectively.

1.2.4.3 sRAGE

sRAGE levels were determined using a commercial ELISA kit (RayBio® Human RAGE, Tebu-bio, UK) specific for human RAGE (MOK protein kinase, Gene ID: 5891). Duplicate samples (diluted 1:5 in PBS) and RAGE standards (0–1.5ng/mL) absorbencies were read at 450nm (Multiskan Spectrum v1.2, Thermo Scientific). The limit of detection of the kit was 3pg/mL (2SD of the blank). Repeatability and reproducibility presented a CV<10%.

1.2.4.4 AGEs quantification by HPLC-fluorescence-MS

1.2.4.4.1 Serum treatment

Serum was prepared as previously described [34]: 600µL of 100mM sodium borohydride dissolved in 200mM borate buffer (pH 9.2) was added to the serum (25µL, 1:5 in water). Proteins were precipitated with trichloroacetic acid (2mL, 200mg/mL). After centrifugation (10min, 2000g), the protein pellet was washed with trichloroacetic acid (1mL, 100mg/mL) and re-centrifuged (10min, 2000g). Hydrochloric acid (400µL, 6M) was added to the pellet, followed by hydrolysis (20h, 110°C), before evaporation to dryness (80°C, under nitrogen). The residue was resuspended in NFPA (500µL, 5mM), and filtered (4mm, 0.22µm PVDF) before HPLC-fluorescence-MS analysis.

1.2.4.4.2 Quality assessment

Spiked serum controls were 0.1µmol/L (low), 1.0µmol/L (medium), and 10µmol/L (high) and CML, CEL and pentosidine standards ranged from 0.01-2.0µmol/L in 5mmol/L NFPA in water. All analysis included a glassware control and reactive control samples. Samples were spiked with the internal standard (CML-d_2, final concentration 1.0µmol/L). The method was assessed for linearity (0–2mmol/L, r ≥.99) and recovery (0.1, 1 and 10µmol/L). The LOQs (signal-to-noise of 10) of CML, CEL and pentosidine were 0.01, 0.015 and 0.005 µmol/L, respectively. Recoveries were 79–115% and relative errors were below 15%. Inter- and intra-variation were below 13%.

1.2.4.4.3 CML & CEL

Analysis was carried out on a Thermo Scientific Exactive™ Plus Orbitrap LCMS Mass Spectrometer, with photodiode array UV –detector coupled to an Orbitrap™ MS analyser; using the Thermo Xcalibur software version Exactive Plus Tune 2.1 (Thermo Scientific Inc, Waltham, MA USA).

Glycated products (10µL) were separated using a Hypersil GOLD C_{18} reversed phase (1.9µm) column (2.1mm id X 100mm column) at 30°C. The mobile phase included H_2O (eluent A) and acetonitrile (eluent B); both with 5mmol/L NFPA, at the following gradient: 0–7min: 10–18% B, 7–9min: 18–50% B, 9–12min: 50–80 % B, 12–13min: 80–100% B, 13–15min: 100% B (0.3mL/min), 15–17min: 100–10% B, and 3min of equilibrium with a flow rate of 0.2mL/min. Mass spectrometry detection was performed using an electron spray ionization interface in the positive ion mode. The ions were analyzed using a scan from 160 to 384m/z (1000msec) with a capillary voltage of 42.5V at 380°C.

1.2.4.4.4 Pentosidine

Pentosidine analysis was carried out on a Thermo Finnigan Surveyor, photodiode array (PDA) detector, coupled to a spectrofluorometer (Jasco FP-920, Jasco Benelux, Maarssen, The Netherlands); using the Thermo Xcalibur software version 1.4 SR1 (Thermo Scientific Inc, Waltham, MA USA). Pentosidine (80µL) was separated on a Synergi MAX-RP 80 Å column (250 x 4.6mm, 4µm) at 30°C. The mobile phase included H_2O (solvent A) and acetonitrile (solvent B); both with 5mmol/L NFPA, at the following gradient: 0–8min: 10–20% B, 8–10min: 20–60% B, 10–13min: 60–94% B, 13–14min: 94–100% B, 14–20min: 100% B (flow rate: 1400mL/min), 20–23min: 100–10% B, and 5min of equilibrium, flow rate of 1mL/min. Fluorometric detection was set at excitation and emission wavelength of 335 and 385nm, respectively.

1.2.4.4.5 AGEs/sRAGE ratio

AGEs levels were divided by sRAGE levels to create a ratio (mmol of AGEs per pmol of sRAGE) that considers AGEs/RAGE interaction in disease previously suggested by others [22-24].

1.2.5 Statistical analysis

Data are presented as mean (SD) for continuous variables, and as frequencies for categorical variables. Data normality was evaluated using the Shapiro-Wilk test. Differences between cases and controls (including levels of AGEs and sRAGE among the stage of the disease; simple liver steatosis and NASH) were determined by Student's t-test (parametric), Mann-Whitney U Test (non-parametric) or chi-squared test (categorical variables). Spearman's or Pearson's correlation coefficients were used to assessing the univariate correlations of AGEs with liver function and inflammation biomarkers. Multinominal logistic regression analysis was used to evaluate the independent contribution of the glycation products/sRAGE axis (as untransformed continuous variable) with liver injury described as AST/ALT (categorical variable). The AST/ALT levels were divided into quartiles (with the highest quartile, >1.72, as the reference group). The independent associations of glycation products/sRAGE with AST/ALT were examined without adjustment (model 1), adjusted for age, abdominal fat level and gender (models 2-3), IL-6 and hsCRP (model 3).

To assess the usefulness of glycation biomarkers in differentiating between healthy controls and NAFLD cases, cut-off values, sensitivity and specificity for each parameter were calculated followed by the construction of receiver operating characteristic (ROC) curve (plotting the sensitivity and reverse specificity at each value) [35]. Binomial logistic regression

analysis was performed to estimate the association between glycation biomarkers (binary variable, according to the cut-off values) and presence of NAFLD (binary variable) adjusted by age, sex, abdominal fat level (modes 1), AST/ALT, γGT, HOMA-IR (model 2), IL-6 and TNF- α (model 3). Additionally, binomial logistic regression analysis was performed to estimate the association of glycation biomarkers (as untransformed continuous variable) with the presence of NASH (vs simple steatosis, binary variable) and fibrosis (defined as LSM>6.6kPa cut-off, binary variable) unadjusted (model 1) and adjusted by age and AST/ALT, (model 2). Reported *p*-values were based on two-sided tests, α=0.05. The SPSS software (IBM® SPSS® Statistics Version 22, 2013, U.S.A) was used.

1.3 Results

1.3.1 General characteristics of the subjects

Subjects were aged 45±12 years, with a BMI of 28.2±3.8kg/m^2, a waist circumference of 98±9.0cm (Table 1). Approximately half (46%) of subjects had increased waist circumference (women >88 cm and men >102 cm).

Table 1. General characteristics of the 58 cases with non-alcoholic fatty liver disease (NAFLD) and their matched healthy controls.

	Cases (n=58)	Controls (n=58)	p value
Gender – female, n (%)	22 (37.9)	22 (37.9)	0.99
Age (years)	45 (12)	45 (12)	0.96
BMI (kg/m^2)	28.7 (4.0)	27.7 (3.6)	0.14
Female	30.8 (5.0)	29.1 (4.6)	0.25
Male	27.5 (2.5)	26.8 (2.5)	0.26
Waist circumference (cm)			
Female	101.0 (10.5)	95.3 (11.0)	0.09
Male	99.1 (7.2)	96.6 (8.0)	0.18
Abdominal fat level (%)	15.0 (4.4)	12.5 (5.1)	0.008
Insulin (IU/mL)	12.5 (8.5 – 16.0)	7.0 (5.0 – 10.0)	<0.001
Glucose (mmol/L)	5.0 (0.7)	4.8 (0.6)	0.07
HOMA-IR [a]	3.1 (2.2)	1.9 (1.5)	<0.001

Data are presented as means (SD), frequencies or median (interquartile range). Differences between cases and their matched controls were observed using X^2 test, 2-samples *t* test or Mann-Whitney U Test.
Abdominal fat levels were assessed by abdominal bioelectrical impedance analysis. BMI, body mass index; HOMA-IR, Homeostasis Model of Assessment of insulin resistance

1.3.2 Glycation biomarkers

Early (fructosamine) and advanced glycation markers (CEL, pentosidine and AGE fluorescence) levels were higher in cases compared to controls (Table 2). A notable exception was CML which

remained similar between groups. Meanwhile, sRAGE serum levels were 1.7-fold lower in cases compared to controls. These differences were maintained when comparing those cases with available biopsy (n=22) and matched healthy controls (n= 22, Supplement 2). From a pathophysiological perspective, there is scope to study the amount of 'free' CML and CEL that could interact with RAGE and activate the AGE/RAGE axis. Moreover, calculating the relative concentration of AGEs to their scavenger sRAGE has been proposed as a proxy of 'free' AGEs. In this study, the CML/sRAGE, CEL/sRAGE and AGEs/sRAGE ratios were four times higher in cases compared to controls (Table 2). Among the 22 NAFLD cases with available biopsies, the serum levels of glycation did not differ according to the grade of necro-inflammation and stage of fibrosis (Brunt [29], Supplement 3 and Supplement 4, respectively), as well as the severity of steatosis according to Kleiner [30] (Supplement 5).

Table 2. Glycation biomarkers levels in the 58 patients with non-alcoholic fatty liver disease (NAFLD) and their matched healthy controls.

	Cases (n=58)	Controls (n=58)	p-value
Fructosamine (mM DMF)	1.1 (0.2)	0.9 (0.2)	<0.001
AGE fluo (AU)[a]	501.4 (411.6 – 595.4)	412.2 (375.2 – 443.2)	<0.001
AGEs (mmol/mol)	135.2 (34.7)	105.3 (29.5)	<0.001
CML (mmol/mol)	10.6 (3.8)	9.9 (2.5)	0.26
CEL (mmol/mol)	122.9 (34.7)	93.9 (30.7)	<0.001
Pentosidine (mmol/mol)[a]	1.6 (1.5 – 1.9)	1.5 (1.4 – 1.7)	0.02
sRAGE (pg/L)[a]	363.0 (252.3 – 513.1)	630.7 (509.8 – 513.1	<0.001
CML/sRAGE (mmol/pmol)	2.5 (5.4)	0.6 (0.3)	0.01
CEL/sRAGE (mmol/pmol)	22.1 (29.2)	5.5 (2.8)	<0.001
AGEs/sRAGE (mmol/pmol)	25.0 (34.3)	6.2 (3.0)	<0.001

Data are presented as means (SD) or [a] median (interquartile range). Differences between cases and their matched controls (match by age, sex and BMI) were observed using 2-samples t test or Mann-Whitney U Test. AGEs: advance glycation end products, CEL: N^{ε}-carboxyethyl-L-lysine, CML: N^{ε}-carboxymethyl-L-lysine, sRAGE: AGEs soluble receptor.

1.3.3 Glycation, hepatic function, inflammation and damage

To explore the role of the AGE-RAGE axis in liver injury, glycation products and their associations with selected liver function, liver damage and inflammation biomarkers in the whole sample of cases and controls (N=116) are presented in Table 3 (and Supplementary Figures 1-9). The association between glycation products and biomarkers among cases only and controls only are presented in Supplements 6 and 7, respectively. Biochemical markers have been previously described [36] and are shown in Supplement 1. No associations were detected between CML and any of the markers studied. Serum levels of all glycation markers, except

CML and AGE fluorescence, were weakly to moderately associated with HOMA-IR; the same was true for AGEs/sRAGE ratios (Table 3).

All glycation markers, but CML, were weakly to moderately positively associated with the transaminase ALT, but not the transaminase AST (weak association with fructosamine and AGE fluorescence) or the transpeptidase γGT (weak association with the sum of the three AGE markers). Stronger negative associations were seen for sRAGE and the AGE/RAGE ratios. Using the AST/ALT liver damage ratio, associations were maintained for all glycation biomarkers (except CML) and all AGEs/sRAGE ratios. However, neither glycation markers, nor sRAGE nor AGE/RAGE ratios did correlate with the non-invasive markers of apoptosis for NASH, fas and m30 (data not shown). Only fructosamine and AGE fluorescence did correlate with liver stiffness (r=-0.32, p=0.04 and r=-0.39, p=0.01, respectively). Early and advanced glycation products were weakly associated to the growth factor TGF-β1 (Table 3) but not with VEGF (data not presented).

Table 3. Spearman (rho) correlations between glycated products and sRAGE, and liver function and inflammation biomarkers in the 116 subjects.

	ALT	AST	AST/ALT	γGT	HOMA-IR	TGF-β1	TNF-α	IL-6	hsCRP
AGEs (sum of)	0.28**	0.16	-0.28**	0.21*	0.26**	0.26**	0.05	0.00	0.06
CML	0.10	0.08	-0.06	0.10	-0.02	0.00	0.05	0.04	-0.15
CEL	0.25**	0.14	-0.20*	0.18	0.26**	0.25**	0.04	-0.07	0.06
Pentosidine	0.20*	0.15	-0.20*	0.12	0.21*	0.05	0.17	-0.13	0.03
Fructosamine	0.33**	0.23*	-0.30**	0.17	0.21*	0.22*	0.02*	-0.21*	-0.12
AGE fluorescence	0.38**	0.20*	-0.32**	0.35**	0.18	0.23*	0.37**	-0.07	0.09
sRAGE	-0.40**	-0.16	0.39**	-0.44**	-0.28**	-0.16	-0.29**	0.01	-0.24*
CML/sRAGE	0.40**	0.19	-0.41**	0.44**	0.20*	0.18	0.27**	0.04	0.17
CEL/sRAGE	0.42**	0.18	-0.45**	0.43**	0.33**	0.26**	0.25**	0.02	0.21*
AGEs/sRAGE	0.43**	0.18	-0.47**	0.44**	0.33**	0.27**	0.25**	0.04	0.21*

*p < 0.05, ** p < 0.01; AGEs,advance glycation end products; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CEL, N^{ε}-carboxyethyl-L-lysine; CML, N^{ε}-carboxymethyl-L-lysine; γGT, γ-glutamyl transpeptidase; HOMA-IR, (log) Homeostasis Model of Assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; IL-6: interleukin 6; sRAGE, AGEs soluble receptor; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor-α.

Associations with inflammatory markers TNF-α, IL6 and hs-CRP were inexistent for glycation products (and weak for fructosamine and AGE fluorescence). Only weak positive associations were observed between sRAGE and TNF-α and hs-CRP (but not IL-6). Calculating glycation products/sRAGE ratios (except CML/sRAGE) also revealed weak positive associations with TNF-α but not hs-CRP (Table 3). No associations were observed for IL-8 or adiponectin levels (data not shown). While lower sRAGE levels alone were not associated with higher level of liver injury

(TGF-β1 and IL-6), the relative ratio of glycation products to sRAGE were. When the aforementioned correlations were carried-out for the cases and controls, separately, only correlations between fructosamine and IL-6 (r=-0.41, p=0.002) and AGEs/sRAGE and TNF-α (r=0.28, p=0.048) persisted in cases (Supplement 6). Among controls only (Supplement 7), AGEs correlated with ALT (r=-0.45, p=0.001), AST (r=-0.43, p=0.001), TNF-α (r=-0.38, p=0.005) and TGF-β1 levels (r=0.36, p=0.007).

Glycation products/sRAGE were evaluated to predict liver injury using the simple predictive model AST/ALT ratio. Having higher levels of any of the AGEs/sRAGE, CEL/sRAGE and AGE fluorescence/sRAGE ratios increased the likelihood of having liver injury (low AST/ALT ratios). For every unit increase, there was a 27-33% increased likelihood of being in the lower quartiles of AST/ALT (higher liver injury) (OR from 1.27, CI 1.1–1.47 to 1.33, CI 1.15–1.55). Surprisingly, this relationship was much stronger for every unit increase of CML/sRAGE, with a 14-fold increase in the likelihood of having liver injury (low AST/ALT) (OR from 13.28, CI 2.35–75.2 to 14.27, CI 2.52–80.77). These associations were maintained/strengthened, especially for the CML/RAGE ratio after adjustment for age, abdominal fat, gender, IL-6 and hs-CRP (models 2 & 3, Supplement 8).

1.3.4 Association of glycation products and likelihood of presenting NAFLD

NAFLD diagnosis and its stages (NASH and simple steatosis) was used as a clinical manifestation of liver injury. Glycation markers were assessed for their capacity to discriminate between controls and cases using ROC curves (Figure 1). CML, CEL, pentosidine and sRAGE presented a poor to fair ability to discriminate with an area under the ROC curve (AUC) below 0.78. The use of AGE-RAGE ratios (except CML/sRAGE), however, was better able to distinguish between case and controls: CEL/sRAGE and AGEs/sRAGE with an AUC of 0.85, and AGE fluorescence/sRAGE with an AUC of 0.83 - considered to present a “good” ability.

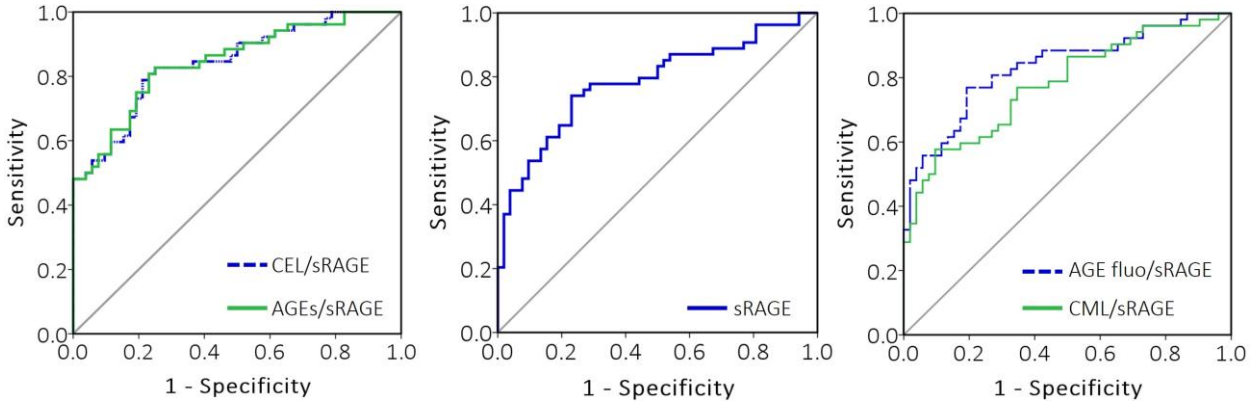

Figure 1. Receiver operating characteristic (ROC) curve for CEL/sRAGE (AUC= 0.844), AGEs/sRAGE (AUC= 0.845), sRAGE (AUC= 0.783), AGE fluorescence/sRAGE (AUC= 0.832) and CML/sRAGE (AUC= 0.779) to discriminate between NALFD patients and healthy controls.

Cut-off values were selected at levels where glycation products yield sensitivities higher than 75% while the corresponding specificities were maintained above 70%. Using a cut-off level of >7.8mmol/pmol for AGEs/sRAGE levels, a sensitivity and specificity of 81% and 77% were yielded. The same accuracy was observed for CEL/sRAGE with a cut-off level of >6.9mmol/pmol. The cut-off for sRAGE was set as <524pg/mL, to provide a sensitivity and specificity of 78% and 71%, respectively. Using a cut-off of >87.4 AU/ng for AGE fluorescence/sRAGE achieved a sensitivity of 80% and a specificity of 79%. Using the cut-off values presented above, the odds ratio (OR) of having NAFLD was calculated adjusting for age, gender, abdominal fat mass, HOMA-IR, AST, γGT, TNF-α and IL-6 (Table 4). Increased likelihood of having NAFLD were seen for sRAGE levels below the cut-off (~1.6-fold), AGEs/sRAGE (11-fold), CEL/sRAGE (~10-fold), and AGE fluorescence/sRAGE (11-fold) above the cut-off. However, the likelihood of having NAFLD was not mantained for sRAGE and AGE fluorescence/sRAGE after adjusting for hepatic function and inflammatory biomarkers.

Table 4. Logistic regression analysis models, exploring the association between cut-offs of glycated products/sRAGE ratio, sRAGE, and the likelihood of the presence of NAFLD (N= 58 cases and 58 controls).

	Model 1[a]			Model 2[b]			Model 3[c]		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
sRAGE <524 pg/mL	1.57	1.04-2.62	<0.001	1.05	052-2.62	0.25	0.52	0.05-2.10	0.33
AGEs/sRAGE >7.8 mmol/pmol	11.00	9.27-13	0.03	4.67	3.34-6.37	0.03	4.94	3.40-7.18	0.04
CEL/sRAGE >6.9 mmol/pmol	9.98	8.3-12	<0.001	4.32	3.07-6.07	0.03	4.65	3.05-7.07	0.04
AGE fluorescence/sRAGE (>87.4 AU/ng)	11.31	10-12.8	<0.001	10.37	0.16-12.53	0.08	10.23	0.17-12.21	0.08

[a] Model 1: adjusted for age, sex and abdominal fat level.
[b] Model 2: adjusted for age, sex, abdominal fat level, AST/ALT, HOMA-IR and γGT.
[c] Model 3: adjusted for age, sex, abdominal fat level, AST/ALT, HOMA-IR, γGT, IL-6 and TNF- α levels.
ALT: AST: aspartate aminotransferase, NAFLD: non-alcoholic fatty liver disease, OR: Odds Ratio, CI: Confidence Interval, CEL: N^{ε}-carboxyethyl-L-lysine, AGEs: N^{ε}-carboxymethyl-L-lysine, CEL and pentosidine advance glycation end products, sRAGE: AGEs soluble receptor, HOMA-IR: Homeostasis Model of Assessment of insulin resistance, IL-6: interleukin 6, γGT: γ-glutamyl transpeptidase, TNF-α: Tumor necrosis factor-α.

Glycation markers were assessed for their capacity to discriminate between simple steatosis and NASH using ROC curves in the sub-sample of patients with biopsy and glycation measurement (n=22). In this small sample, CML, CEL, pentosidine and AGE fluorescence were considered as a non-valid instrument to discriminate between simple steatosis and NASH (AUC <0.60). Fructosamine, sRAGE, CML/sRAGE and CEL/sRAGE presented a "poor" ability to discriminate between simple steatosis and NASH (AUC=0.60-0.67). However, AGE fluorescence/sRAGE (AUC= 0.73) was considered to present a "fair" ability (AUC=0.73). Hence, no threshold values could be calculated for NASH and simple steatosis. To predict the likelihood of NASH vs simple steatosis, the ORs were calculated using glycation products/sRAGE levels as untransformed continuous variables (unadjusted and after adjustment for age and AST/ALT). None of the glycation biomarkers predicted the likelihood of having NASH, compared to simple steatosis (Supplement 9). Additionally, none of the markers or ratios did predict the likelihood of having fibrosis (defined as LSM>6.6kPa cut-off, Supplement 10).

1.4 Discussion

AGEs can exert pathological effects extracellularly, by inducing modifications of proteins, and intracellularly, by binding to the AGEs receptors (RAGE) [11]. The AGE/RAGE axis activates a positive feedback loop, which in turn lead to a cascade of events from chemotaxis, oxidative

stress and inflammation to cell dysfunction, fibrosis and apoptosis, ultimately manifesting as end-organ damage [13, 14]. Conversely, the decoy receptors sRAGE and esRAGE offer protection by preventing AGEs/RAGE interaction and removal of AGE-modified products [11]. Therefore, evaluating together glycation products with their decoy receptor sRAGE is an important strategy to better understand the development and progression of NAFLD.

To the best of our knowledge, this is the first study to simultaneously explore the role of multiple glycation biomarkers (including early and advanced products) and their receptors (sRAGE) in NAFLD normo-glycemic patients as components of the multiple hits model of NAFLD, and the ratio of glycation products/sRAGE as a proxy for AGE/RAGE axis activation. An important finding was that the combination of AGEs with their scavenger receptor (sRAGE) as ratios made the associations between glycation markers and biochemical parameters of liver injury stronger than studying AGEs alone. Early and advanced glycation end-products (except CML) were 10-30% higher and CML/sRAGE, CEL/sRAGE and AGEs/sRAGE ratios 4-times higher in NAFLD cases compared to controls. However, ratios did not differ according to the presence of fibrosis or the stage of NAFLD (different levels of liver injury).

CML is the most studied glycation product and is commonly used as a sole proxy for all AGEs. AGEs are formed under different pathways: CML is formed by the oxidative degradation of fructosamine, CEL can derive from a broad range of precursors (such as methylglyoxal), and pentosidine is derived from the glycation and oxidation of lysine and arginine. AGEs involvement in disease pathogenesis may also differ with CEL and CML acting through interaction with RAGE [37], while pentosidine and AGE fluorescence products acting through their cross-linking properties [37]. Our findings agree with the lack of difference observed between CML levels and hepatic impairment (with simple liver steatosis or NASH, and with NAFLD or normal liver function) in patients and age-, sex- and BMI-matched controls [38-41].

The lower serum sRAGE levels in NAFLD cases compared to controls observed in this study are in agreement with previous reports. In cross-sectional studies of NAFLD cases, esRAGE [42] (57 NAFLD subjects vs 14 controls) and sRAGE [43] (60 cases with familial combined hyperlipidemia and/or metabolic syndrome vs 50 controls) levels were 40% lower in cases with NAFLD (esRAGE= 663pg/mL and sRAGE= 1065pg/mL), compared to control subjects (esRAGE= 897 pg/mL and sRAGE= 1480pg/mL). Similar to our findings, sRAGE levels were not different between the stages of NAFLD. In obese prepuberal children [44] (n=140, aged 6-10y), lower esRAGE and sRAGE were observed in individuals with liver steatosis compared to healthy controls (esRAGE, 790 vs 1500pg/ml; sRAGE, 1000 vs. 1350pg/mL).

Accurate noninvasive diagnosis for NAFLD (and stages) is of great relevance to identified patients at higher risk of liver-related morbidity and mortality [45]. AGEs/sRAGE ratios give an indication of the relative proportion of AGEs not binded to the decoy receptors and hence available to stimulate the intracellular events by AGE/RAGE axis activation. For instance, sRAGE (and esRAGE) are reported to be reduced in certain diseases (such as coronary artery disease) and elevated in others (such as diabetes and renal impairment) [22]. Similarly, CML levels are lower as BMI, waist circumference and body fat mass increase [38-41]. In this study, CML/sRAGE, CEL/sRAGE and AGEs/sRAGE ratios were used as proxies of the free glycation fraction able to elicit further molecular events. We observed higher ratios among cases compared to controls, without detectable differences between simple liver steatosis and NASH. Higher ratios of glycated products/sRAGE were associated with increased liver injury (lower AST/ALT ratios) in the ordinal regression models, even after adjustment for age, abdominal fat and inflammation. AGEs/sRAGE and CEL/sRAGE ratio presented a good ability to discriminate NAFLD from a normal liver function, as shown by ROC curves (AUC=0.85). Considering that AGEs quantification by HPLC-MS is not always possible, and adipose tissue may be a preferred tissue to measure CML as opposed to plasma, a simple fluorescence analysis to serum and a sRAGE measurement via ELISA presented similar capacity to detect liver damage and inflammation to their more sophisticated AGEs/sRAGE ratio. In fact, AGE fluorescence/sRAGE ratio analysis presented an AUC similar to CEL/sRAGE and AGEs/sRAGE. Due to its metabolic pathway AGE fluorescence could also indicate levels of cross-linked products formation which are more likely to represent liver damage and inflammation. Glycation products other than CML should be considered since obesity synergistically with metabolic syndrome are associated with 7-10 fold increased risk of hepatic steatosis and fibrosis [46, 47]. However, AGE fluorescence is a non-specific method where any fluorescent adduct could be interfering in the analysis. Also, sRAGE levels should be interpreted accordingly with the physiological conditions (i.e. glycemic levels and renal function).

Although histological samples are not always available, analysis of AGE and sRAGE with biopsies remain of interest. *In vitro* and animal models have shown that as AGEs accumulates in the liver, inflammation occurs followed by macro- and micro- vesicular steatosis. However, when AGE/RAGE interaction is blocked, the pro-inflammatory environment is suppressed [48-50]. This highlights the relevance of AGEs/RAGE interaction in NAFLD progression. From the 22 biopsies that were available from the NAFLD cases in this study, AGEs/sRAGE, CEL/sRAGE, sRAGE and AGE fluorescence/sRAGE were not associated with the likelihood of having simple liver steatosis or NASH. However, the small number of biopsies limited our study to fully

explore the relationship between glycation products and the presence of NASH. Limited number of studies, with 8 to 74 participants, have evaluated serum CML and sRAGE levels according to stages of NAFLD, with no differences observed between simple steatosis, borderline NASH and define NASH [38, 43, 50]. However, 30-50% higher CML levels in the liver have been observed in obese subjects (n=74) with moderate-severe steatosis and lobular inflammation (stage 1-3) compared to those with low grade of steatosis and no inflammation (stage 0) [50]. So far, there is no validated non-invasive biomarkers or predictive model to detect or distinguish NASH [51]. The marker of apoptosis, m30, is considered as promising non-invasive biomarkers to predict the presence of NASH (sensitivity of 60-91% and specificity of 77-96%). However the cut-off levels of m30 vary between studies (121-380 U/L) [52]. Different predictive models of NASH have been developed such as the HAIR score [53] and NASH test [54]. However, validation is needed for both non-invasive biomarkers and predicted models for clinical practices.

In the present study, we also evaluated the association between AGEs/sRAGE and downstream RAGE inflammatory regulation (possibly via reduction of intracellular activation) [55]. Notable aspects include the absence of association between CML and any of the markers considered, the weak to inexistent relationships between inflammation markers IL-6 and hs-CRP with the glycation markers studied (possibly due to the exogenous origin of CML and/or deposition in the adipose tissue, reducing our ability to detect an effect). Stronger associations were seen between sRAGE and the liver function/injury markers, which was also reflected in the association between ratios and the liver function/injury markers. These associations are relevant since ALT, HOMA-IR and the AST/ALT ratio are the first signs of the presence of NAFLD. However, several correlations between glycation products and liver markers were not maintained when cases and controls were analyzed independently, while some new association appeared significant in one of the subgroup only. This could be a product of the variability in data in each subset (case or control) and the smaller sample sizes when subsets are considered, this is especially true considering the narrower range of values for liver enzymes and glycation products in the controls. The inverse association of sRAGE with ALT, γGT and HOMA-IR was in accordance with Yilmaz et al. [43], Santilli et al. [42, 56] and D'adamo et al. [44] in patients with hyperlipidemia with or without NAFLD. Conversely, positive associations have been previously reported between liver function and sRAGE in diabetic patients [57]. To our advantage, only normo-glycemic subjects were included in the study, eliminating this confounding factor from the analysis. These findings strengthen the involvement of AGEs-RAGE axis in the pathogenesis

of NAFLD, whereas no associations were evident for markers of apoptosis, liver stiffness or NAFLD score.

The current study has limitations that should be mentioned. Given the cross-sectional design of our study, no causal relations can be established. This matched case-control study sample size is similar to others in the field, but remains small when considering the staging of NAFLD – this might have limited our results to fully explore the relationship between glycation products and disease stages, and the correlations between glycation products and liver/inflammation biomarkers, according to the disease stage, especially when cases and controls were considered separately. As this was a single-centre study, results and glycation levels should be extrapolated to other population carefully. On the other hand, one strength of the study is the wide type of early and advanced glycation end-products included, analyzed using an accurate and precise method by ultra-high resolution LC-MS, to explore associations with NAFLD characteristics. Similarly, the serum levels of sRAGE included both sRAGE and esRAGE in the same ELISA kit. The inclusion of normo-glycaemic subjects only is an additional strength, excluding the effect of hyperglycemia on AGEs formation and sRAGE levels.

In conclusion, these findings support the hypothesis that AGE/RAGE activation is involved in the development of liver injury as part of the multiple hit model and add information regarding the pathophysiology of the disease. The measurement of an AGEs/sRAGE ratio shows better capacity to detect end organ damage and downstream organ function (liver in our case) and is hence to be preferred versus studying AGEs or sRAGE levels in isolation. Evaluating AGEs/sRAGE ratio provides a new translational and clinical perspective to determine disease progression (related to NAFLD pathogenesis). Although glycation products/sRAGE ratios discriminated between healthy and NAFLD patients, reference values would need to be established and validated in different NAFLD populations. Given that our sample of patients with available biopsies was small, future studies may focus on whether the evaluation of protein glycation/sRAGE could discriminate between the stages of NAFLD and to evaluate their usefulness as a non-invasive way of staging NAFLD. Moreover, potential modulation of AGEs/RAGE axis through diet could be the aim of future studies.

1.5 References

[1] Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology. 2003;37:1202-19.

[2] Hourigan SK, Abrams S, Yates K, Pfeifer K, Torbenson M, Murray K, et al. Relation between vitamin D status and nonalcoholic fatty liver disease in children. J Pediatr Gastroenterol Nutr. 2015;60:396-404.

[3] Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. J Hepatol. 2010;53:372-84.

[4] Moore JB. Non-alcoholic fatty liver disease: the hepatic consequence of obesity and the metabolic syndrome. Proc Nutr Soc. 2010;69:211-20.

[5] Ray K. NAFLD—the next global epidemic. Nature Reviews. 2013;10:621-.

[6] Reccia I, Kumar J, Akladios C, Virdis F, Pai M, Habib N, et al. Non-alcoholic fatty liver disease: A sign of systemic disease. Metabolism. 2017;72:94-108.

[7] Day CP, James OF. Steatohepatitis: a tale of two “hits”? Gastroenterology. 1998;114:842-5.

[8] Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 2016.

[9] Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, et al. Nonalcoholic fatty liver disease a feature of the metabolic syndrome. Diabetes. 2001;50:1844-50.

[10] Santos JCdF, Valentim IB, de Araújo OR, Ataide TdR, Goulart MO. Development of nonalcoholic hepatopathy: contributions of oxidative stress and advanced glycation end products. International Journal of Molecular Science. 2013;14:19846-66.

[11] Matafome P, Rodrigues T, Sena C, Seiça R. Methylglyoxal in Metabolic Disorders: Facts, Myths, and Promises. Med Res Rev. 2016.

[12] Thomas MC, Forbes J. The Maillard Reaction: Interface Between Aging, Nutrition, and Metabolism: Royal Society of Chemistry; 2010.

[13] Thornalley PJ. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. Cell Mol Biol (Noisy-le-grand). 1998;44:1013-23.

[14] Sadowska-Bartosz I, Bartosz G. Effect of glycation inhibitors on aging and age-related diseases. Mech Ageing Dev. 2016.

[15] Pan MH, Lai CS, Tsai ML, Ho CT. Chemoprevention of nonalcoholic fatty liver disease by dietary natural compounds. Mol Nutr Food Res. 2014;58:147-71.

[16] Akhlaghi M. Non-alcoholic Fatty Liver Disease: Beneficial Effects of Flavonoids. Phytother Res. 2016;30:1559-71.

[17] Uribarri J, Woodruff S, Goodman S, Cai W, Chen X, Pyzik R, et al. Advanced glycation end products in foods and a practical guide to their reduction in the diet. J Am Diet Assoc. 2010;110:911-6. e12.

[18] Goldberg T, Cai W, Peppa M, Dardaine V, Baliga BS, Uribarri J, et al. Advanced glycoxidation end products in commonly consumed foods. J Am Diet Assoc. 2004;104:1287-91.

[19] Basta G, Sironi AM, Lazzerini G, Del Turco S, Buzzigoli E, Casolaro A, et al. Circulating soluble receptor for advanced glycation end products is inversely associated with glycemic control and S100A12 protein. The Journal of Clinical Endocrinology & Metabolism. 2006;91:4628-34.

[20] Geroldi D, Falcone C, Emanuele E, D'Angelo A, Calcagnino M, Buzzi MP, et al. Decreased plasma levels of soluble receptor for advanced glycation end-products in patients with essential hypertension. J Hypertens. 2005;23:1725-9.

[21] Šebeková K, Krivošíková Z, Gajdoš M. Total plasma Nε-(carboxymethyl) lysine and sRAGE levels are inversely associated with a number of metabolic syndrome risk factors in non-diabetic young-to-middle-aged medication-free subjects. Clin Chem Lab Med. 2014;52:139-49.

[22] Prasad K. Low levels of serum soluble receptors for advanced glycation end products, biomarkers for disease state: myth or reality. Int J Angiol. 2014;23:011-6.

[23] Šebeková Kn, Kupčová V, Schinzel R, Heidland A. Markedly elevated levels of plasma advanced glycation end products in patients with liver cirrhosis–amelioration by liver transplantation. J Hepatol. 2002;36:66-71.

[24] Takino J-i, Nagamine K, Hori T, Sakasai-Sakai A, Takeuchi M. Contribution of the toxic advanced glycation end-products-receptor axis in nonalcoholic steatohepatitis-related hepatocellular carcinoma. World J Hepatol. 2015;7:2459.

[25] Biswas SK, Mohtarin S, Mudi SR, Anwar T, Banu LA, Alam SM, et al. Relationship of Soluble RAGE with Insulin Resistance and Beta Cell Function during Development of Type 2 Diabetes Mellitus. Journal of diabetes research. 2015;2015:150325.

[26] Georgoulis M, Kontogianni MD, Tileli N, Margariti A, Fragopoulou E, Tiniakos D, et al. The impact of cereal grain consumption on the development and severity of non-alcoholic fatty liver disease. Eur J Nutr. 2014;53:1727-35.

[27] Kontogianni MD, Tileli N, Margariti A, Georgoulis M, Deutsch M, Tiniakos D, et al. Adherence to the Mediterranean diet is associated with the severity of non-alcoholic fatty liver disease. Clin Nutr. 2014;33:678-83.
[28] Georgoulis M, Kontogianni M, Margariti A, Tiniakos D, Fragopoulou E, Zafiropoulou R, et al. Associations between dietary intake and the presence of the metabolic syndrome in patients with non-alcoholic fatty liver disease. J Hum Nutr Diet. 2015;28:409-15.
[29] Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. The American Journal of Gastroenterology. 1999;94:2467-74.
[30] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313-21.
[31] Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28:412-9.
[32] Georgoulis M, Kontogianni MD, Tileli N, Margariti A, Fragopoulou E, Tiniakos D, et al. The impact of cereal grain consumption on the development and severity of non-alcoholic fatty liver disease. Eur J Nutr. 2014;53:1727-35.
[33] Vlassopoulos A, Lean M, Combet E. Role of oxidative stress in physiological albumin glycation: A neglected interaction. Free Radic Biol Med. 2013;60:318-24.
[34] Teerlink T, Barto R, Herman J, Schalkwijk CG. Measurement of Nε-(carboxymethyl) lysine and Nε-(carboxyethyl) lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. Clin Chem. 2004;50:1222-8.
[35] Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem. 1993;39:561-77.
[36] Georgoulis M, Fragopoulou E, Kontogianni MD, Margariti A, Boulamatsi O, Detopoulou P, et al. Blood redox status is associated with the likelihood of nonalcoholic fatty liver disease irrespectively of diet's total antioxidant capacity. Nutrition research. 2015;35:41-8.
[37] Xue J, Rai V, Singer D, Chabierski S, Xie J, Reverdatto S, et al. Advanced glycation end product recognition by the receptor for AGEs. Structure. 2011;19:722-32.
[38] Hyogo H, Yamagishi Si, Iwamoto K, Arihiro K, Takeuchi M, Sato T, et al. Elevated levels of serum advanced glycation end products in patients with non-alcoholic steatohepatitis. J Gastroenterol Hepatol. 2007;22:1112-9.
[39] Butscheid M, Schäfer C, Brenner S, Alscher D, Mürdter T, Niwa T, et al. Unchanged serum levels of advanced glycation endproducts in patients with liver disease. Naunyn-Schmiedeberg's Arch Pharmacol. 2007;375:401-6.
[40] Semba RD, Arab L, Sun K, Nicklett EJ, Ferrucci L. Fat mass is inversely associated with serum carboxymethyl-lysine, an advanced glycation end product, in adults. The Journal of Nutrition. 2011;141:1726-30.
[41] Gaens KH, Ferreira I, Van De Waarenburg MP, van Greevenbroek MM, van der Kallen CJ, Dekker JM, et al. Protein-Bound Plasma Nε-(Carboxymethyl) lysine Is Inversely Associated With Central Obesity and Inflammation and Significantly Explain a Part of the Central Obesity–Related Increase in Inflammation The Hoorn and CODAM Studies. Arterioscler Thromb Vasc Biol. 2015;35:2707-13.
[42] Santilli F, Blardi P, Scapellato C, Bocchia M, Guazzi G, Terzuoli L, et al. Decreased plasma endogenous soluble RAGE, and enhanced adipokine secretion, oxidative stress and platelet/coagulative activation identify non-alcoholic fatty liver disease among patients with familial combined hyperlipidemia and/or metabolic syndrome. Vascul Pharmacol. 2015;72:16-24.
[43] Yilmaz Y, Ulukaya E, Gul OO, Arabul M, Gul CB, Atug O, et al. Decreased plasma levels of soluble receptor for advanced glycation endproducts (sRAGE) in patients with nonalcoholic fatty liver disease. Clin Biochem. 2009;42:802-7.
[44] D'Adamo E, Giannini C, Chiavaroli V, de Giorgis T, Verrotti A, Chiarelli F, et al. What is the significance of soluble and endogenous secretory receptor for advanced glycation end products in liver steatosis in obese prepubertal children? Antioxid Redox Signal. 2011;14:1167-72.
[45] Alkhouri N, Feldstein AE. Noninvasive diagnosis of nonalcoholic fatty liver disease: Are we there yet? Metabolism. 2016;65:1087-95.
[46] Huh JH, Kim KJ, Kim SU, Han SH, Han K-H, Cha B-S, et al. Obesity is more closely related with hepatic steatosis and fibrosis measured by transient elastography than metabolic health status. Metabolism. 2017;66:23-31.
[47] Käräjämäki AJ, Bloigu R, Kauma H, Kesäniemi YA, Koivurova O-P, Perkiömäki J, et al. Non-alcoholic fatty liver disease with and without metabolic syndrome: Different long-term outcomes. Metabolism. 2017;66:55-63.
[48] Patel R, Baker SS, Liu W, Desai S, Alkhouri R, Kozielski R, et al. Effect of dietary advanced glycation end products on mouse liver. PLoS One. 2012;7:e35143.
[49] Cai W, He JC, Zhu L, Chen X, Wallenstein S, Striker GE, et al. Reduced oxidant stress and extended lifespan in mice exposed to a low glycotoxin diet: association with increased AGER1 expression. The American Journal of Pathology. 2007;170:1893-902.

[50] Gaens KH, Niessen PM, Rensen SS, Buurman WA, Greve JW, Driessen A, et al. Endogenous formation of Nepsilon-(carboxymethyl)lysine is increased in fatty livers and induces inflammatory markers in an in vitro model of hepatic steatosis. J Hepatol. 2012;56:647-55.
[51] Fazel Y, Koenig AB, Sayiner M, Goodman ZD, Younossi ZM. Epidemiology and natural history of non-alcoholic fatty liver disease. Metabolism. 2016;65:1017-25.
[52] Musso G, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. Ann Med. 2011;43:617-49.
[53] Dixon JB, Bhathal PS, O'brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. Gastroenterology. 2001;121:91-100.
[54] Poynard T, Ratziu V, Charlotte F, Messous D, Munteanu M, Imbert-Bismut F, et al. Diagnostic value of biochemical markers (NashTest) for the prediction of non alcoholo steato hepatitis in patients with non-alcoholic fatty liver disease. BMC Gastroenterol. 2006;6:34.
[55] Xie J, Méndez JD, Méndez-Valenzuela V, Aguilar-Hernández MM. Cellular signalling of the receptor for advanced glycation end products (RAGE). Cell Signal. 2013;25:2185-97.
[56] Santilli F, Bucciarelli L, Noto D, Cefalù AB, Davì V, Ferrante E, et al. Decreased plasma soluble RAGE in patients with hypercholesterolemia: effects of statins. Free Radic Biol Med. 2007;43:1255-62.
[57] Nakamura K, Yamagishi S-i, Adachi H, Kurita-Nakamura Y, Matsui T, Yoshida T, et al. Serum levels of sRAGE, the soluble form of receptor for advanced glycation end products, are associated with inflammatory markers in patients with type 2 diabetes. Mol Med. 2007;13:185.

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Conflict of interest statement

Authors declare no conflict of interest. AV work in this study was performed as part of his previous contract with the University of Glasgow and his current employer (Nestle SA) has no role in the analysis, interpretation of results and the writing of the manuscript.

Authors' Contribution

EC, MK and AV conceived and designed the present study; AM and MG were responsible for recruitment, sample collection and data acquisition in Greece; SP and SZ performed the experiments and analysed the data, under the supervision of EC; SP wrote the original draft of the paper under the supervision of EC, GP supervised data collection and critically revised the manuscript. All authors contributed to and approved the final manuscript.

615

616 1.6 Supplements

617 Tables

618 **Supplement 1. Liver, biochemical and inflammatory markers in NAFLD patients and their matched**
619 **healthy controls**

	Cases (n= 58)	Controls (n=58)	p-value
ALT (IU/L)	76.3 (41.3)	15.0 (5.9)	<0.001
AST (IU/L)	42.1 (19.1)	24.7 (6.8)	<0.001
γGT (IU/L)	98.7 (124.5)	20.2 (12.1)	<0.001
IL-6 (pg/mL)	2.3 (3.9)*	3.7 (9.3)	0.191
IL-8 (pg/mL)	32.5 (51.4)*	33.8 (84.8)	0.089
TNF-α (pg/mL)	4.8 (3.7)	2.5 (3.6)	<0.001
hsCRP (mg/L)	1.8 (2.0)*	1.2 (1.1)	0.027
Adiponectin (mg/mL)	6.6 (4.7)	10.0 (7.7)	0.007
VEGF (pg/mL)	317.7 (161.4)*	383.9 (231.9)	0.150
TGF-β1 (mg/mL)	36.3 (12.9)	25.3 (14.4)	0.001

Differences between cases and their matched controls (match by age, sex and BMI) were observed using Mann-Whitney U test. ALT, alanine aminotransferase; AST, Aspartate aminotransferase; γGT, γ-glutamyl transpeptidase; hsCRP, high sensitive C reactive protein; IL-6, interleukin 6; IL-8, interleukin 8; TGF-β1; transforming growth factor beta-1; TNF-α, tumor necrosis factor-α, VEGF; vascular endothelial factor.

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621 **Supplement 2. Sensitive analysis of glycation biomarkers levels among the non-alcoholic fatty**
622 **liver disease (NAFLD) patients with available biopsy (n=22) and healthy controls (n=22).**

	Cases (n=22)	Controls (n=22)	p-value
Fructosamine (mM DMF)	1.2 (0.2)	1.0 (0.2)	0.009
AGE fluo (AU)[a]	501.7 (438.3 – 584.8)	404.8 (377.9 – 429.9)	0.001
AGEs (mmol/mol)	131.6 (34.0)	112.2 (25.6)	0.038
CML mmol/mol	10.9 (4.3)	9.3 (2.3)	0.133
CEL mmol/mol	119.0 (34.2)	101.4 (26.5)	0.06
Pentosidine mmol/mol[a]	1.6 (1.5 – 1.9)	1.6 (1.4 – 1.6)	0.07
sRAGE (pg/L)[a]	322.8 (186.9 – 438.8)	602.7 (519.7 – 764.8)	0.001
CML/sRAGE (mmol/pmol) [a]	1.2 (0.7 – 1.5)	0.5 (0.4 – 0.8)	0.001
CEL/sRAGE (mmol/pmol)	19.6 (18.7)	6.3 (2.6)	0.002
AGEs/sRAGE (mmol/pmol)	22.4 (22.9)	7.0 (2.8)	0.004

Data are presented as means (SD) or [a] median (interquartile range). Differences between cases and their matched controls (match by age, sex and BMI) were observed using 2-samples *t* test or Mann-Whitney U Test. AGEs (advance glycation end products) sum of CEL (N^{ε}-carboxyethyl-L-lysine), CML (N^{ε}-carboxymethyl-L-lysine) and pentosidine. sRAGE, AGEs soluble receptor.

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626 **Supplement 3. Glycation biomarkers levels according to the necro-inflammatory grade score among patients with available biopsies (n=22).**

	Grade 0 (n=10)	Grade 1 (n=4)	Grade 2 (n=7)	Grade 3 (n=1)	p-value
Fructosamine (mM DMF)	1.20 (0.99-1.39)	1.16 (1.03-1.45)	0.97 (0.93-1.23)	1.34	0.226
AGE fluo (AU)	501.16 (474.97-549.97)	558.15 (438.28-634.17)	542.72 (414.55-586.84)	613.87	0.711
AGEs (mmol/mol)	133.36 (99.78-167.11)	136.44 (121.64-142.64)	137.25 (103.84-138.34)	124.56	0.987
CML mmol/mol	10.72 (5.73-13.22)	13.39 (12.17-14.93)	8.65 (6.62-11.03)	15.34	0.166
CEL mmol/mol	121.50 (93.08-148.41)	120.58 (106.17-127.13)	124.64 (94.71-130.14)	107.02	0.902
Pentosidine mmol/mol	1.65 (1.50-1.92)	1.85 (1.70-2.17)	1.56 (1.45-1.58)	2.19	0.139
sRAGE (pg/L)	327.22 (285.20-388.62)	419.65 (310.56-927.74)	186.85 (157.10-442.09)	474.72	0.529
CML/sRAGE (mmol/pmol)	1.06 (0.56-1.41)	1.29 (0.84-5.60)	1.17 (0.95-2.07)	1.13	0.773
CEL/sRAGE (mmol/pmol)	13.06 (8.91-19.58)	10.65 (6.36-45.33)	22.35 (10.47-28.99)	7.89	0.533
AGEs/sRAGE (mmol/pmol)	14.59 (10.02-21.42)	12.12 (7.30-51.83)	23.62 (11.58-30.82)	9.18	0.533

Data are presented as median (interquartile range). Differences according to the grade of necro-inflammation (Brunt [29]) were performed among the groups with at least two patients, using Kruskal-Wallis Test. AGEs (advance glycation end products) is the sum of CEL (N^{ε}-carboxyethyl-L-lysine), CML (N^{ε}-carboxymethyl-L-lysine) and pentosidine. sRAGE, AGEs soluble receptor.

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628 **Supplement 4. Glycation biomarkers levels according to fibrosis stage among patients with available biopsies (n=22).**

	Grade 0 (n=9)	Grade 1 (n=4)	Grade 2 (n=5)	Grade 3 (n=4)	p-value
Fructosamine (mM DMF)	1.10 (0.99-1.41)	1.31 (1.16-1.39)	1.05 (1.01-1.23)	0.95 (0.91-1.16)	0.332
AGE fluo (AU)	496.66 (431.50-529.91)	549.29 (438.28-600.07)	542.72 (491.86-546.01)	589.77 (478.62-603.28)	0.742
AGEs (mmol/mol)	118.16 (99.78-148.55)	147.57 (121.64-163.72)	138.06 (137.25-138.08)	121.19 (110.83-131.45)	0.240
CML mmol/mol	10.62 (5.73-12.27)	13.38 (12.17-15.61)	11.03 (10.68-14.78)	7.64 (5.69-12.00)	0.790
CEL mmol/mol	109.25 (93.08-133.75)	133.74 (106.49-147.49)	124.64 (120.25-125.55)	109.26 (100.47-120.82)	0.784
Pentosidine mmol/mol	1.58 (1.57-1.78)	1.85 (1.67-1.92)	1.58 (1.46-1.82)	1.57 (1.41-1.89)	0.759
sRAGE (pg/L)	328.01 (319.14)	419.65 (310.56-438.77)	186.85 (95.57-442.09)	244.24 (165.86-394.29)	0.624
CML/sRAGE (mmol/pmol)	0.70 (0.51-1.16)	1.35 (1.27-1.50)	2.07 (1.17-3.91)	1.05 (0.96-1.30)	0.212
CEL/sRAGE (mmol/pmol)	10.21 (5.19-18.06)	12.73 (9.76-16.60)	23.35 (14.60-45.98)	16.41 (9.18-25.67)	0.465
AGEs/sRAGE (mmol/pmol)	10.94 (6.48-18.77)	14.26 (11.19-18.31)	25.71 (15.89-50.56)	17.60 (10.38-27.22)	0.465

Data are presented as median (interquartile range). Differences according to stage of fibrosis (Brunt [29]) were performed using Kruskal-Wallis Test. AGEs (advance glycation end products) is the sum of CEL (N^{ε}-carboxyethyl-L-lysine), CML (N^{ε}-carboxymethyl-L-lysine) and pentosidine. sRAGE, AGEs soluble receptor.

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Supplement 5. Glycation biomarkers levels according to the stage of NAFLD based on the Kleiner score among patients with available biopsies.

	Simple steatosis (n=10)	NASH (n=12)	p-value
Fructosamine (mM DMF)	1.21 (0.28)	1.10 (0.18)	0.25
AGE fluo (AU)[a]	533.08 (124.93)	511.22 (88.15)	0.67
AGEs (mmol/mol)	127.49 (40.68)	135.01 (28.61)	0.62
CML mmol/mol	11.28 (4.82)	10.57 (3.91)	0.71
CEL mmol/mol	114.55 (41.42)	122.70 (28.27)	0.59
Pentosidine mmol/mol[a]	1.67 (0.28)	1.74 (0.34)	0.67
sRAGE (pg/L)[a]	524.98 (396.65)	323.32 (243.49)	0.17
CML/sRAGE (mmol/pmol)	2.95 (6.44)	2.09 (2.60)	0.68
CEL/sRAGE (mmol/pmol)	15.58 (16.35)	23.00 (20.57)	0.37
AGEs/sRAGE (mmol/pmol)	18.80 (23.00)	25.44 (23.44)	0.51

Differences between cases and their matched controls (match by age, sex and BMI) were observed using 2-samples *t* test or Mann-Whitney U Test [a] Data are not normally distributed. AGEs (advance glycation end products) sum of CEL (N^{ϵ}-carboxyethyl-L-lysine), CML (N^{ϵ}-carboxymethyl-L-lysine) and pentosidine. sRAGE, AGEs soluble receptor; NASH, non-alcoholic steatohepatitis.

Supplement 6. Spearman (rho) correlations between glycated products and sRAGE, and liver function and inflammation biomarkers in the cases (n=58).

	ALT	AST	AST/ALT	γGT	HOMA-IR	TGF-β1	TNF-α	IL-6	hsCRP
AGEs (sum of)	0.09	0.08	-0.03	-0.07	0.04	-0.18	0.10	<0.01	-0.08
CML	-0.05	-0.01	0.09	-0.17	-0.15	-0.13	-0.06	-0.06	-0.09
CEL	0.08	0.05	-0.03	0.09	0.05	-0.17	0.10	<0.01	-0.05
Pentosidine	0.10	0.11	-0.06	-0.07	0.10	0.11	0.02	0.09	-0.06
Fructosamine	-0.02	-0.02	0.03	-0.22	-0.06	0.07	<0.01	**-0.41***	-0.22
AGE fluorescence	-0.14	-0.18	0.14	0.10	-0.08	0.09	0.05	0.11	0.05
sRAGE	-0.01	0.22	-0.06	-0.26	-0.15	-0.14	-0.24	-0.16	-0.12
CML/sRAGE	-0.03	-0.16	0.02	0.13	0.04	0.10	0.18	0.12	0.06
CEL/sRAGE	-0.06	-0.24	<0.01	0.15	0.13	0.06	**0.28***	0.22	0.14
AGEs/sRAGE	-0.05	-0.24	-0.02	0.16	0.12	0.06	**0.28***	0.21	0.13

*p = 0.002. AGEs (advance glycation end products) sum of CEL (N^{ε}-carboxyethyl-L-lysine), CML (N^{ε}-carboxymethyl-L-lysine) and pentosidine. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGT, γ-glutamyl transpeptidase; HOMA-IR, (log) Homeostasis Model of Assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; IL-6: interleukin 6; sRAGE, AGEs soluble receptor; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor-α.

Supplement 7. Spearman (rho) correlations between glycated products and sRAGE, and liver function and inflammation biomarkers in the controls (n=58).

	ALT	AST	AST/ALT	γGT	HOMA-IR	TGF-β1	TNF-α	IL-6	hsCRP
AGEs (sum of)	**-0.45****	**-0.43****	0.16	-0.18	0.11	**0.36****	**-0.38****	-0.24	-0.05
CML	0.16	0.26	0.03	0.18	0.07	-0.09	0.10	0.12	-0.26
CEL	**-0.43****	**-0.45****	0.13	-0.18	0.10	**0.36****	**-0.35***	-0.26	-0.02
Pentosidine	-0.11	-0.08	0.10	-0.06	0.16	-0.16	0.20	0.10	0.02
Fructosamine	0.13	0.14	-0.11	-0.13	0.18	0.07	**-0.31***	-0.21	-0.20
AGE fluorescence	0.01	-0.07	0.01	0.03	0.03	-0.07	**0.33****	-0.20	-0.10
sRAGE	0.10	0.19	-0.03	-0.10	0.05	0.05	0.05	0.16	0.26
CML/sRAGE	-0.08	-0.11	0.08	0.17	-0.05	-0.06	0.03	-0.08	0.13
CEL/sRAGE	**-0.33***	**-0.39****	0.14	-0.03	0.05	0.17	-0.27	-0.23	0.12
AGEs/sRAGE	**-0.32***	**-0.39****	0.13	-0.02	0.04	0.16	-0.26	-0.21	0.14

*p = 0.02, ** p < 0.01; AGEs (advance glycation end products) sum of CEL (N^{ε}-carboxyethyl-L-lysine), CML (N^{ε}-carboxymethyl-L-lysine) and pentosidine. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGT, γ-glutamyl transpeptidase; HOMA-IR, (log) Homeostasis Model of Assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; IL-6: interleukin 6; sRAGE, AGEs soluble receptor; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor-α.

Supplement 8. Multinominal logistic regression analysis (OR, 95%CI), exploring the association between sRAGE and glycated products/sRAGE ratio with AST/ALT (N= 58 cases and 58 controls).

	AST/ALT quartiles	Model 1 [a]	Model 2 [b]	Model 3 [c]
sRAGE				
	Q1 (<0.55)	1.00 (0.99 – 1.00)	1.00 (0.99 – 1.00)	1.00 (0.99 – 1.00)
	Q2 (0.55 – 0.95)	1.00 (0.99 – 1.00)	1.00 (0.99 – 1.00)	1.00 (0.99 – 1.00)
	Q3 (0.96 – 1.72)	1.00 (0.99 – 1.00)	1.00 (0.99 – 1.00)	1.00 (0.99 – 1.00)
AGEs/sRAGE				
	Q1 (<0.55)	1.27 (1.10 – 1.47)*	1.31 (1.10 – 1.57)*	1.35 (1.10 – 1.65)*
	Q2 (0.55 – 0.95)	1.27 (1.10 – 1.47)*	1.31 (1.10 – 1.57)*	1.35 (1.10 – 1.64)*
	Q3 (0.96 – 1.72)	1.02 (0.87 – 1.19)	1.08 (0.89 – 1.30)	1.13 (0.92 – 1.40)
CML/sRAGE				
	Q1 (<0.55)	13.28 (2.35 – 75.20)*	26.64 (2.74 – 258.79)*	28.18 (2.80 – 283.72)*
	Q2 (0.55 – 0.95)	14.27 (2.52 – 80.77)*	27.57 (2.84 – 267.99)*	29.00 (2.88 – 292.16)*
	Q3 (0.96 – 1.72)	1.79 (0.27 – 12.08)	3.12 (0.28 – 34.97)	4.35 (0.36 – 52.94)
CEL/sRAGE				
	Q1 (<0.55)	1.29 (1.10 – 1.50)*	1.33 (1.10 – 1.60)*	1.37 (1.11 – 1.69)*
	Q2 (0.55 – 0.95)	1.29 (1.10 – 1.50)*	1.32 (1.10 – 1.60)*	1.36 (1.10 – 1.68)*
	Q3 (0.96 – 1.72)	1.01 (0.86 – 1.20)	1.08 (0.88 – 1.31)	1.14 (0.91 – 1.42)
AGE fluorescence/sRAGE				
	Q1 (<0.55)	1.32 (1.14 – 1.54)*	1.33 (1.12 – 1.58)*	1.35 (1.13 – 1.61)*
	Q2 (0.55 – 0.95)	1.33 (1.15 – 1.55)*	1.34 (1.13 – 1.59)*	1.35 (1.13 – 1.61)*
	Q3 (0.96 – 1.72)	1.08 (0.92 – 1.26)	1.09 (0.91 – 1.31)	1.12 (0.93 – 1.34)

The reference value is an AST/ALT (aspartate aminotransferase/alanine aminotransferase) ratio higher than 1.72(Q4). *p < 0.01.

[a] Model 1: univariate, unadjusted

[b] Model 2: adjusted for age, gender, abdominal fat level.

[c] Model 3: adjusted for age, gender, abdominal fat level, interleukin 6 and high-sensitive C-reactive protein.

Advance glycation end products (AGEs) including N^{ϵ}-carboxymethyl-L-lysine (CML), N^{ϵ}-carboxyethyl-L-lysine (CEL) and pentosidine, and sRAGE (AGEs soluble receptor) are expressed as continuous variables.

Supplement 9. Logistic regression analysis models, exploring the association between glycated products/sRAGE ratio, sRAGE, and the likelihood of NASH (n=12) against simple steatosis (n=10).

	Model 1[a]			Model 2[b]		
	OR	95% CI	P	OR	95% CI	P
sRAGE	0.99	0.99-1.00	0.17	0.99	0.99-1.00	0.17
AGEs/sRAGE	1.01	0.97-1.06	0.50	1.01	0.97-1.06	0.53
CEL/sRAGE	1.03	0.97-1.08	0.37	1.02	0.97-1.08	0.40
AGE fluorescence/sRAGE	1.12	0.97-1.30	0.11	1.15	0.97-1.35	0.10

[a] Model 1: unadjusted.

[b] Model 2: adjusted for age and AST/ALT.

NAFLD: non-alcoholic fatty liver disease, OR: Odds Ratio, CI: Confidence Interval, CEL: N^{ε}-carboxyethyl-L-lysine, AGEs: N^{ε}-carboxymethyl-L-lysine, CEL and pentosidine advance glycation end products, sRAGE: AGEs soluble receptor, HOMA-IR: Homeostasis Model of Assessment of insulin resistance, γGT: γ-glutamyl transpeptidase.

Supplement 10. Logistic regression analysis models, exploring the association between glycated products/sRAGE ratio, sRAGE, and the likelihood of having fibrosis (defined as LSM>6.6 kPa cut-off).

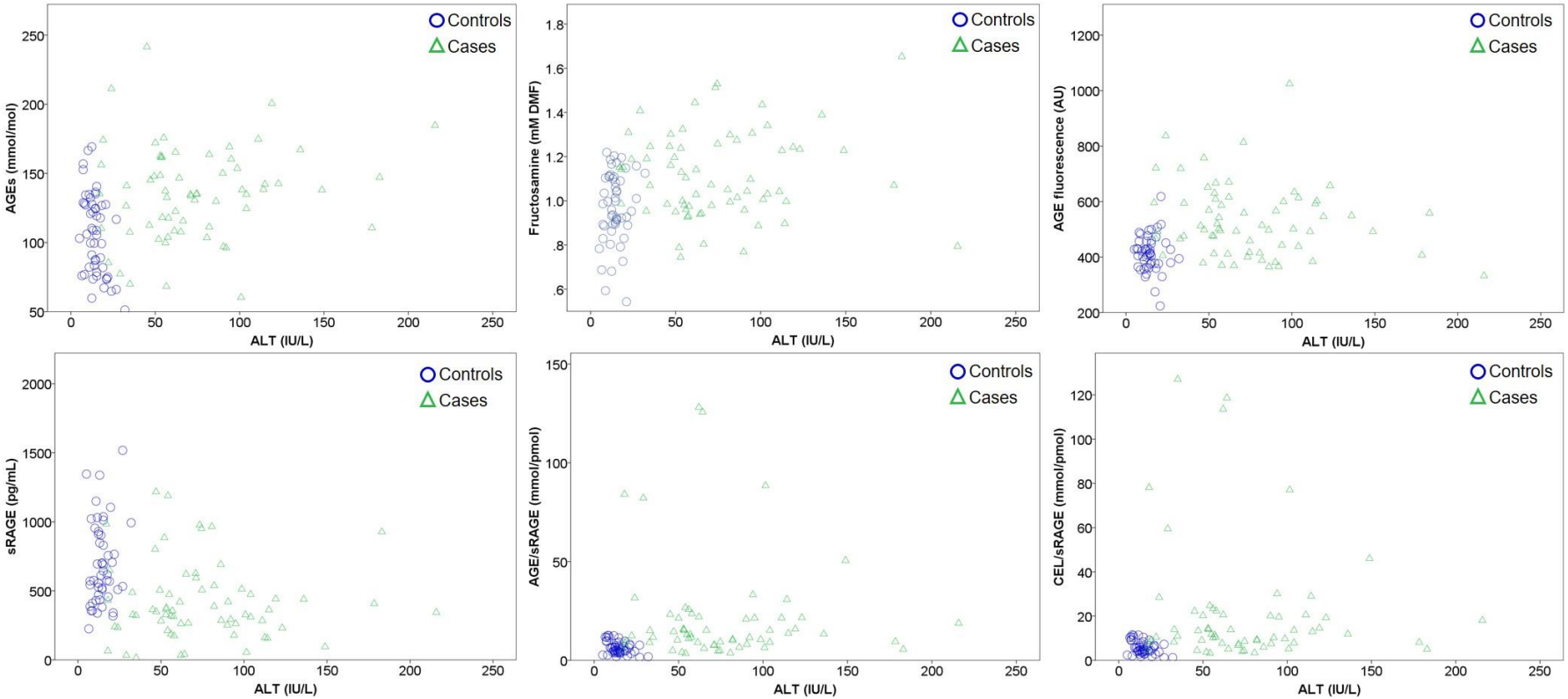
	Model 1[a]			Model 2[b]		
	OR	95% CI	P	OR	95% CI	P
sRAGE	0.99	0.99-1.00	0.15	0.99	0.99-1.00	0.09
AGEs/sRAGE	0.99	0.98-1.02	0.86	1.00	0.98-1.02	0.99
CEL/sRAGE	1.00	0.98-1.02	0.96	1.00	0.98-1.03	0.90
AGE fluorescence/sRAGE	0.99	0.97-1.01	0.48	0.99	0.99-1.02	0.57

[a] Model 1: unadjusted.

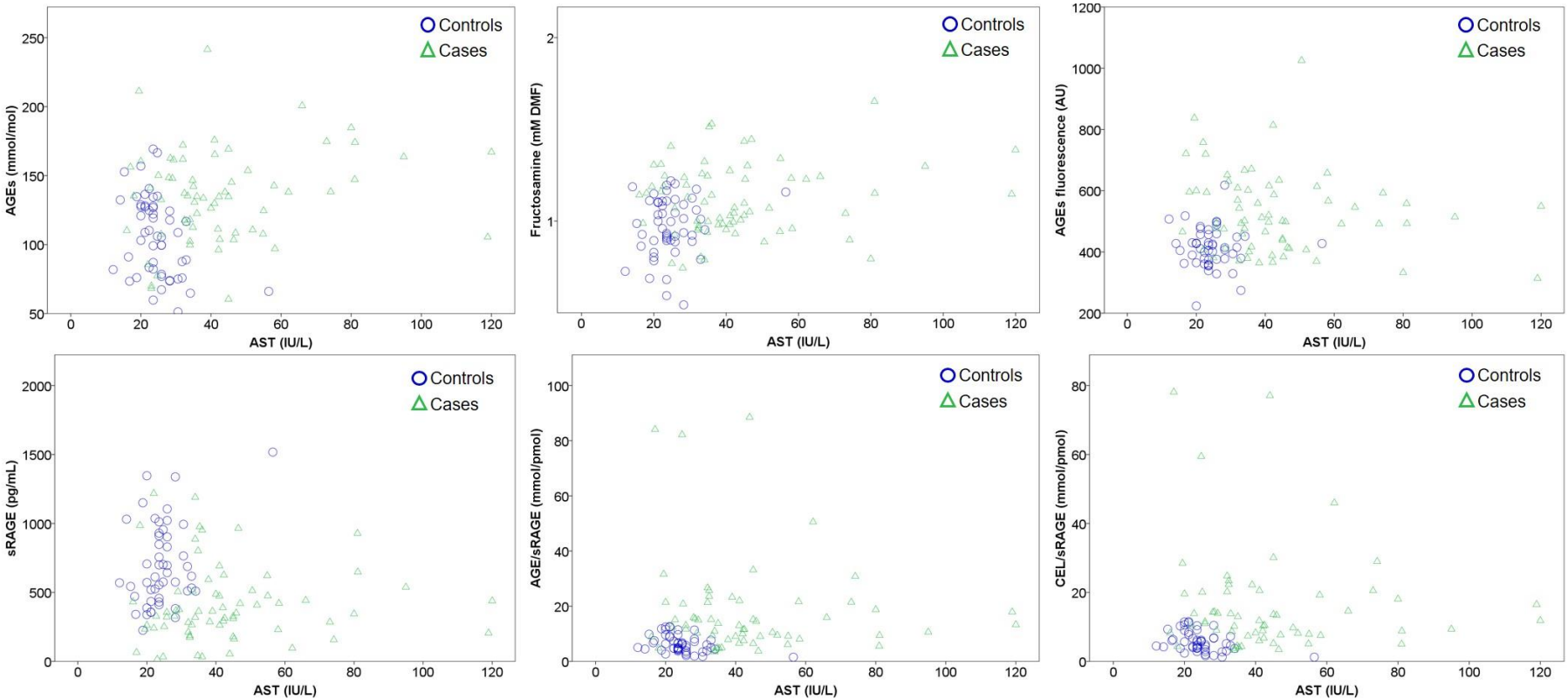
[b] Model 2: adjusted for age and AST/ALT.

Twenty-seven NAFLD patients with a liver stiffness measurement (LSM) <6.6 kPa and 18 NAFLD patients with LSM > 6.6 kPa. NAFLD: non-alcoholic fatty liver disease, OR: Odds Ratio, CI: Confidence Interval, AGEs (advance glycation end products) includes: N^{ε}-carboxymethyl-L-lysine, N^{ε}-carboxyethyl-L-lysine (CEL) and pentosidine, sRAGE: AGEs soluble receptor, HOMA-IR: Homeostasis Model of Assessment of insulin resistance, γGT: γ-glutamyl transpeptidase.

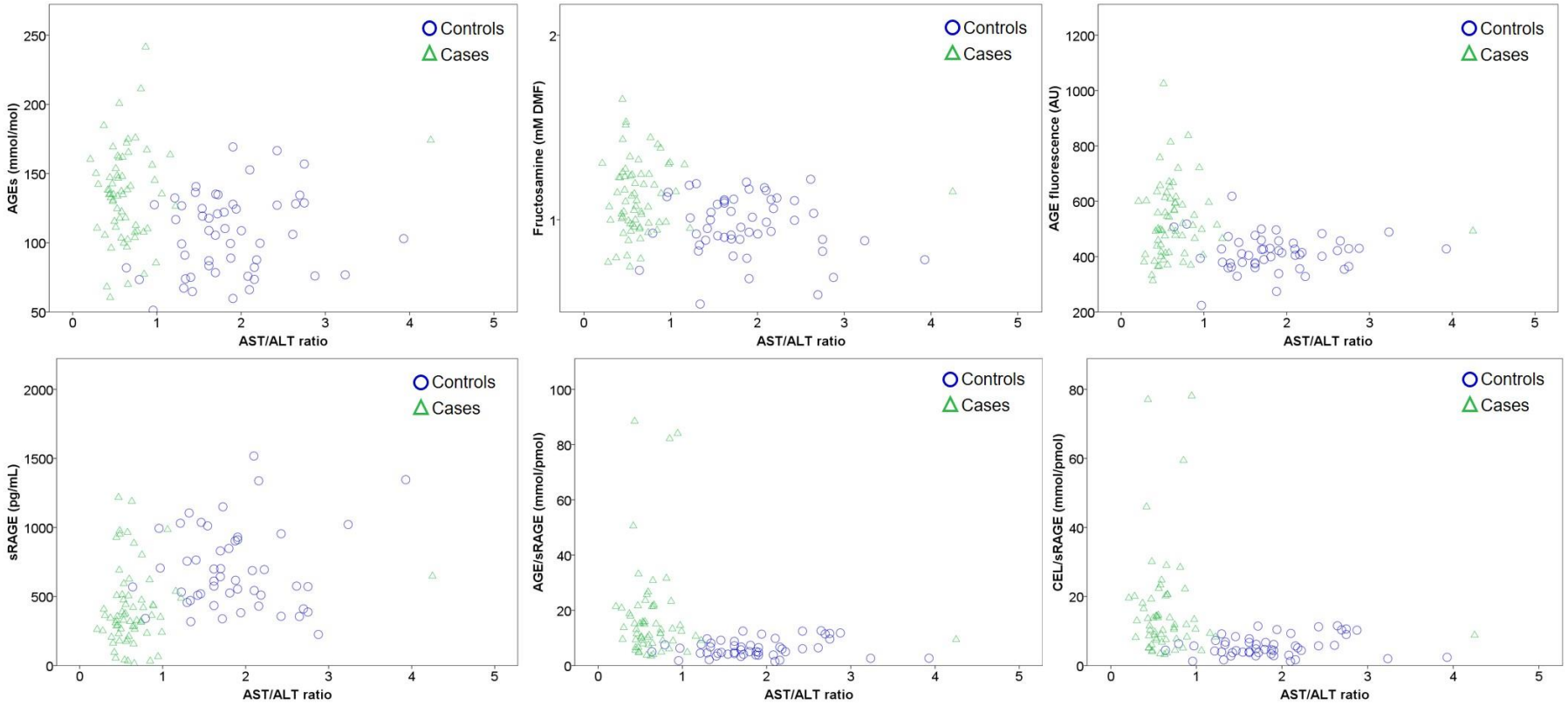
Figures



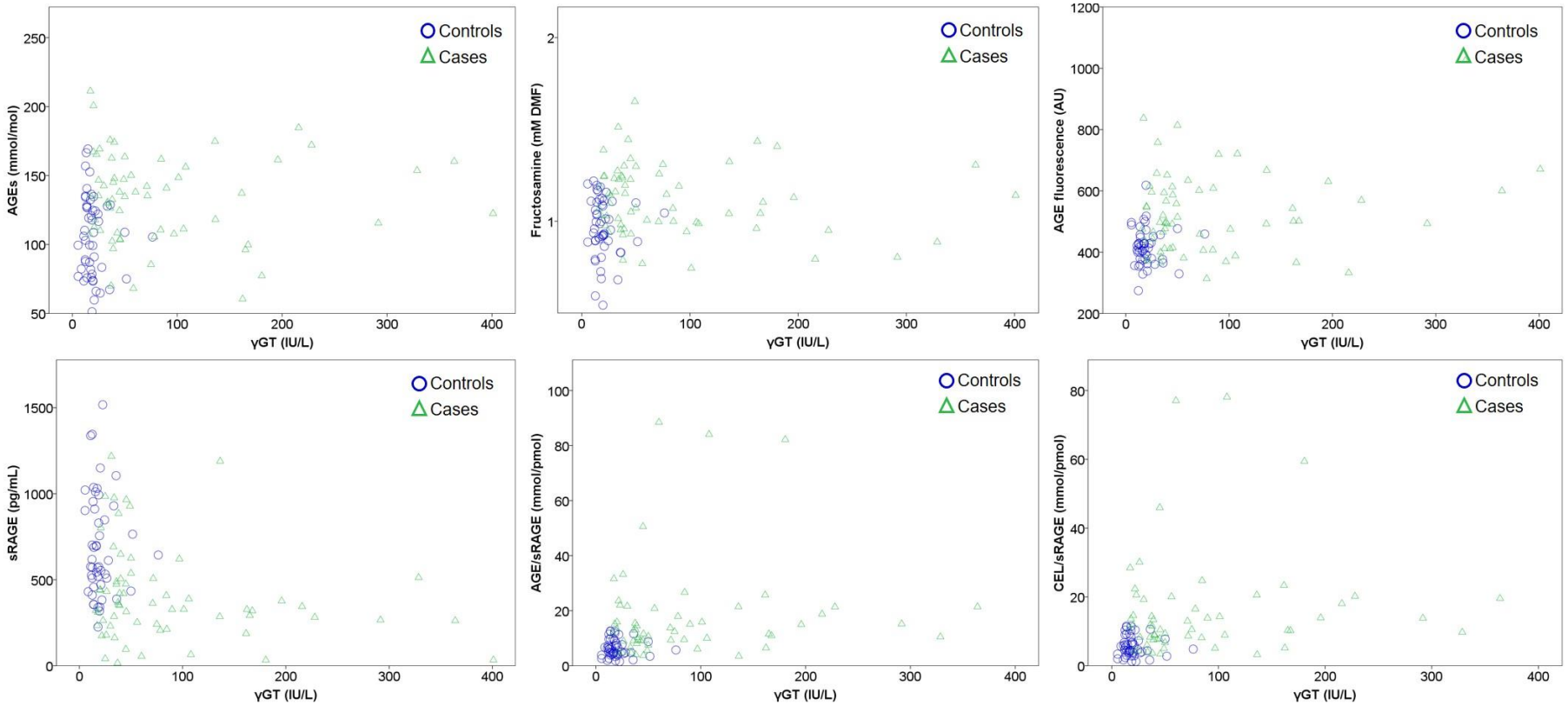
Supplementary figure 1. Correlation between glycated products and sRAGE (AGEs soluble receptor), and alanine aminotransferase (ALT) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (N$^{\varepsilon}$-carboxyethyl-L-lysine), CML (N$^{\varepsilon}$-carboxymethyl-L-lysine) and pentosidine.



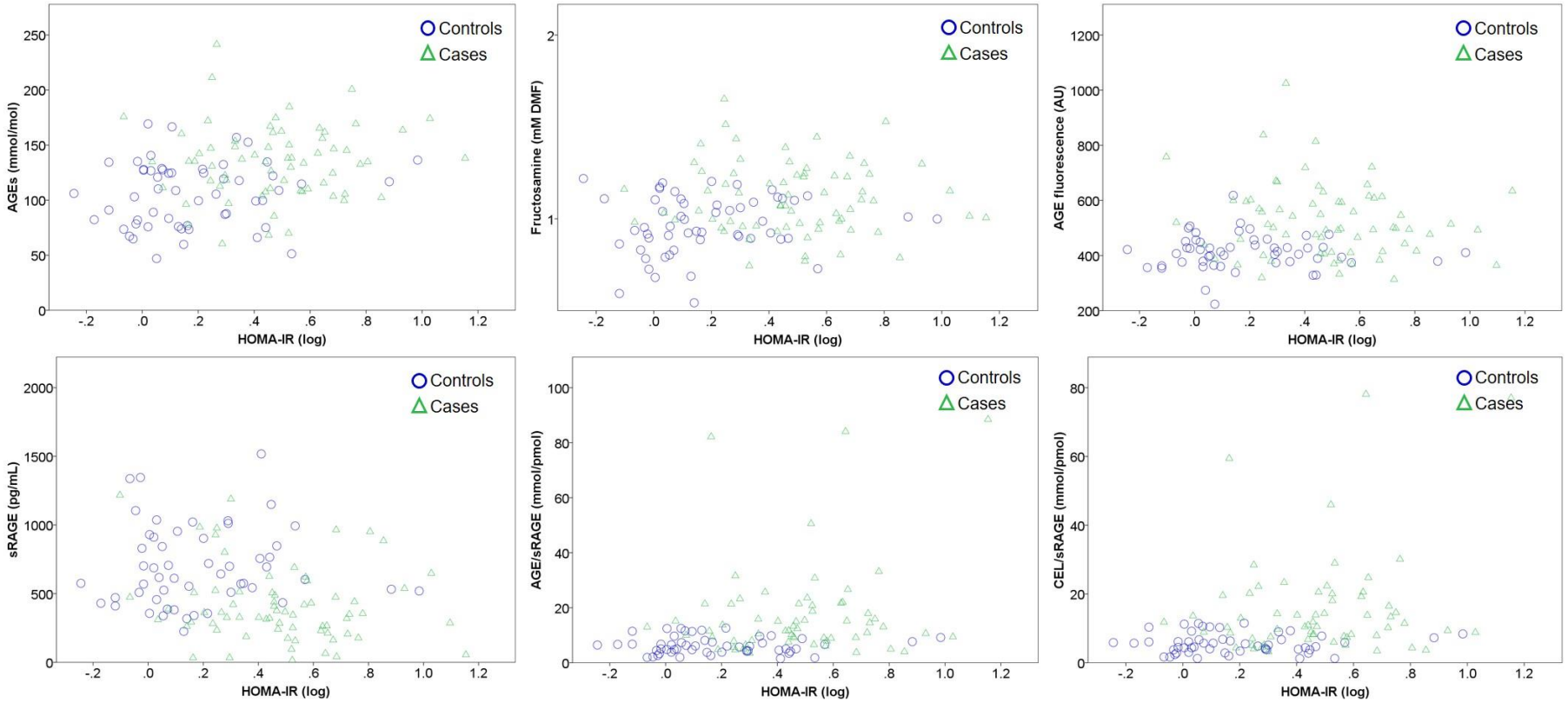
Supplementary figure 2. Correlation between glycated products and sRAGE (AGEs soluble receptor), and aspartate aminotransferase (AST) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.



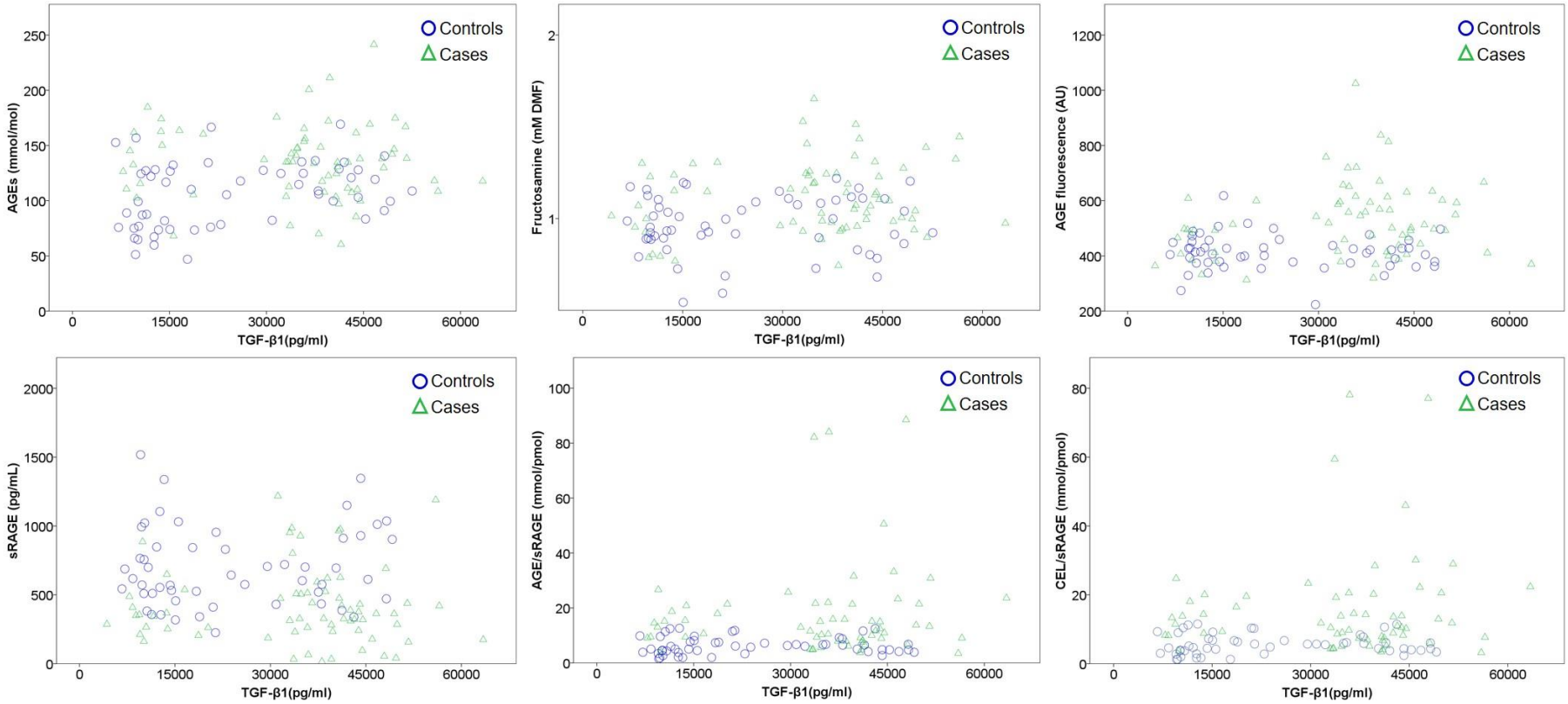
Supplementary figure 3. Correlation between glycated products and sRAGE (AGEs soluble receptor), and aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) ratio in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.



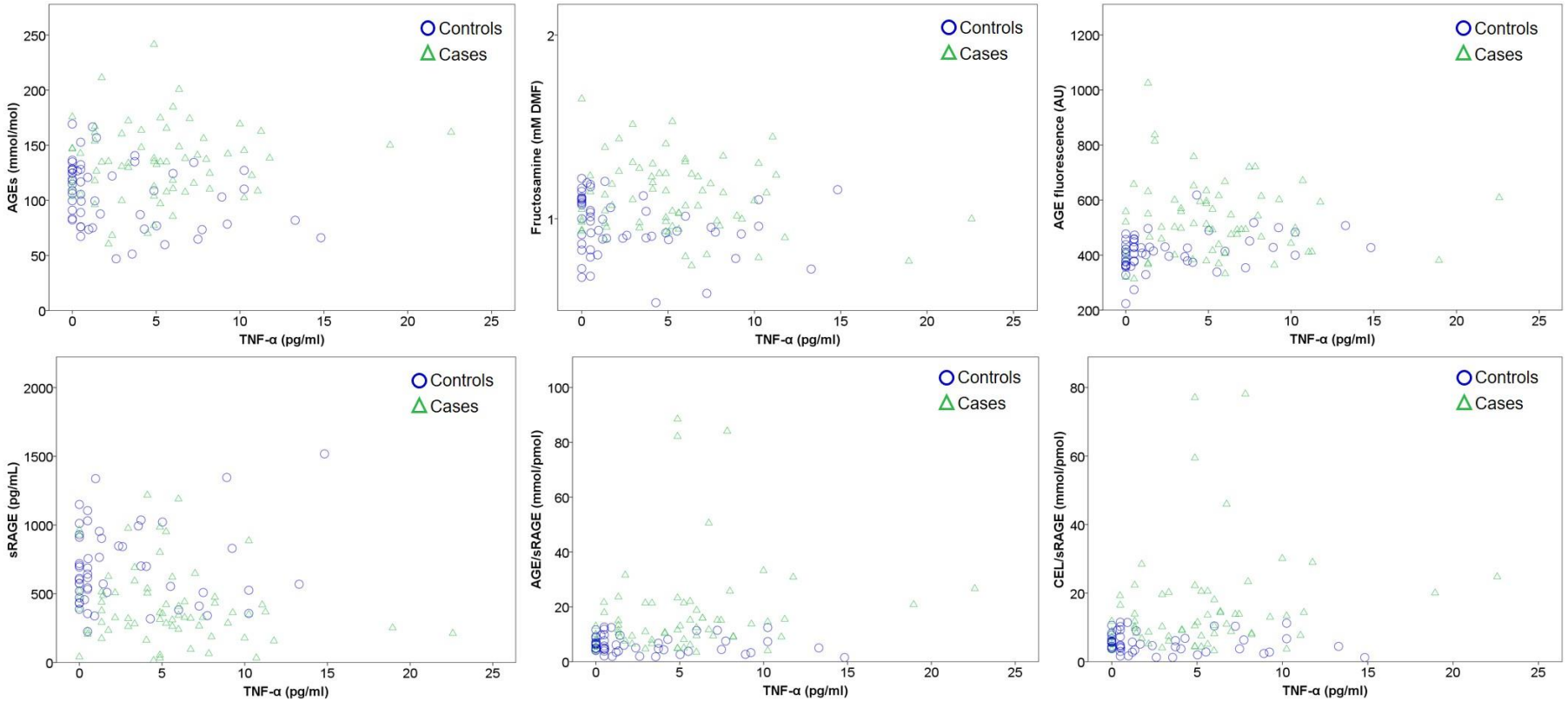
Supplementary figure 4. Correlation between glycated products and sRAGE (AGEs soluble receptor), and γ-glutamyl transpeptidase (γGT) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.



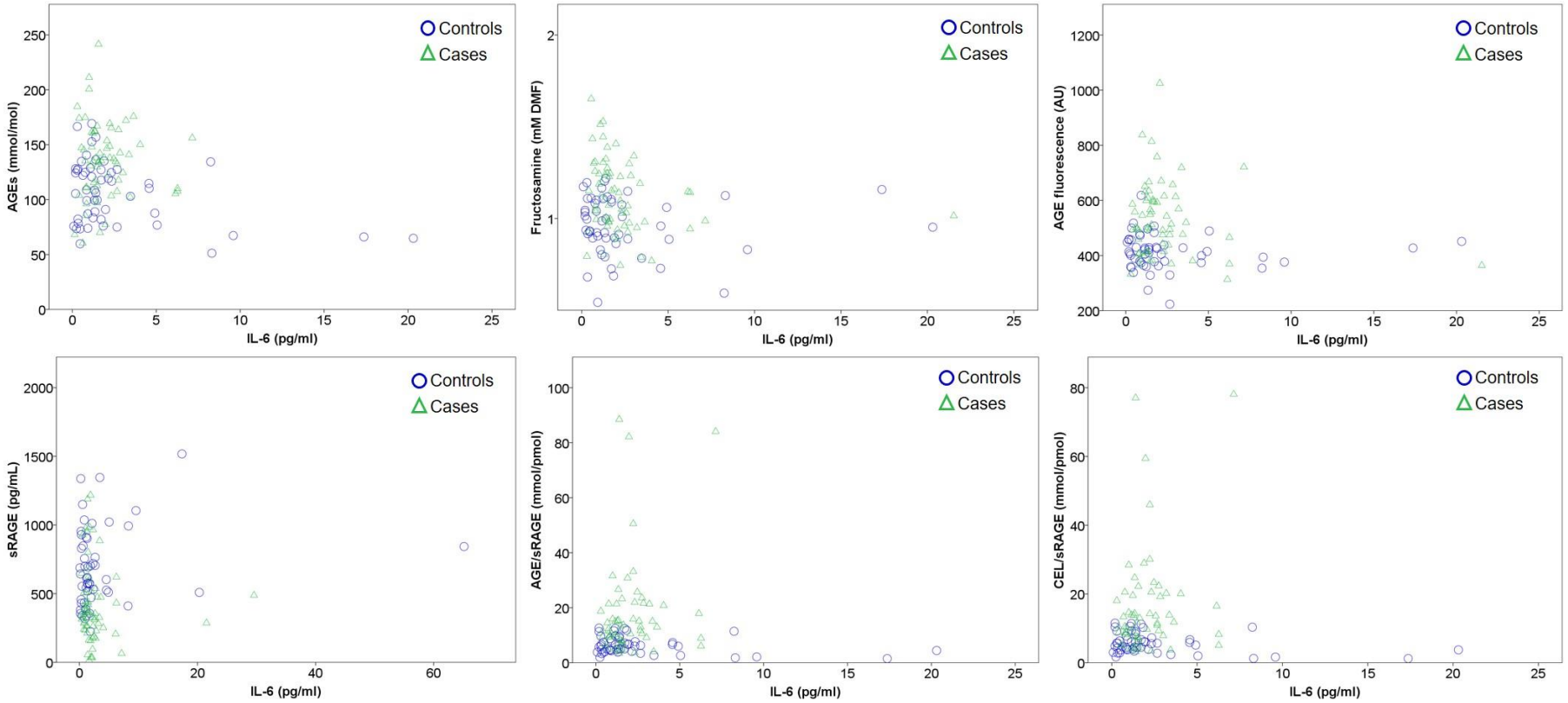
Supplementary figure 5. Correlation between glycated products and sRAGE (AGEs soluble receptor), and homeostasis Model of Assessment of insulin resistance (HOMA-IR, log) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.



Supplementary figure 6. Correlation between glycated products and sRAGE (AGEs soluble receptor), and Transforming growth factor beta 1 (TGF-β1) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.



Supplementary figure 7. Correlation between glycated products and sRAGE (AGEs soluble receptor), and Tumor necrosis factor-α (TNF-α) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.



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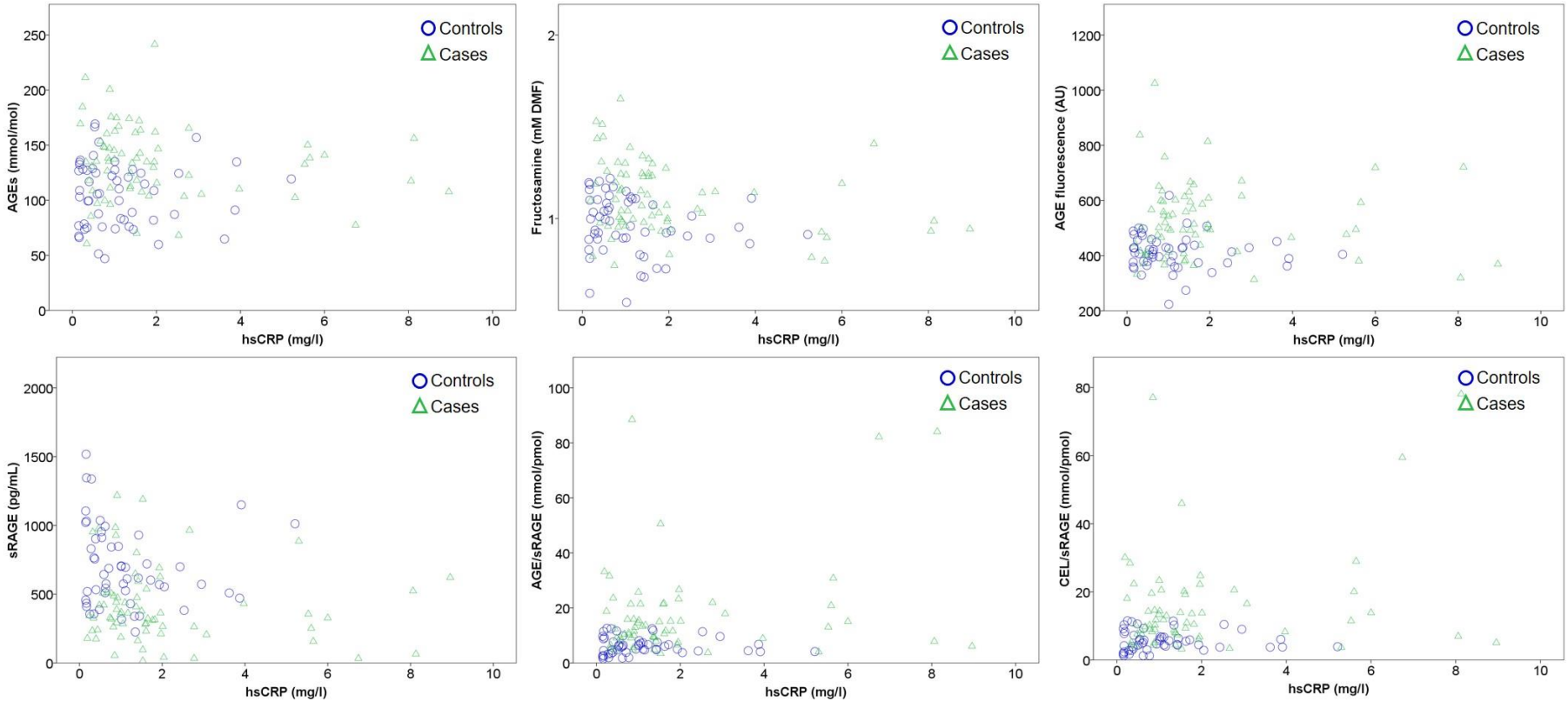
679 **Supplementary figure 8. Correlation between glycated products and sRAGE (AGEs soluble receptor), and interleukin 6 (IL-6) in cases (green triangles) and**
680 **controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.**



Supplementary figure 9. Correlation between glycated products and sRAGE (AGEs soluble receptor), high-sensitivity C-reactive protein (hsCRP) in cases (green triangles) and controls (blue circles). AGEs (advance glycation end products) sum of CEL (Nε-carboxyethyl-L-lysine), CML (Nε-carboxymethyl-L-lysine) and pentosidine.