



S100 Genes are Highly Expressed in Peripheral Leukocytes of Type 2 Diabetes Mellitus Patients Treated with Dietary Therapy

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Abstract

Background and Objectives We demonstrated that the mRNA induction of S100s in rat peripheral leukocytes by severe hyperglycemia was reduced by inhibiting postprandial hyperglycemia. Here, we compared inflammatory gene expression in peripheral leukocytes between type 2 diabetes mellitus (T2DM) patients undergoing dietary therapy alone and healthy volunteers, and between T2DM patients undergoing dietary therapy alone and those undergoing such therapy in combination with drug therapy using the α -glucosidase inhibitor miglitol.

Methods T2DM patients who had undertaken dietary therapy alone or in combination with drug therapy using miglitol for ≥ 8 weeks and healthy volunteers were subjected to a meal tolerance test and glucose concentration, neutrophil elastase concentration, and mRNA expression analyses of peripheral leukocytes by microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) immediately before and 180 min after a meal.

Results Blood glucose concentrations 60 min after a meal were lower in T2DM patients with dietary + miglitol therapy than in those with dietary therapy alone. Neutrophil elastase concentrations at 60 and 120 min after a meal were lower in T2DM patients with dietary + miglitol therapy than in those with dietary therapy alone. Expression levels of *S100A8* in a fasting state and *S100A6*, *S100A8*, and *S100A9* 180 min after a meal were higher in T2DM patients with dietary therapy alone than in healthy volunteers. Expression levels of *S100A12* in a fasting state and 180 min after a meal were higher in T2DM patients with dietary therapy alone than in T2DM patients with dietary + miglitol therapy.

Conclusions S100 genes were more highly expressed in T2DM patients with dietary therapy than in healthy volunteers.

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Key Points

Inflammatory gene levels in peripheral leukocytes were measured.

S100 genes were more highly expressed in type 2 diabetes mellitus (T2DM) patients than in healthy volunteers.

Expression of the *S100I2* gene was lower in T2DM patients with miglitol therapy.

1 Introduction

A double-blind, randomized, placebo-controlled trial (RCT), called the Study to Prevent Non-Insulin-Dependent Diabetes Mellitus (STOP-NIDDM), including 1429 participants aged 40–70 years, showed that the administration of the α -glucosidase inhibitor (α -GI) acarbose, which suppresses blood glucose increases after meal intake by inhibiting α -glucosidases in the small intestine, tracked for an average of 3.3 years, significantly reduced the incidence of cardiovascular disease (CVD) and hypertension [1]. In the Acarbose Cardiovascular Evaluation (ACE) double-blind RCT, a phase 4 trial for 176 patients in China with coronary heart disease and impaired glucose tolerance, acarbose reduced the incidence of diabetes, but not major CVD events [2]. Regarding food intake, a cohort study of 75,521 middle-aged American women also showed that higher intake of foods with a lower glycemic index, an indicator of an increase in blood glucose concentration after a meal compared with reference foods, such as white bread and white rice, reduced the incidence of CVD [3]. Therefore, the suppression of postprandial hyperglycemia after meal intake in people with impaired glucose tolerance or type 2 diabetes mellitus (T2DM) can reduce the development and progression of T2DM and associated complications such as CVD. However, the mechanism by which repeated blood glucose increases after meal intake induce the onset of arteriosclerosis-related diseases in T2DM patients remains unclear.

Our recent studies have demonstrated that inflammatory genes such as interleukin-1 β (*Il1b*) and tumor necrosis factor- α (*Tnfa*) as well as S100 protein family members such as *S100a4*, *S100a6*, *S100a8*, *S100a9*, and *S100a11* were highly expressed in peripheral leukocytes in streptozotocin (STZ)-induced severely hyperglycemic rats. It was also indicated that such expression was reduced by inhibiting the postprandial hyperglycemia associated with dietary intake via the administration of the α -GI miglitol for 20 days [4]. Additionally, repeated single-dose daily sucrose oral loading for 4 days led to increased expression of *Il1b* and *Tnfa* in peripheral leukocytes of moderately postprandially

hyperglycemic rats [5]. The reduction of glucose fluctuations by switching the α -GI from acarbose or voglibose to miglitol, the latter of which strongly reduces postprandial hyperglycemia compared with the other two, in 43 T2DM patients for 3 months reduced the mRNA expression of *IL1B*, *TNFA*, and *S100A4/6/9/10/11/12* in peripheral leukocytes [6]. These results indicate that the repeated induction of postprandial hyperglycemia by meal intake may induce the development of T2DM and subsequent arteriosclerosis-related diseases including CVD by enhancing the expression of inflammatory genes in peripheral leukocytes. It has been reported that proinflammatory cytokines such as *IL1B* and *TNFA*, as well as chemokines, which are frequently induced by oxidative stress, promote the migration of leukocytes of the innate immune system, such as neutrophils, monocytes, and macrophages [7]. Leukocyte migration is the first step of atherosclerosis because the neutrophils secrete reactive oxygen species (ROS) and proteolytic enzymes, resulting in damage to blood vessels. Subsequently, macrophages, which are differentiated from monocytes, promote atheroma formation by incorporating cholesterol at the sites of the vessels damaged by neutrophils [8–10]. Regarding the S100 protein family, several studies have demonstrated that the heterodimer S100A8–S100A9 induces the expression of inflammatory chemokines, macrophage activation, and infiltration of macrophages into the vascular endothelium [11–13]. Additionally, S100A12 binds receptors for advanced glycation end products and enhances *TNFA* and vascular cell adhesion molecule 1 (VCAM1) expression in mice [14]. It is very likely that these S100 proteins are related to the progression of T2DM and arteriosclerosis-related diseases, including CVD, and can be more predictive of the development and progression of these diseases than proinflammatory cytokines because S100 proteins are located upstream of proinflammatory cytokine expression, as mentioned above. However, it remains unclear whether the expression levels of S100 family proteins in peripheral leukocytes are higher in T2DM patients than in healthy individuals and whether such expression is induced by meal intake in T2DM patients and healthy individuals. Additionally, it remains unclear whether the expression of S100 family proteins in peripheral leukocytes is lower in T2DM patients administered drugs for reducing postprandial hyperglycemia than in those without this.

Therefore, in the present study, a meal tolerance test (MTT) was conducted in healthy volunteers and in T2DM patients with dietary therapy alone for 8 weeks and in those with this therapy in combination with drug therapy using miglitol for 8 weeks. Using blood samples from these participants, we examined whether the mRNA expression of inflammatory genes including those encoding S100 proteins in the peripheral leukocytes differed among the groups.

2 Material and Methods

2.1 Ethical Approval of the Study Protocol

This study was a prospective exploratory trial conducted in a hospital setting (Naka Kinen Clinic, Ibaraki) in Japan. The University Hospital Medical Information Network registration number for the trial is UMIN000018370 (<http://www.umin.ac.jp/english/>). The study protocol was approved by the Ethics Committee of Naka Kinen Clinic. Study participants provided written informed consent for the use of their personal and health information in our analysis.

2.2 Inclusion and Exclusion Criteria

We reviewed the clinical records of potential participants and identified those who met the inclusion criteria but not the exclusion criteria for our study.

2.2.1 Type 2 Diabetes Mellitus Patients

Inclusion criteria were male and female T2DM patients, aged 20–74 years, with glycosylated hemoglobin (HbA1c) (National Glycohemoglobin Standardization Program) of 6.5–8.5%. Those treated with dietary therapy alone and those treated with drug therapy using miglitol (50 or 75 mg) at each meal in combination with dietary therapy for ≥ 8 weeks before study commencement in the clinic were included (those with dietary therapy alone: $N = 125$; those with dietary + miglitol therapy: $N = 28$). In accordance with the exclusion criteria, we excluded individuals who were or could have been pregnant or were trying to become pregnant; those with severe nephropathy; hepatic disorders; CVD (diagnosed within 24 weeks before study commencement); impaired pulmonary function, pancreatopathy, cancer, myocardial infarction, or a cardiac arrest or stroke within 24 weeks of study commencement; diabetic ketosis or were in a diabetic coma; those with inadequate management of blood pressure; a need for an immediate change of medications; those undergoing insulin therapy; and those who required modification of medications within 24 weeks of study commencement. We also excluded T2DM patients with severe diabetic complications (neural disorder, retinopathy, nephropathy); severe endocrine disorders, severe anemia, and diseases of the intestine with malfunction of digestion and absorption; a history of surgery within the digestive system (excluding appendectomy and cholecystectomy) > 1 year before study commencement; malfunction of the pituitary gland; dysfunction of the adrenal glands; a history of hypersensitivity to miglitol, or lactic acidosis or digestion problems; and those who were alcoholics; malnourished, undernourished,

or did not eat meals regularly; those who had not altered their diet and/or exercise for ≥ 8 weeks before study commencement; had severe infectious diseases; had an external injury; those who would be undergoing surgery; and those who had gastrointestinal disorders (e.g., diarrhea, vomiting). We also excluded patients who were considered unsuitable for the trial by doctors. In total, 30 patients in the group with dietary therapy alone and six patients with miglitol therapy were removed. Using these inclusion and exclusion criteria, we recruited patients based on numerical targets ($N = 15$ for each group) (Fig. 1).

2.2.2 Healthy Participants

We recruited healthy volunteers in the hospital. The volunteers, who were relatives of patients receiving medical treatment in the hospital, were approached, and we enrolled the participants in order. The inclusion criteria for healthy volunteers were a body mass index (BMI) of 18.5–24.9 kg/m² and aged 20–59 years. These volunteers were among those who visited the hospital between July 2015 and November 2015. Regarding the healthy volunteers, we excluded those who could not provide their medical history; had a surface wound; had impaired mental capacity; were dependent upon alcohol or drugs; were addicted to drugs; had a food allergy; smoked > 10 cigarettes per day, and were positive for human immunodeficiency virus antigen and antibody, hepatitis B antigen, hepatitis C virus antibody, or syphilis.

2.3 Study Cohort

This study involved open-labeled exploratory research. Therefore, we did not calculate the sample size. However, our previous study demonstrated that a single dose of acarbose in a meal test reduced postprandial hyperglycemia and tended to reduce *IL1B* expression in peripheral leukocytes in 16 Japanese diabetic patients with similar HbA1c levels [15]. Therefore, in this study, we set a target sample size of 15 in each group (healthy volunteers, T2DM patients with dietary therapy alone, and T2DM patients treated with miglitol therapy). Finally, we successfully recruited 15 participants in each group. We recruited 30 T2DM patients and 15 healthy volunteers. Participants were grouped as healthy volunteers (C group), T2DM patients with dietary therapy alone (N group), and T2DM patients treated with miglitol therapy (M group). All patients and healthy volunteers were enrolled before starting the trial. Patients had been undergoing stable therapy (including miglitol treatment) for ≥ 8 weeks before entering the study. All 45 individuals completed the trial, in whom, we analyzed the relationship between blood glucose concentrations and mRNA levels in peripheral leukocytes. Among the participants, one individual in the

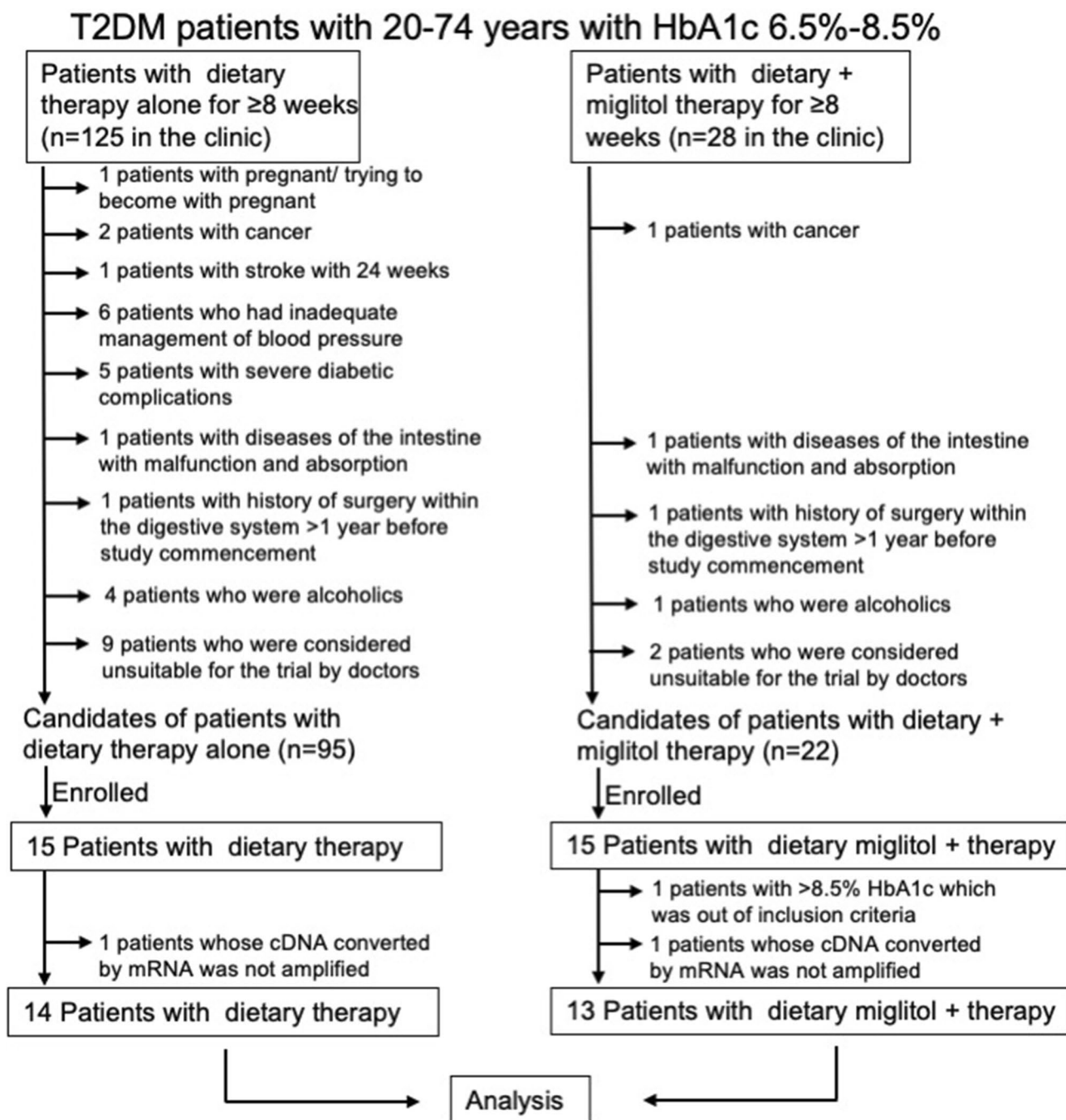


Fig. 1 Flow chart of enrollment. *HbA1c* glycated hemoglobin, *T2DM* type 2 diabetes mellitus

M group was removed because of an HbA1c level of over 8.5% at the trial and enrollment periods, which failed to meet the inclusion criteria at enrollment. Additionally, one participant in each group was removed because of poor amplification of mRNA. Therefore, we finally analyzed the data of 14 individuals in the C group, 14 in the N group, and 13 in the M group (Table 1).

2.4 Meal Tolerance Test

The height (AD-6225A; A&D Medical, Tokyo, Japan) and body weight (AD-6207A; A&D Medical) of the participants were measured (X-SCAN PLUS; Owa Medical, Fukuoka, Japan), and HbA1c levels as well as serum concentrations of glycoalbumin (GA) were determined using

Table 1 Baseline characteristics of the study cohort

	Healthy participants (C group)		T2DM patients with dietary therapy alone (N group)		T2DM patients with dietary + miglitol therapy (M group)	
	<i>n</i>		<i>n</i>		<i>n</i>	
Sex (male/female)	14	6/8	14	12/2	13	8/5
Age (years)	14	33.1±8.85a	14	56.7±10.3b	13	60.5±9.16b
BMI (kg/m ²)	14	20.3±1.30a	14	26.8±3.45b	13	25.0±3.03b
HbA1c (%)	–		–	7.26±0.751	–	7.21±0.530
GA (%)	–		–	18.4±2.03	–	19.1±2.11
Systolic blood pressure (mmHg)	14	113±11.4a	14	129±14.1b	13	130±12.2b
Diastolic blood pressure (mmHg)	14	70.4±9.48a	14	83.9±10.8b	13	79.5±7.84b
Total cholesterol (mg/dL)	9	179±22.5	13	195±34.4	11	187±29.5
HDL cholesterol (mg/dL)	9	74.6±21.2b	13	56.6±15.6a	11	64.3±9.28ab
Uric acid (mg/dL)	9	4.33±1.13a	13	5.74±1.11b	11	5.12±1.24ab
Duration of diabetes mellitus (years)	–			4.28±3.26		5.58±4.53
Diabetic complications						
Retinopathy (<i>n</i>)	–			3		3
Nephropathy (<i>n</i>)	–			1		2
Neuropathy (<i>n</i>)	–			5		6
Drug prescribed						
Hypertension (<i>n</i>)	–			8		6
Hyperlipidemia (<i>n</i>)	–			10		7
Hyperuricemia (<i>n</i>)	–			0		2
Miglitol dose /day (150 mg/225 mg)	–			–		9/4
Glucose (mg/dL)						
Fasting	14	92.6±8.6a	14	137.1±21.4b	13	146.7±22.2b
After a meal: 60 min	14	102.7±35.4a	14	240.5±32.8c	13	201.2±34.3b
After a meal: 120 min	14	94.4±19.4a	14	211.8±36.6b	13	223.0±33.2b
After a meal: 180 min	14	89.6±9.6a	14	172.2±50.5b	13	194.5±35.1b
Neutrophil elastase (µg/mL)						
Fasting	14	57.4±39.8	14	40.2±22.6	13	33.1±17.1
After a meal: 60 min	14	60.5±41.1b	14	29.7±17.7a	13	43.6±21.6ab
After a meal: 120 min	14	53.1±26.9b	14	31.7±23.8a	13	36.9±14.9ab
After a meal: 180 min	14	62.4±38.2	14	35.8±29.9	13	39.7±16.6

Values are the mean ± SD or number (*n*)

Tukey–Kramer multiple range test for comparison among three groups or Student's *t* test for comparison between two groups was performed.

a, b, c: Different letters indicate significant differences by Tukey–Kramer multiple range test

Chi-squared tests were performed between N and M groups of each diabetic complication (retinopathy, nephropathy, or neuropathy) and among drugs prescribed (for hypertension, hyperlipidemia, or hyperuricemia); no significant differences were detected

BMI body mass index, *CRP* C-reactive protein, *GA* glycoalbumin, *HbA1c* glycated hemoglobin, *HDL* high-density lipoprotein, *T2DM* type 2 diabetes mellitus

blood samples obtained in the morning after an overnight fast.

MTTs were undertaken using a test meal (total energy of 505 kcal, comprising 64% carbohydrate, 12.5% protein, and 23.5% fat) with/without a single dose of miglitol. The miglitol was taken within 1 min before meal intake. The miglitol doses in the test meal were the same as the doses

used before the trial. Anthropometric data were obtained just before the MTT in the morning, and the blood glucose concentration was measured at 60, 120, and 180 min after the meal using an automatic glucose analyzer (GA-1171; Arkray, Kyoto, Japan). Serum elastase concentrations were measured by a kit measuring granulocyte elastase (Fagnos Elastase Dip kit; Sanwa Kagaku Kenkyusho Co., Ltd.).

Blood samples were collected immediately before (0 h) and 180 min after consuming the meal. Blood samples were taken for measurement of the blood glucose concentration and mRNA expression in peripheral leukocytes.

2.5 Microarray Analysis and Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses including S100 genes were performed with approval from the Ethics Committee of Naka Kinen Clinic to determine gene expression (3 h before meal and after meal) of the peripheral blood leukocyte fraction. In this study, we assessed mRNA levels of inflammatory genes in peripheral leukocytes upon fasting and at 180 min after a meal because our previous animal studies demonstrated that induction of the expression of inflammatory genes in peripheral leukocytes in rats by single postprandial hyperglycemia can be assessed at 180 min after oral glucose loading [5, 16, 17]. Blood samples were obtained in a fasting state and at 180 min after a meal for total RNA extraction from peripheral leukocytes. Immediately after collection, these samples were fixed in a PAXgene RNA tube (Qiagen/BD, Hombrechtikon, Switzerland), which included fixation buffer, for 4 h at room temperature as per the manufacturer's directions. Total RNA from the fixed blood samples was extracted using an RNeasy Kit purchased from Qiagen.

In each microarray, eight samples in each group (C group at 0 min and N group at 0 min) were selected at random from a random number table. Aliquots containing 100 ng of total RNA were converted individually to cDNA, fractionated, and labeled with a Gene ChIP[®] Poly-A RNA Control kit, WT Amplification kit, and Gene ChIP[®] WT Terminal Labeling kit (Affymetrix, Santa Clara, CA, USA), in accordance with the manufacturer's instructions. Hybridization, washing, and staining were carried out using Affymetrix[®] HuGene2.1 ST Array Strips and a GeneAtlas[®] Hybridization Wash and Stain kit for WT Assay Strips (Affymetrix), in accordance with the manufacturer's instructions. After washing, the strips were analyzed using a GeneAtlas Imaging Station (Affymetrix). Data analyses were performed using Expression Console (Affymetrix), Transcriptome Analysis Console (Affymetrix), and Excel[™] (Microsoft, Redmond, WA, USA) software. We considered significant differences between two groups to be those with $P < 0.05$ and a fold change greater than ± 1.3 . The microarray data were confirmed by qRT-PCR, and the primer sequences used in this study are listed in S-Table 1 (see the electronic supplementary material).

Total RNA samples were converted to cDNA by reverse transcription using SuperScript[™] III RT (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's

instructions. Samples of total RNA were converted to cDNA by reverse transcription using SuperScript III RT (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. To estimate mRNA expression of the selected genes, PCR amplification using a universal probe was undertaken on a LightCycler[™] instrument (Roche Diagnostics, Rotkreuz, Switzerland). The CT (Threshold Cycle) values of each gene determined by real-time RT-PCR were converted into signal intensities based on the $\Delta\Delta CT$ method, which recognizes a difference of one CT value as a twofold difference between samples. The RNA levels were normalized by the level of transcription factor II B (*Tf2B*). The primer sequences used in this study are listed in S-Table 1 (see the electronic supplementary material).

2.6 Statistical Analyses

Values are presented as the mean \pm standard deviation (SD) or number (n). Statistical analyses were undertaken using JMP version 15 (SAS Institute Inc., Cary, NC, USA). The significance of differences among the three groups and between two groups was determined using unpaired Tukey–Kramer's multiple range test and unpaired Student's t test, respectively, as appropriate. Chi-squared tests were performed between N and M groups of each diabetic complication (retinopathy, nephropathy, or neuropathy) and between the N and M groups of each category of "drugs prescribed" (i.e., those for hypertension, hyperlipidemia, or hyperuricemia). $P < 0.05$ was considered significant.

3 Results

3.1 Basic Clinical Parameters

The healthy volunteers (C group) comprised six men and eight women, the T2DM patients with dietary therapy alone (N group) comprised 12 men and two women, and the T2DM patients with dietary + miglitol therapy (M group) comprised eight men and five women. The mean age (years) in the C group was 33.1 ± 8.85 , whereas it was 56.7 ± 10.3 in the N group and 60.5 ± 9.16 in the M group.

There was no significant difference in the duration of diabetes mellitus (weeks) between the N and M groups. The BMI, systolic blood pressure, diastolic blood pressure, uric acid, and blood glucose concentration at the fasting and postprandial periods (60–180 min) were significantly lower in the C group than those in the N group. The fasting high-density lipoprotein (HDL) cholesterol concentrations were significantly higher in the C group than in the N group. The BMI, HbA1c level, GA level, and fasting blood glucose concentration did not differ significantly between the N and M groups. No differences were observed between

the N and M groups for each diabetic complication (retinopathy, nephropathy, or neuropathy) or between the N and M groups for each drugs prescribed category (i.e., those for hypertension, hyperlipidemia, or hyperuricemia). The serum concentration of neutrophil elastase was higher in the C group than in the N group at 60 and 120 min after a meal. The blood glucose concentration at 60 min after a meal in the MTT was significantly lower in the M group than that in the N group (Table 1).

3.2 Microarray and qRT-PCR Analyses in Peripheral Leukocytes

mRNA expression in peripheral leukocytes was compared between the C and N groups in a fasting state, using microarray analyses. Genes with upregulated expression in the N group compared with the levels in the C group upon fasting were *S100A12*, *CLEC17*, *CD22*, *CLEC10*, *F5*, *PLCG2*, *MMP9*, and *CLCO4C1* (S-Table 2, see the electronic supplementary material).

qRT-PCR was undertaken on the upregulated and down-regulated genes detected in microarray analyses and genes related to them. Differences of *S100A12* between C and N groups were not observed, but the *S100A12* mRNA level was higher in the N group than in the M group. Other genes such as *CLEC17*, *CD22*, and *F5* were not significantly differently expressed between the C and N groups or between the N and M groups.

We performed qRT-PCR for S100 genes and proinflammatory cytokines in peripheral leukocytes because our previous animal studies showed their higher expression in diabetic animals [5, 16, 17]. *S100A6* in a fasting state, *S100A8* in a fasting state and at 180 min after a meal, and *S100A9* at 180 min after a meal showed higher mRNA expression in the N group than in the M group 180 min after a meal, but not between the N group and the M group. Regarding the proinflammatory cytokines, there were no significant differences in mRNA expression for *interferon-γ* (*IFNG*), *IL1B*, *TNFA*, or *IL8* between the N and C groups, or between the N and M groups (Table 2).

4 Discussion

The blood glucose concentration at 60 min after a meal in the MTT was significantly lower in T2DM patients with dietary + miglitol therapy than in those with dietary therapy alone. Researchers have reported that miglitol administration in T2DM patients reduces blood glucose concentrations markedly at 30 and 60 min after a meal [18]. In this study, we first demonstrated that the mRNA expression of *S100A6*, *S100A8*, *S100A9*, and *S100A12* in peripheral leukocytes at fasting and/or 180 min after a meal was higher in T2DM

patients undergoing dietary therapy alone than in healthy volunteers (*S100A6*, *S100A8*, and *S100A9*) and/or T2DM patients with dietary + miglitol therapy (*S100A12*). *S100A8* and *S100A9* have been reported to be elevated in the plasma of those with type 1 diabetes [11]. A population-based cohort study following up 106 patients who survived without amputation and 93 who survived without signs of peripheral arterial disease showed that higher plasma protein concentrations of *S100A12* were associated with an increased risk of amputation or death in patients with type 2 diabetes [19]. Additionally, a cohort study following 264 patients with diabetes revealed that an increased plasma *S100A12* concentration was associated with subsequent death from any cause and cerebrovascular events in diabetic retinopathy patients without a previous diagnosis of macrovascular disease [20]. Furthermore, reducing glucose fluctuations by switching the α -GI from acarbose or voglibose to miglitol in T2DM patients has been shown to reduce the mRNA expression of *S100A6/9/11/12* in peripheral leukocytes in a fasting state [6]. Considering these results, the higher expression of *S100A6*, *S100A8*, *S100A9*, and *S100A12* in T2DM patients with dietary therapy alone than in healthy volunteers and/or T2DM patients with dietary + miglitol therapy may contribute to the higher risk of CVDs.

Notably, *IFNG*, *IL1B*, *IL8*, and *TNFA*, which are major proinflammatory cytokines, were not altered between healthy volunteers and T2DM patients with dietary therapy alone, or between T2DM patients with dietary therapy alone and those with dietary + miglitol therapy. Previous studies have demonstrated that *IL1B* mRNA expression in peripheral leukocytes was higher in T2DM patients than in healthy participants [21]. One reason why proinflammatory cytokines were not altered between T2DM participants with dietary therapy and healthy volunteers or between T2DM participants with dietary therapy alone and T2DM participants with dietary + miglitol therapy is that fasting blood glucose was higher in T2DM participants in the previous study (179.8 ± 71.46 mg/mL) than in this study (137.21 ± 49 mg/mL). Additionally, the diabetic background, such as duration of diabetes and prescribed drugs, was not described in that study. The T2DM patients with dietary therapy in the present study had undergone an MTT without taking antidiabetic drugs, but those with dietary + miglitol therapy had undergone an MTT upon taking a single dose of miglitol. The reason why the inhibitory action of miglitol was mild in the present study is that the duration of T2DM and fasting blood glucose concentration tended to be greater in patients with dietary + miglitol therapy than in those with dietary therapy, although these differences were not significant. In this study, we cannot consider the effects of a single dose of miglitol on gene expression in peripheral leukocytes because the miglitol group was treated with miglitol for over 8 weeks. Therefore, in further study, the expression of these inflammation-related

Table 2. Comparison of proinflammatory genes at fasting and 180 min after a meal by qRT-PCR among healthy participants (C group), T2DM patients with dietary therapy (N group), and T2DM patients with miglitol therapy (M group)

	C (n = 14)	N (n = 14)	M (n = 13)
Genes with higher expression detected in microarray analysis in T2DM patients than in healthy participants at fasting state			
<i>S100A12</i>			
Fasting	5.99±3.13ab	8.85±4.41b	5.17±2.81a
180 min	5.61±3.65ab	8.14±3.46b	4.40±1.88a
<i>CLEC17A</i>			
Fasting	0.0416±0.0207	0.0526±0.0208	0.0499±0.0310
180 min	0.0638±0.0343	0.0734±0.0366	0.0481±0.0291
<i>CD22</i>			
Fasting	0.797±0.418	1.076±0.367	1.079±0.805
180 min	0.984±0.548	1.355±0.623	0.902±0.411
<i>F5</i>			
Fasting	1.11±0.430	1.49±0.398	1.47±0.549
180 min	1.40±0.482	1.67±0.848	1.20±0.339
Expression of S100 proteins and inflammatory cytokines			
<i>S100a4</i>			
Fasting	6.01±1.37	7.08±1.40	6.16±1.39
180 min	5.66±1.38	6.90±1.44	6.46±1.61
<i>S100a6</i>			
Fasting	17.3±4.19	19.6±4.37	16.8±4.62
180 min	16.8±4.19a	21.3±4.12b	17.7±4.77ab
<i>S100a8</i>			
Fasting	123±41.8a	206±91.0b	143±70.9ab
180 min	130±46.9a	206±84.0b	154±56.9ab
<i>S100a9</i>			
Fasting	4.25±1.69	5.90±3.37	5.17±2.58
180 min	4.41±2.10a	6.13±3.81b	5.02±1.95ab
<i>S100a10</i>			
Fasting	7.06±1.83	7.80±1.65	8.75±2.84
180 min	6.69±1.12	8.01±1.90	7.74±2.74
<i>IFNG</i>			
Fasting	0.000660±0.000316	0.001022±0.000610	0.001180±0.000791
180 min	0.000515±0.000306	0.001099±0.001145	0.000976±0.000365
<i>IL1B</i>			
Fasting	2.62±0.963	2.85±0.924	3.12±1.37
180 min	3.22±1.35	3.30±0.803	3.17±1.29
<i>TNF</i>			
Fasting	0.511±0.171	0.523±0.158	0.435±0.192
180 min	0.532±0.133	0.567±0.242	0.695±0.328
<i>IL8</i>			
Fasting	0.450±0.343	0.434±0.197	0.774±1.468
180 min	0.377±0.237	0.371±0.152	0.360±0.207

Values are the mean ± SD

a, b: Different letters indicate significant differences by Tukey–Kramer multiple range test

qRT-PCR quantitative reverse-transcription polymerase chain reaction, T2DM type 2 diabetes mellitus

genes should be examined in peripheral leukocytes and compared between healthy participants and T2DM patients without pharmacological therapy who have visited the hospital for diabetes therapy for the first time and been diagnosed at

that time. It should also be examined whether such expression and the incremental data in peripheral leukocytes are reduced by treatment with a single dose of miglitol. In addition, it should be examined whether the protein expression

of these inflammatory genes in peripheral leukocytes and the serum protein concentrations differ between healthy participants and T2DM patients without pharmacological therapy, and whether such expression is reduced by treatment with longer or a single dose of miglitol. Interestingly, we found that the expression of *S100A6*, *S100A8*, and *S100A10* was higher in T2DM patients with dietary therapy alone than in healthy volunteers, despite the expression of proinflammatory cytokine genes in peripheral leukocytes not differing between the two groups. Additionally, the expression of *S100A12* was lower in T2DM patients with dietary + miglitol therapy than in those with dietary therapy alone. These results indicate that the mRNA levels of S100 proteins in peripheral leukocytes are responsive to repeated postprandial hyperglycemia rather than proinflammatory cytokine mRNA levels in peripheral leukocytes. Since S100 genes are related to inflammation and/or atherosclerosis, as mentioned above, and control blood glucose concentration, even if proinflammatory cytokine expression is not higher in T2DM patients than in healthy participants, reduction of S100 genes in peripheral leukocytes may lead to reducing complications related to inflammation and/or atherosclerosis such as macrovascular and microvascular diseases. Further studies should examine whether the mRNA levels of the S100 protein family in peripheral leukocytes predict the development and progression of T2DM and related complications including CVD.

It should be noted that there were significant differences in age, BMI, systolic blood pressure, diastolic blood pressure, HDL cholesterol, and uric acid as well as blood glucose concentration between healthy participants and T2DM patients with dietary therapy alone. In addition, the recruited healthy participants were younger than the T2DM patients. This was because we recruited healthy volunteers who visited the hospital and enrolled the participants in the order in which they appeared. Regarding the other parameters besides age, in general, T2DM patients tended to have higher BMI, blood pressure, and uric acid, and lower HDL cholesterol. Therefore, in this study, it is impossible to assess confounding effects of blood glucose, blood pressure, and/or lipid profiles on the expression of inflammatory genes in peripheral leukocytes. Previous studies demonstrated that the expression of S100 genes in peripheral leukocytes was higher in diabetic rats than in healthy rats and that reducing postprandial hyperglycemia in diabetic rats by miglitol suppressed expression of the S100 genes [4]. In addition, reducing glucose fluctuation in T2DM patients by changing acarbose to miglitol reduced the expression of S100 genes in peripheral leukocytes [6]. In this study, we found that T2DM patients with dietary + miglitol therapy had lower expression of *S100A12* in peripheral leukocytes at fasting and 180 min after a meal and tended to have lower expression of *S100A6* at 180 min ($P = 0.0987$) and *S100A8*

in the fasting period ($P = 0.0692$) than T2DM patients with dietary therapy. Therefore, the higher expression of S100 genes in T2DM patients with dietary therapy than in T2DM patients with miglitol could in part have been caused by elevation of blood glucose concentrations. It should be examined whether double-blind RCTs for reducing postprandial hyperglycemia using miglitol can reveal a reduction in the expression of inflammatory genes including S100 genes. Further studies are also needed to examine whether the expression of genes related to inflammation in peripheral leukocytes is higher in T2DM patients than in age-matched healthy participants. It should also be examined whether anti-hyperglycemic drugs besides miglitol, anti-hyperlipidemic drugs, and anti-hyperuricemic drugs affect the expression of genes related to inflammation in peripheral leukocytes in T2DM patients with hyperlipidemia and/or hyperuricemia.

It should be mentioned that the serum neutrophil elastase concentration was lower in T2DM patients with dietary therapy alone at 60 and 120 min after a meal than in healthy participants at the same time points. A recent animal study demonstrated that knockout of neutrophil elastase reduced Western diet-induced nonalcoholic steatohepatitis in mice [22]. Our results appear to be the opposite of those observed in the animal study. However, in general, the functions of neutrophils, including chemotaxis, phagocytosis, and adhesion, were reported to be lower in diabetic patients than in healthy participants [23]. Neutrophil elastase has also been shown to contribute to the neutrophil extracellular traps [24]. Therefore, a decrease of neutrophil elastase concentration in T2DM patients with dietary therapy may contribute to an increased risk of infectious diseases. This should also be investigated in further studies.

5 Conclusion

S100 genes such as *S100A12*, *S100A6*, *S100A8*, and *S100A9* were more highly expressed in T2DM patients with dietary therapy than in healthy volunteers and/or T2DM patients undergoing drug therapy using miglitol.

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Author Contributions SK carried out most of the experiments and wrote the manuscript. NH assisted with the experiments. MS, SD, and TO recruited the participants and carried out the trial in the clinic. KM helped to draft the manuscript. TO and KM organized the study. All authors approved the final version of the manuscript.

Declarations

Conflict of interest This study was supported by Sanwa Kagaku Kenkyusho Co., Ltd. KM and TO received research grants from Sanwa Kagaku Kenkyusho Co., Ltd. The other authors have no conflicts of interest.

Ethical approval Ethics approval in this study was performed at the Ethics Committee of Naka Kinen Clinic.

Consent to participate and publication consent to participate and consent for publication from participants in this study were obtained at Naka Kinen Clinic. The is not applicable in this study.

Code availability Not applicable.

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