



# The Impact of Salsalate Treatment on Serum Levels of Advanced Glycation End Products in Type 2 Diabetes

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## OBJECTIVE

Salsalate is a nonacetylated salicylate that lowers glucose levels in people with type 2 diabetes (T2D). Here we examined whether salsalate also lowered serum-protein-bound levels of early and advanced glycation end products (AGEs) that have been implicated in diabetic vascular complications.

## RESEARCH DESIGN AND METHODS

Participants were from the Targeting Inflammation Using Salsalate for Type 2 Diabetes (TINSAL-T2D) study, which examined the impact of salsalate treatment on hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) and a wide variety of other parameters. One hundred eighteen participants received salsalate, 3.5 g/day for 48 weeks, and 109 received placebo. Early glycation product levels (HbA<sub>1c</sub> and fructoselysine [measured as furosine]) and AGE levels (glyoxal and methylglyoxal hydroimidazolones [G<sup>-1</sup>H, MG<sup>-1</sup>H], carboxymethyllysine [CML], carboxyethyllysine [CEL], pentosidine) were measured in patient serum samples.

## RESULTS

Forty-eight weeks of salsalate treatment lowered levels of HbA<sub>1c</sub> and serum furosine ( $P < 0.001$ ) and CML compared with placebo. The AGEs CEL and G<sup>-1</sup>H and MG<sup>-1</sup>H levels were unchanged, whereas pentosidine levels increased more than twofold ( $P < 0.001$ ). Among salsalate users, increases in adiponectin levels were associated with lower HbA<sub>1c</sub> levels during follow-up ( $P < 0.001$ ). Changes in renal and inflammation factor levels were not associated with changes in levels of early or late glycation factors. Pentosidine level changes were unrelated to changes in levels of renal function, inflammation, or cytokines.

## CONCLUSIONS

Salsalate therapy was associated with a reduction in early but not late glycation end products. There was a paradoxical increase in serum pentosidine levels suggestive of an increase in oxidative stress or decreased clearance of pentosidine precursor.

Diabetes Care 2014;37:1083–1091 | DOI: 10.2337/dc13-1527

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Received 28 June 2013 and accepted 14 November 2013.

This article contains Supplementary Data online at <http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc13-1527/-/DC1>.

\*A list of members of the TINSAL-T2D Research Consortium appears in the Supplementary Data online.

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The diabetic milieu of hyperglycemia leads to nonenzymatic glycation of long-lived proteins (1,2). Early reaction products produced by these reactions, called Amadori products, include hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), an adduct on the hemoglobin molecule, and fructoselysine (fructosamine), a glucose adduct in blood and tissue proteins. Late-stage products, called advanced glycation end products (AGEs), are brought about by glycooxidative/lipoxidative processes in the presence of reactive oxygen and nitrosylating species, free metals, and rearrangements of the Amadori products (3). AGEs include modifications of arginine residues by glyoxal and methylglyoxal (G<sup>-1</sup>H and MG<sup>-1</sup>H, respectively), lysine adducts such as N<sup>ε</sup>-carboxymethyllysine (CML) and N<sup>ε</sup>-carboxyethyllysine (CEL), and pentosidine. The latter is a lysine-arginine AGE crosslink derived from oxidized glucose or dehydroascorbic acid, an oxidation product of vitamin C. The formation and accumulation of AGEs are implicated in the progression of age-related diseases and the microvascular and macrovascular complications of type 2 diabetes (T2D) (4,5). These glycation products, in addition to causing structural changes in long-lived proteins, increase vascular permeability, interfere with nitric oxide-mediated vasodilation, oxidize LDL, and bind to surface receptors for AGEs on macrophages and endothelial cells to induce the secretion of cytokines, growth factors, and reactive oxygen species (5).

Pilot studies have shown that salsalate, a nonacetylated salicylate, lowers blood glucose levels in patients with T2D (6,7). The Targeting Inflammation Using Salsalate for Type 2 Diabetes (TINSAL-T2D) trials were larger studies conducted to determine the efficacy of salsalate as a treatment modality for T2D, as well as to assess parameters of safety. Stage 1 of TINSAL-T2D was a dose-ranging study that treated patients with T2D for 14 weeks (*n* = 128); all three doses (3.0, 3.5, and 4.0 g/day) of salsalate showed decreased HbA<sub>1c</sub> and fasting glucose levels (8). Stage 2 of TINSAL-T2D (*n* = 286) was conducted for 48 weeks to

assess the magnitude and durability of glycemic efficacy over 1 year, tolerability, and an array of safety parameters relevant to patients with diabetes. It showed decreased HbA<sub>1c</sub> and fasting glucose levels as well as markers of inflammation (9). In this ancillary study of stage 2 TINSAL-T2D, we measured levels of early and late serum glycation products in a subset of subjects enrolled in the parent trial. We reasoned that if salsalate reduces levels of glucose and early glycation products as well as markers of inflammation, then levels of AGEs might also be lower. We also examined if changes in markers of inflammation, many of which are related to oxidative stress, were related to changes in levels of early glycation-reaction products and AGEs.

## RESEARCH DESIGN AND METHODS

Stage 2 of TINSAL-T2D (9) was a single-masked placebo lead-in, randomized, double-masked, placebo-controlled clinical trial of adult patients ≤75 years old with HbA<sub>1c</sub> levels of 7.0 to 9.5% at screening. Treatment included 1 week of screening, a 4-week single-masked placebo run-in, pretreatment baseline evaluation, and a 48-week treatment period. Salsalate was administered at 3.0 g/day for 2 weeks, then escalated to 3.5 g/day, as tolerated, divided into three daily doses, or a matching placebo. During the initial 24 weeks of the trial, it was suggested that patients maintain stable dosages of diabetes, lipid-lowering, and hypertension medications to assess drug efficacy. Reductions in doses of diabetes medications were made, however, if the patient experienced hypoglycemia. Subsequent adjustments followed good clinical practice with planned “rescue therapy” for poorly controlled diabetes.

All participating institutions in the study obtained institutional review board approval, and all participants signed informed consent.

### Measurement of AGEs

Serum AGEs, early and advanced (CML, CEL, furosine, G<sup>-1</sup>H, and MG<sup>-1</sup>H), were determined in acid hydrolyzed fractions essentially as previously published (10), except that quantitation was performed using liquid chromatography (LC) mass spectrometry (MS) as described by

Fan et al. (11). Briefly, an aliquot of serum (90 μL) was mixed with 90 μL cold trichloroacetic acid and left on ice for 20 min. Samples were centrifuged at 15,000 rpm for 10 min, and the pellet was washed three times with cold ether. The pellet was transferred to a Pyrex tube with Teflon cap, and 3 mL of deoxygenated 6 N HCl was added. Test tubes were blanketed with argon and heated to 110°C for 17 h. Samples were dried under vacuum and reconstituted in 1 mL of water. Samples were cleaned over Spin-X filters, and ninhydrin assay was used for estimation of 100 μg aliquots of sample using leucine content. Aliquots of 100 μg were combined with added isotopically labeled internal standards, and 40 μg was injected into the LC-MS/MS instrument.

Pentosidine was also measured in the acid hydrolysate (12) for selected serum samples (*n* = 12) that were increased from baseline to weeks 12 and 48 of treatment. This was done to validate the fluorescence method. First, we synthesized D<sub>8</sub>-labeled pentosidine as an internal standard as follows. N $\alpha$ -t-BOC-L-arginine (~3.0 mmol, 880 mg) and DL-lysine-3,3,4,4,5,5,6,6,-d<sub>8</sub> 2HCl (~1 mmol, 227 mg) along with D-ribose (5 mmol, 750 mg) were dissolved in 2 mL of 1 mol/L ammonium bicarbonate (pH ~8.0). O<sub>2</sub> was bubbled overnight into the solution held in a water bath at 50°C. The reaction mixture was frozen and lyophilized. After 25 mL of 3 mol/L HCl was added, the mixture was brought to 100°C with a stream of N<sub>2</sub> to evaporate the HCl. The residue was reconstituted with 50 mL H<sub>2</sub>O and passed over a Dowex cation W50 exchange resin in H<sup>+</sup> form. Elution was initiated by adding two bed volumes of 100 mmol/L HCl and two bed volumes of 500 mmol/L HCl followed by pyridine acetate 0.5 mol/L pH 5.1. Fractions of 15 mL were collected. Fluorescent material was monitored by thin-layer chromatography with a Woods ultraviolet lamp. Fractions corresponding to product were pooled, evaporated, and reconstituted in 1.0 mL of water and injected for pentosidine analysis.

D<sub>8</sub>-pentosidine purification was carried using a Discovery C18 BIO Wide Pore 25 cm × 10 mm, 5 μm semipreparative

column. The mobile phase was 1% trifluoroacetic acid, 2.5% acetonitrile in water (0–2 min) with a linear gradient (2–25 min) to 47% solvent B (60% acetonitrile). The flow rate was 2.0 mL/min, and eluate fluorescence was monitored at 335/385 nm. The material eluting between 20 and 28 min was, repetitively, collected and lyophilized to dryness. It was reconstituted in 1 mL H<sub>2</sub>O to make stock. Stock was calibrated by fluorescence high-performance LC using unlabeled pentosidine standard.

We performed pentosidine analysis by LC-MS/MS using isotope dilution technique as follows. Samples were assayed by LC-MS/MS using a 2690 separation module with a Quattro Ultima triple quadrupole mass spectrometric detector (Waters-Micromass). A 5- $\mu$ m, 2.1  $\times$  50-mm<sup>2</sup> Hypercarb (Thermo Scientific) column was used. The mobile phase was 0.1% trifluoroacetic acid with a linear gradient of 10–50% acetonitrile from 0 to 15 min and isocratic 50% acetonitrile thereafter. The flow rate was 0.2 mL/min. The ionization source temperature was 120°C, and the desolvation gas temperature was 350°C. The cone gas and desolvation gas flow rates were 200 and 600 L/h, respectively. The capillary voltage was 3.20 kV, and the cone voltage was 60 V. Argon gas (2.7  $\times$  10<sup>-3</sup>) was in the collision cell. Eighty micrograms of protein equivalent was injected with 2 pmol of internal standard spike. Native pentosidine MS/MS fragment was monitored for the transition *m/z* 379.48 > 250.46 at retention time of 24.79 min. D<sub>8</sub>-pentosidine fragment was monitored at 387.7 > 250.68 at retention time of 24.74 min. Comparison of pentosidine values measured by LC/MS versus fluorescence had a linear correlation with regression equation  $y = 1.056 \times -0.338$  ( $P < 0.0001$ ), validating thereby the fluorescence measurements.

#### Statistical Analysis

Baseline characteristics between the salsalate and placebo groups were compared using *t* tests for continuous traits and  $\chi^2$  or Fisher exact tests for categorical traits. The log transform of triglyceride was used because this variable is not normally distributed.

General estimating equations were used to compare the baseline characteristics of this subsample to the parent TINSAL-T2D cohort.

In intention-to-treat analysis, we estimated mean treatment group differences for each AGE measurement (CML, CEL, furosine, G<sup>-1</sup>H, MG<sup>-1</sup>H, and pentosidine) in linear regression mixed models, adjusted for baseline levels over the 48-study-week period. Values were adjusted for the presence of retinopathy and coronary artery disease (CAD). Under such a model, we assumed that the correlation within each participant follows an autoregressive covariance pattern. This pattern assumes that the variability in AGE is constant regardless of when it was measured. It also assumes that the closer in time two AGE values are measured, the correlation will be higher than from time points farther apart in time. This method allows use of data for all patients until the point of dropping out of the trial or completing the trial.

We used linear regression models to test the association between changes in early and late glycation product levels with changes in inflammation factor, renal function, albumin-to-creatinine ratio, and lipid-level changes. Repeated measures mixed models were used to test associations between changes in AGE levels with markers of inflammation and renal function within each treatment group.

All analyses were done at the George Washington University Biostatistics Center using SAS version 9.2. A *P* value  $\leq 0.05$  was considered statistically significant.

#### RESULTS

There were 227 participants from the 286 stage 2 TINSAL-T2D participants who had fructoselysine (as furosine) and AGE levels measured. These 227 participants were older (56.4  $\pm$  9.6 vs. 55.8  $\pm$  9.6 years;  $P = 0.04$ ), were more likely to be white (57.3 vs. 52.8%;  $P = 0.004$ ) and less likely to be black (28.6 vs. 33.2%;  $P = 0.004$ ), were more likely to have hypertension (76.7 vs. 72.7%;  $P = 0.01$ ), and were more likely to be using lifestyle only for the treatment of T2D (5.7 vs. 4.5%;  $P = 0.0006$ ) than the

cohort as a whole (Supplementary Table 1).

Baseline characteristics of the substudy cohort, categorized by treatment, are shown in Table 1. The average age was 56 years, 43% were female, and 57% were white. There were few differences between participants who did and did not receive salsalate therapy except that those who received salsalate had a higher heart rate (74.1  $\pm$  10.8 vs. 71.0  $\pm$  9.1;  $P = 0.02$ ) and used less non-ACE/angiotensin receptor blocker hypertension medications (33.1 vs. 46.8%;  $P = 0.03$ ) than those who received placebo. There were no statistically significant differences between the treatment groups with respect to levels of creatinine, cystatin C, cystatin-C-based estimated glomerular filtration rate (eGFR), or albumin-to-creatinine ratios.

The association of AGEs with baseline measures of inflammation, cytokines, and renal function is shown in Supplementary Table 2. There were no significant associations other than CEL levels with adiponectin. Given the multiplicity of analyses, this finding should be interpreted with caution.

The changes in selected variables from baseline to the end of follow-up between participants who received salsalate or a placebo are shown in Table 2. In participants receiving salsalate, levels of creatinine rose, while cystatin C levels decreased. The cystatin-based eGFR change was not significantly different between those receiving salsalate and those receiving placebo. As previously reported (9), salsalate therapy was associated with increased urine albumin-to-creatinine ratio levels ( $P < 0.001$ ), LDL cholesterol ( $P < 0.001$ ), and total cholesterol ( $P = 0.043$ ) compared with levels in those receiving placebo.

Mean treatment group differences, using linear regression mixed models adjusted for baseline levels, retinopathy, and CAD, are shown in Table 3. As previously reported (9), participants who received salsalate had significantly lowered HbA<sub>1c</sub>. Compared with participants who received placebo, participants who received salsalate also had significantly lower furosine ( $P < 0.001$ ) and CML

**Table 1—Baseline characteristics of the TINSAL-T2D subcohort that had AGE testing stratified into those receiving salsalate therapy or placebo**

	Total <i>n</i> = 227	Salsalate <i>n</i> = 118	Placebo <i>n</i> = 109	<i>P</i> value
Baseline characteristics				
Age (years)	56.4 ± 9.6	56.1 ± 9.3	56.7 ± 9.9	0.64
Female sex, <i>n</i> (%)	97 (42.7)	47 (39.8)	50 (45.9)	0.36
Race/ethnicity, <i>n</i> (%)				
White	130 (57.3)	64 (54.2)	66 (60.6)	0.34
Black	65 (28.6)	35 (29.7)	30 (27.5)	0.72
Other	32 (14.1)	19 (16.1)	13 (11.9)	0.37
Weight (kg)	96.7 ± 22.8	97.7 ± 23.2	95.5 ± 22.4	0.47
BMI (kg/m <sup>2</sup> )	33.0 ± 7.0	33.0 ± 7.0	33.0 ± 7.0	0.88
Time since diabetes diagnosis (years)	6.9 ± 6.0	6.9 ± 6.3	6.9 ± 5.7	0.95
Medical history, <i>n</i> (%)				
Established CAD	27 (11.9)	15 (12.7)	12 (11.0)	0.69
Hypertensive	174 (76.7)	90 (76.3)	84 (77.1)	0.89
Dyslipidemia	162 (71.4)	83 (70.3)	79 (72.5)	0.72
Retinopathy requiring laser therapy	39 (17.2)	22 (18.6)	17 (15.6)	0.54
Family history of type 1 diabetes	11 (4.8)	7 (5.9)	4 (3.7)	0.54#
Family history of T2D	149 (65.6)	79 (66.9)	70 (64.2)	0.80
Family history of cardiovascular disease	129 (56.8)	63 (61.2)	66 (66.0)	0.28
Blood pressure (mmHg)				
Systolic	127 ± 13.0	126 ± 13.0	127 ± 14.0	0.66
Diastolic	77.0 ± 8.0	76.0 ± 9.0	77.0 ± 8.0	0.33
Heart rate (bpm)	72.6 ± 10.1	74.1 ± 10.8	71.0 ± 9.1	<b>0.02</b>
Laboratory values				
Creatinine (μmol/L)	73.5 (14.4)	73.2 (14.8)	73.8 (14.0)	0.74
Cystatin (μg/mL)	0.96 ± 0.20	0.96 ± 0.20	0.97 ± 0.20	0.69
Cystatin-based eGFR (mL/min/1.73 m <sup>2</sup> )	90.6 (17.8)	89.6 (17.6)	90.6 (17.8)	0.67
Albumin (g/dL)	4.4 ± 0.27	4.4 ± 0.27	4.4 ± 0.27	0.32
Log urinary albumin-to-creatinine ratio (μg/mg)	9.6 (2.8)	9.9 (3.0)	9.2 (2.6)	0.60
HbA <sub>1c</sub> (%)	7.7 ± 0.7	7.7 ± 0.7	7.7 ± 0.7	0.81
Fasting glucose (mg/dL)	151 ± 39.0	153 ± 39.0	149 ± 39.0	0.39
Triglycerides (mmol/L)*	142 (486)	138 (468)	143 (465)	<b>0.88**</b>
Cholesterol (mmol/L)	165 ± 39.0	163 ± 37.0	167 ± 41.0	0.51
HDL (mmol/L)	47. ± 13.0	46.0 ± 13.0	48.0 ± 12.0	0.35
LDL (mmol/L)	102 ± 33.0	100 ± 31.0	103 ± 34.0	0.50
Medications, <i>n</i> (%)				
Metformin	197 (86.8)	101 (85.6)	96 (88.1)	0.58
Insulin secretagogue	121 (53.3)	69 (58.5)	52 (47.7)	0.10
α-Glucosidase inhibitor	1 (0.4)	1 (0.8)	0 (0.0)	1.00#
Dipeptidyl peptidase-4 inhibitor	33 (14.5)	18 (15.3)	15 (13.8)	0.75
Monotherapy	90 (39.6)	45 (38.1)	45 (41.3)	0.63
Dual therapy	110 (48.5)	57 (48.3)	53 (48.6)	0.96
Lifestyle only	13 (5.7)	6 (5.1)	7 (6.4)	0.78#
On lipid medication, <i>n</i> (%)				
On statin	138 (60.8)	74 (62.7)	64 (58.7)	0.54
On other lipid medication	23 (10.1)	12 (10.2)	11 (10.1)	0.98
On antihypertensive medication, <i>n</i> (%)				
On ACE/angiotensin receptor blocker	132 (58.1)	69 (58.5)	63 (57.8)	0.92
On other antihypertensive	90 (39.6)	39 (33.1)	51 (46.8)	<b>0.03</b>
On low-dose aspirin, <i>n</i> (%)	93 (41.0)	46 (39.0)	47 (43.1)	0.53

Boldface data indicate statistical significance at the *P* < 0.05 level. #Fisher exact test. \*Median (interquartile range). \*\*Based on a *t* test using log triglyceride.

(*P* = 0.022) levels, while CEL, G<sup>-1</sup>H, and MG<sup>-1</sup>H levels remained unchanged. Pentosidine levels increased significantly (*P* < 0.001). In order to

exclude the presence of a fluorescent artifact, the 12 highest values of pentosidine (Supplementary Figure 1) were verified by LC/MS/MS using

isotope dilution with standard <sup>13</sup>C<sub>6</sub>-pentosidine prepared from <sup>13</sup>C<sub>6</sub>-lysine and D-ribose as described in the Supplementary Data. The results were

**Table 2—Mean differences in selected laboratory values from baseline to the end of follow-up of TINSAL-T2D participants who had AGE testing stratified by receiving salsalate therapy or placebo**

	Salsalate		Placebo		Mean difference (95% CI)**	
		<i>P</i> value*		<i>P</i> value*		<i>P</i> value*
Creatinine (μmol/L)	0.92 (−0.55 to 2.38)	0.22	−1.20 (−2.69 to 0.29)	0.11	2.12 (0.03–4.21)	<b>0.047</b>
Cystatin (μg/L)	−0.023 (−0.42 to −0.004)	<b>0.018</b>	−0.14 (−0.34 to 0.005)	0.14	−0.009 (−0.31 to 0.02)	0.527
Cystatin C eGFR (mL/min/1.73 m <sup>2</sup> )	2.6 (0.70–4.40)	<b>0.008</b>	1.1 (−0.80 to 3.00)	0.28	1.5 (−1.2 to 4.1)	0.28
Urinary albumin-to-creatinine ratio (μg/mg)	8.0 (−112.0 to 257)#	<b>&lt;0.001</b> §	1.0 (−106 to 479)#	0.18§		<b>&lt;0.001</b> §
LDL (direct) (mmol/L)	0.24 (0.15–0.33)	<b>&lt;0.001</b>	−0.03 (−0.11 to 0.06)	0.57	0.27 (0.14–0.39)	<b>&lt;0.001</b>
HDL (mmol/L)	0.02 (−0.01 to 0.05)	0.18	0.04 (0.01–0.06)	<b>0.012</b>	−0.02 (−0.06 to 0.02)	0.38
Total cholesterol (mmol/L)	0.17 (0.07–0.27)	<b>&lt;0.001</b>	0.03 (−0.07 to 0.13)	0.58	0.14 (0.00–0.29)	<b>0.043</b>

Boldface data indicate statistical significance at the *P* < 0.05 level. \*\*3.5 g salsalate minus placebo. \*H<sub>0</sub>, *u* = 0. #Median (minimum–maximum). §Wilcoxon rank test.

consistent with the fluorescence high-performance LC findings.

The changes in early and late glycation products in relation to changes in levels of adiponectin, inflammation factor levels, renal function, albuminuria, and lipid levels are shown in Table 4. Owing to the many analyses, a *P* value of <0.05 should be interpreted with caution. Changes in HbA<sub>1c</sub> levels were significantly and inversely related to

adiponectin and cystatin C levels in salsalate users. Thus for every 1 μg/mL increase in adiponectin, the HbA<sub>1c</sub> level declined 0.07% (95% CI −0.12 to −0.02; *P* = 0.003) during follow-up. There was a positive (*P* = 0.04) association of HbA<sub>1c</sub> change with C-reactive protein (CRP) change and a negative association (*P* = 0.02) with HDL cholesterol change. Changes in furosine levels among salsalate users were positively related to

CRP and tumor necrosis factor (TNF)-α level changes and trended inversely with changes in adiponectin. CML levels trended positively in relation to increased CRP levels. There were no other significant relationships between changes in other glycation product level and changes in levels of inflammation factors, adiponectin, lipids, renal function, and albuminuria, including pentosidine.

**Table 3—Mean differences in early glycation product and AGE levels in TINSAL-T2D participants treated with salsalate or who received placebo over 48 weeks**

AGE outcome by treatment group	Mean Δ*** (95% confidence limits)	<i>P</i> value*	Mean difference*** between salsalate and placebo groups (95% confidence limits)#	<i>P</i> value**
HbA <sub>1c</sub> (%)			−0.37 (−0.54 to −0.19)	<b>&lt;0.001</b>
Placebo	0.01 (−0.12 to 0.13)	0.89		
3.5 g	−0.36 (−0.48 to −0.24)	<b>&lt;0.001</b>		
Furosine (mmol/mol)			−0.11 (−0.16 to −0.05)	<b>&lt;0.001</b>
Placebo	0.06 (0.02–0.10)	<b>0.002</b>		
3.5 g	−0.05 (−0.08 to −0.01)	<b>0.018</b>		
CML (μmol/mol)			−7.63 (−14.2 to −1.04)	<b>0.023</b>
Placebo	4.95 (0.24–9.65)	<b>0.04</b>		
3.5 g	−2.68 (−7.26 to 1.90)	0.25		
CEL (μmol/mol)			−0.24 (−0.60 to 0.12)	0.20
Placebo	0.12 (−0.14 to 0.038)	0.37		
3.5 g	−0.12 (−0.37 to 0.13)	0.35		
G <sup>−1</sup> H (mmol/mol)			1.15 (−0.24 to 2.54)	0.11
Placebo	−0.27 (−1.26 to 0.72)	0.59		
3.5 g	0.87 (−0.10 to 1.85)	0.08		
MG <sup>−1</sup> H (mmol/mol)			7.66 (−10.3 to 25.63)	0.40
Placebo	3.81 (−8.97 to 16.58)	0.56		
3.5 g	11.46 (−1.18 to 24.11)	0.08		
Pentosidine (nmol/mol)			553.2 (408.1–698.3)	<b>&lt;0.001</b>
Placebo	−4.00 (−108 to 99.87)	0.94		
3.5 g	549.2 (448.1–650.4)	<b>&lt;0.001</b>		

Boldface data indicate statistical significance at the *P* < 0.05 level. Models are adjusted for retinopathy and CAD. \*\*\*Testing the null hypothesis that Δ (change from baseline) = 0. \*Models are adjusted for history of CAD and retinopathy. \*\*AGEs are expressed per mol amino acid modified. #Testing the null hypothesis that salsalate minus placebo = 0.

**Table 4—Change in early glycation product and AGE levels over 48 weeks of follow-up as related to changes in inflammation factors (CRP, TNF- $\alpha$ , WBC, and types of WBC), free fatty acid, adiponectin, cystatin C, albumin-to-creatinine levels, and cholesterol and its subfractions categorized by treatment**

AGE measurement ( $\Delta$ )	Placebo		3.5 g	
	Estimate (95% CI)	P value	Estimate (95% CI)	P value
<b>HbA<sub>1c</sub> (%)</b>				
CRP ( $\mu\text{g/mL}$ )	0.00 (−0.01 to 0.01)	0.97	0.01 (0.00–0.03)	0.04
Adiponectin ( $\mu\text{g/mL}$ )	−0.03 (−0.10 to 0.04)	0.34	−0.07 (−0.12 to −0.02)	0.003
Cystatin C ( $\mu\text{g/mL}$ )	−0.93 (−1.73 to −0.14)	0.02	−1.27 (−1.95 to −0.58)	<0.001
Cystatin C eGFR (mL/min)	0.01 (0.00–0.02)	0.04	0.01 (0.01–0.02)	<0.0001
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	−0.00 (−0.00 to 0.00)	0.77	0.00 (−0.00 to 0.00)	0.94
FFA conc. (mmol/L)	−0.04 (−0.38 to 0.30)	0.81	0.08 (−0.21 to 0.37)	0.59
TNF- $\alpha$ (pg/mL)	0.01 (−0.22 to 0.25)	0.91	0.07 (−0.20 to 0.34)	0.61
WBC ( $\times 10^9$ cells/L)	0.03 (−0.06 to 0.12)	0.51	−0.04 (−0.13 to 0.05)	0.37
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	0.19 (−0.04 to 0.42)	0.11	0.04 (−0.21 to 0.29)	0.75
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	−0.01 (−0.11 to 0.09)	0.87	−0.03 (−0.12 to 0.06)	0.51
LDL (mmol/L)	0.11 (−0.05 to 0.27)	0.16	0.03 (−0.14 to 0.20)	0.71
HDL (mmol/L)	0.20 (−0.46 to 0.86)	0.55	−0.81 (−1.48 to −0.14)	0.02
Cholesterol (mmol/L)	0.14 (0.01–0.26)	0.04	0.05 (−0.09 to 0.20)	0.47
<b>Furosine (mmol/mol)</b>				
CRP ( $\mu\text{g/mL}$ )	−0.00 (−0.01 to 0.00)	0.46	0.00 (0.00–0.01)	0.04
Adiponectin ( $\mu\text{g/mL}$ )	0.00 (−0.03 to 0.03)	0.92	−0.01 (−0.02 to 0.00)	0.10
Cystatin C ( $\mu\text{g/mL}$ )	−0.16 (−0.48 to 0.16)	0.33	0.07 (−0.13 to 0.27)	0.49
Cystatin C eGFR (mL/min)	0.01 (−0.01 to 0.02)	0.39	0.00 (−0.01 to 0.01)	0.56
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	0.00 (−0.00 to 0.00)	0.62	−0.00 (−0.00 to 0.00)	0.65
FFA conc. (mmol/L)	0.13 (−0.01 to 0.26)	0.07	0.04 (−0.05 to 0.12)	0.37
TNF- $\alpha$ (pg/mL)	−0.03 (−0.12 to 0.07)	0.57	0.08 (0.00–0.15)	0.05
WBC ( $\times 10^9$ cells/L)	−0.01 (−0.04 to 0.03)	0.70	−0.01 (−0.03 to 0.01)	0.44
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	0.07 (−0.01 to 0.16)	0.10	0.01 (−0.06 to 0.08)	0.79
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	−0.02 (−0.06 to 0.01)	0.19	−0.01 (−0.04 to 0.01)	0.31
LDL (mmol/L)	−0.01 (−0.06 to 0.05)	0.86	0.04 (−0.00 to 0.09)	0.08
HDL (mmol/L)	0.00 (−0.25 to 0.25)	0.99	−0.14 (−0.32 to 0.04)	0.14
Cholesterol (mmol/L)	−0.00 (−0.05 to 0.05)	0.85	0.04 (−0.00 to 0.08)	0.05
<b>CML (<math>\mu\text{mol/mol}</math>)</b>				
CRP ( $\mu\text{g/mL}$ )	0.14 (−0.40 to 0.68)	0.60	0.49 (−0.02 to 1.00)	0.06
Adiponectin ( $\mu\text{g/mL}$ )	2.53 (−0.29 to 5.36)	0.08	−0.27 (−2.00 to 1.47)	0.76
Cystatin C ( $\mu\text{g/mL}$ )	8.78 (−24.60 to 42.17)	0.60	−2.54 (−27.84 to 22.76)	0.84
Cystatin C eGFR (mL/min)	−0.10 (−0.44 to 0.25)	0.58	−0.06 (−0.33 to 0.21)	0.64
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	−0.03 (−0.12 to 0.06)	0.52	0.00 (−0.04 to 0.05)	0.88
FFA conc. (mmol/L)	4.42 (−9.73 to 18.57)	0.54	1.98 (−8.76 to 12.73)	0.72
TNF- $\alpha$ (pg/mL)	−2.31 (−11.99 to 7.37)	0.64	2.41 (−7.07 to 11.90)	0.61
WBC ( $\times 10^9$ cells/L)	−1.01 (−4.60 to 2.58)	0.58	−1.66 (−4.69 to 1.38)	0.28
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	1.85 (−7.36 to 11.06)	0.69	3.88 (−4.70 to 12.46)	0.37
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	−1.32 (−5.00 to 2.36)	0.48	−2.20 (−5.29 to 0.90)	0.16
LDL (mmol/L)	0.28 (−5.76 to 6.33)	0.93	−3.88 (−9.65 to 1.90)	0.19
HDL (mmol/L)	18.60 (−8.04 to 45.24)	0.17	−8.49 (−31.38 to 14.39)	0.46
Cholesterol (mmol/L)	−0.10 (−5.09 to 4.89)	0.97	−1.39 (−6.29 to 3.52)	0.58
<b>CEL (<math>\mu\text{mol/mol}</math>)</b>				
CRP ( $\mu\text{g/mL}$ )	0.01 (−0.03 to 0.05)	0.74	0.01 (−0.04 to 0.05)	0.79
Adiponectin ( $\mu\text{g/mL}$ )	0.02 (−0.19 to 0.24)	0.82	0.07 (−0.08 to 0.23)	0.37
Cystatin C ( $\mu\text{g/mL}$ )	−1.28 (−3.71 to 1.14)	0.30	−1.55 (−3.79 to 0.68)	0.17
Cystatin C eGFR (mL/min)	0.01 (−0.02 to 0.03)	0.49	0.02 (−0.01 to 0.04)	0.20
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	0.00 (−0.00 to 0.01)	0.55	−0.00 (−0.01 to 0.00)	0.40
FFA conc. (mmol/L)	0.30 (−0.73 to 1.33)	0.56	−0.38 (−1.36 to 0.59)	0.44
TNF- $\alpha$ (pg/mL)	0.03 (−0.66 to 0.72)	0.93	−0.48 (−1.32 to 0.36)	0.26
WBC ( $\times 10^9$ cells/L)	−0.15 (−0.40 to 0.11)	0.27	−0.03 (−0.31 to 0.25)	0.82
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	−0.08 (−0.74 to 0.58)	0.82	0.14 (−0.65 to 0.93)	0.73
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	−0.14 (−0.41 to 0.13)	0.30	−0.04 (−0.33 to 0.25)	0.80
LDL (mmol/L)	−0.34 (−0.78 to 0.11)	0.14	−0.33 (−0.86 to 0.19)	0.21
HDL (mmol/L)	−0.60 (−2.54 to 1.34)	0.54	−0.64 (−2.68 to 1.40)	0.53
Cholesterol (mmol/L)	−0.30 (−0.67 to 0.07)	0.11	−0.30 (−0.75 to 0.15)	0.19
<b>G<sup>−1</sup>H (mmol/mol)</b>				
CRP ( $\mu\text{g/mL}$ )	0.09 (−0.04 to 0.22)	0.19	0.05 (−0.10 to 0.20)	0.54

Continued on p. 1089

Table 4—Continued

AGE measurement ( $\Delta$ )	Placebo		3.5 g	
	Estimate (95% CI)	<i>P</i> value	Estimate (95% CI)	<i>P</i> value
Adiponectin ( $\mu\text{g/mL}$ )	-0.07 (-0.78 to 0.64)	0.85	0.33 (-0.15 to 0.81)	0.17
Cystatin C ( $\mu\text{g/mL}$ )	1.57 (-6.47 to 9.61)	0.70	0.36 (-6.65 to 7.38)	0.92
Cystatin C eGFR (mL/min)	-0.01 (-0.10 to 0.07)	0.75	-0.04 (-0.12 to 0.04)	0.29
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	0.00 (-0.02 to 0.03)	0.72	-0.00 (-0.01 to 0.01)	0.70
FFA conc. (mmol/L)	3.68 (0.33 to 7.03)	0.03	0.22 (-2.90 to 3.34)	0.89
TNF- $\alpha$ (pg/mL)	-1.31 (-3.69 to 1.07)	0.28	0.29 (-2.16 to 2.75)	0.81
WBC ( $\times 10^9$ cells/L)	0.02 (-0.78 to 0.82)	0.96	-0.38 (-1.24 to 0.47)	0.38
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	-0.58 (-2.61 to 1.45)	0.57	2.19 (-0.22 to 4.59)	0.07
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	0.03 (-0.81 to 0.87)	0.95	-0.62 (-1.52 to 0.27)	0.17
LDL (mmol/L)	1.21 (-0.26 to 2.68)	0.11	0.10 (-1.60 to 1.79)	0.91
HDL (mmol/L)	6.81 (0.50–13.12)	0.04	-5.75 (-11.85 to 0.35)	0.06
Cholesterol (mmol/L)	0.89 (-0.33 to 2.11)	0.15	-0.60 (-2.06 to 0.86)	0.42
Log MG- <sup>1</sup> H (mmol/mol)				
CRP ( $\mu\text{g/mL}$ )	-0.00 (-0.01 to 0.01)	0.60	-0.00 (-0.02 to 0.01)	0.67
Adiponectin ( $\mu\text{g/mL}$ )	-0.03 (-0.08 to 0.03)	0.29	-0.01 (-0.05 to 0.04)	0.80
Cystatin C ( $\mu\text{g/mL}$ )	-0.35 (-0.97 to 0.28)	0.28	0.02 (-0.57 to 0.61)	0.95
Cystatin C eGFR (mL/min)	0.00 (-0.01 to 0.01)	0.70	-0.00 (-0.01 to 0.00)	0.50
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	-0.00 (-0.00 to 0.00)	0.72	-0.00 (-0.00 to 0.00)	0.24
FFA conc. (mmol/L)	-0.09 (-0.35 to 0.18)	0.51	-0.03 (-0.30 to 0.23)	0.82
TNF- $\alpha$ (pg/mL)	0.06 (-0.12 to 0.24)	0.50	-0.06 (-0.26 to 0.14)	0.57
WBC ( $\times 10^9$ cells/L)	-0.02 (-0.08 to 0.05)	0.64	0.01 (-0.06 to 0.08)	0.79
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	-0.05 (-0.22 to 0.12)	0.58	0.18 (-0.02 to 0.39)	0.08
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	-0.01 (-0.08 to 0.06)	0.78	-0.03 (-0.11 to 0.04)	0.42
LDL (mmol/L)	0.03 (-0.08 to 0.15)	0.58	0.05 (-0.09 to 0.20)	0.46
HDL (mmol/L)	0.16 (-0.34 to 0.65)	0.53	0.13 (-0.42 to 0.67)	0.65
Cholesterol (mmol/L)	0.03 (-0.06 to 0.13)	0.50	0.07 (-0.06 to 0.19)	0.30
Log pentosidine (nmol/mol)				
CRP ( $\mu\text{g/mL}$ )	-0.24 (-2.43 to 1.95)	0.83	-0.09 (-1.42 to 1.25)	0.90
Adiponectin ( $\mu\text{g/mL}$ )	0.78 (-10.75 to 12.32)	0.89	-1.76 (-9.36 to 5.84)	0.65
Cystatin C ( $\mu\text{g/mL}$ )	-3.87 (-151.54 to 143.79)	0.96	-10.72 (-76.77 to 55.33)	0.75
Cystatin C eGFR (mL/min)	0.10 (-1.40 to 1.61)	0.89	0.15 (-0.79 to 1.09)	0.75
Albumin-to-creatinine ratio ( $\mu\text{g/mg}$ )	0.01 (-0.28 to 0.30)	0.95	-0.01 (-0.21 to 0.18)	0.89
FFA conc. (mmol/L)	14.87 (-52.14 to 81.89)	0.66	-3.91 (-42.05 to 34.24)	0.84
TNF- $\alpha$ (pg/mL)	16.47 (-39.95 to 72.89)	0.56	-3.21 (-38.61 to 32.19)	0.86
WBC ( $\times 10^9$ cells/L)	-0.17 (-13.38 to 13.05)	0.98	-0.21 (-9.56 to 9.15)	0.97
Lymphocytes ( $\mu\text{L} \times 10^{-3}$ )	-11.90 (-60.95 to 37.15)	0.63	-9.47 (-60.12 to 41.18)	0.71
Neutrophils ( $\mu\text{L} \times 10^{-3}$ )	1.67 (-13.33 to 16.67)	0.83	-0.90 (-18.72 to 16.92)	0.92
LDL (mmol/L)	-1.61 (-36.35 to 33.13)	0.93	3.02 (-20.57 to 26.61)	0.80
HDL (mmol/L)	17.80 (-100.08 to 135.68)	0.76	-10.94 (-100.45 to 78.56)	0.81
Cholesterol (mmol/L)	2.24 (-18.42 to 22.90)	0.83	0.23 (-16.88 to 17.34)	0.98

Given the multiplicity of tests ( $n = 182$ ), a Bonferroni-adjusted significant *P* value ( $0.05/182$ ) is  $<0.001$ . Boldface data indicate statistical significance at the  $P < 0.001$  level. FFA conc., free fatty acid concentration.

## CONCLUSIONS

Stage 2 of the TINSAL-T2D trial showed that treatment with salsalate reduced HbA<sub>1c</sub> levels over the 48 weeks of the study (9). Anti-inflammatory effects of salsalate were evident by reductions in white blood cell (WBC), neutrophil, and lymphocyte counts. In this substudy of stage 2 of TINSAL-T2D, we report lower HbA<sub>1c</sub> and furosine levels at 12 and 48 weeks of therapy as compared with baseline levels and as compared with levels in participants who received placebo. Both HbA<sub>1c</sub> and furosine are

early glucose-derived factors (Amadori products) that are directly influenced by glycemia. Reductions in levels of these adducts, we reasoned, together with lower levels of inflammation factors, could lead to lower serum AGE levels downstream. We found that most AGE levels did not change with salsalate therapy. Plasma CML levels were lower in salsalate compared with placebo-treated groups. Change in CEL, G-<sup>1</sup>H, and MG-<sup>1</sup>H levels did not differ between treatment groups. On the other hand, pentosidine levels unexpectedly rose at both time points studied. Changes in

HbA<sub>1c</sub> levels were strongly and inversely related to cystatin C and adiponectin level changes, and furosine and CML levels had a weaker positive association with changes in CRP levels.

Most serum-protein-bound AGE levels (with the exception of CML) did not change significantly despite salsalate therapy's glycemia-lowering effect. It may be argued that the duration of the trial was too short to expect changes in AGE levels. However, Ahmed et al. (13) found a correlation between lowered HbA<sub>1c</sub> and plasma protein levels of CML,

G<sup>-1</sup>H, and hemoglobin-linked CML and pentosidine in type 1 diabetes after only 2 months of intensified insulin therapy. The reason we did not find a change in late glycation products is uncertain. One possibility is that metformin, which was taken by more than 85% of participants, was scavenging the AGE oxoaldehyde precursors glyoxal and methylglyoxal, thereby masking further decreases in CML and CEL/MG-<sup>1</sup>H levels, respectively (14). In vitro, metformin can trap glyoxal to form guanidine-glyoxal adducts (15). High doses of metformin, taken for 2 months, are reported to lower methylglyoxal plasma levels in people with diabetes (16). Another explanation is that AGEs in serum may originate from multiple pathways that are only indirectly glucose dependent, such as glyoxal for CML and G<sup>-1</sup>H and dehydroascorbic acid for pentosidine. Last, reductions in antihyperglycemic medications in salsalate-treated patients for mild hypoglycemic events and addition of antihyperglycemic treatments in placebo-treated patients for exceeding hyperglycemic safety thresholds may have diminished glycaemic and thus AGE differences between groups.

The increase in pentosidine levels was unexpected. While salicylates have intrinsic fluorescence, we do not believe that this confounded measures of pentosidine levels, as we confirmed higher values in some samples by LC/MS methods. One might anticipate reductions in this AGE based on the results of one study in which HbA<sub>1c</sub> was reduced 40% over a 2-month period of time, and pentosidine and other AGE levels were decreased by 12.2 and 25.2%, respectively (17,18). Possible explanations for the rise in pentosidine include excess production of a pentosidine precursor, such as the vitamin C oxidation product dehydroascorbate. This pathway was recently shown to explain pentosidine formation in mice in which the human vitamin C transporter had been expressed in the lens (10). There is both chemical and biological precedence for the possibility that antioxidants can paradoxically increase AGE levels. Glycation reactions that are carried out in the presence of antioxidants or

aminoguanidine can increase pentosidine and CML formation (19,20). Similarly, salicylates have been shown to increase mitochondrial hydrogen peroxide formation (21), i.e., a mechanism that could explain increased ascorbate oxidation and pentosidine formation. We note that in one clinical study, ingestion of acetylsalicylate (aspirin) inhibited intestinal absorption of exogenous ascorbic acid, possibly altering ascorbic acid metabolism (22). We also note that people with diabetes relative to people without diabetes have lower ascorbate levels as a consequence of the disease itself (23). Thus increased pentosidine level could be due to an increased dehydroascorbate-over-ascorbate ratio. Also, in the course of a clinical trial, many factors may change, such as use of over-the-counter antioxidant medications and diet. It is known that dietary factors (which were not collected in the study) can impact AGE levels (24). However, there is no reason to believe that these factors should have differed between the two study groups. Last, few studies, to our knowledge, have examined simultaneously as many AGEs as were done here, so it is not known for certain whether digression in AGE levels with a certain intervention is the exception or the rule.

Stage 2 of TINSAL-T2D trial reported that salsalate leads to decreases in the WBC count and lymphocyte and neutrophil numbers plus an increase in adiponectin levels (9) and that the reduction in HbA<sub>1c</sub> level was positively related to reduction in CRP and inversely with adiponectin change. Here we show that changes in furosine levels from baseline to the end of follow-up were positively associated with changes in CRP and TNF- $\alpha$  levels, and CML levels trended to being positively associated with CRP levels. Changes in other AGE levels were not related to changes in these inflammation markers and cytokine. This latter finding may be explained by the fact that serum inflammation markers are indirect markers of oxidative stress (25), which drive AGE formation in the tissue and thus do not necessarily reflect systemic oxidative stress (26). Finally, we note that changes in renal function and

albuminuria were not associated with changes in late glycation factor levels.

In conclusion, salsalate therapy was associated with a reduction in early glycation end product levels of HbA<sub>1c</sub> and furosine and the later product CML as compared with placebo. In contrast, other AGE products did not change, while pentosidine levels increased. The clinical significance of the increase in the biomarker pentosidine remains uncertain.

**Funding.** This study was supported by grants 1R01DK-081373-01A2 (J.I.B.) and U01 DK-74556 (S.E.S. and A.B.G.) from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** J.I.B. wrote the manuscript, reviewed the statistical analyses, and researched the data. K.A.J. did the statistical analysis and reviewed the paper for intellectual content. A.B.G. was the co-principal investigator for the TINSAL-T2D study, researched the data, helped analyze the data, and participated in the writing of the paper. S.E.S. was the principal investigator for the TINSAL-T2D study, researched the data, helped analyze the data, and participated in the writing of the paper. V.F. researched the data and reviewed the paper for intellectual content. C.S. performed the AGE assays. V.M.M. oversaw the performance of the AGE assays, reviewed the data, and participated in the writing of the manuscript. J.I.B. and V.M.M. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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