

Osteoarthritis patients with high haemoglobin A1c have increased Toll-like receptor 4 and matrix metalloprotease-13 expression in the synovium

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Kosuke Murata¹
Kentaro Uchida¹
Shotaro Takano¹
Shintaro Shoji¹
Dai Iwase¹
Gen Inoue¹
Jun Aikawa¹
Yuji Yokozeki¹
Hiroyuki Sekiguchi²
Masashi Takaso¹

¹Department of Orthopedic Surgery, Kitasato University School of Medicine, Sagami City, Kanagawa 252-0374, Japan; ²Shonan University of Medical Sciences Research Institute, Chigasaki City, Kanagawa 253-0083, Japan

Purpose: While research has identified diabetes mellitus (DM) as a risk factor for knee osteoarthritis (KOA), the underlying mechanisms are not fully understood. Studies suggest that Toll-like receptor 4 (TLR4) expression is elevated in osteoarthritic lesions of OA patients and in target tissues of insulin resistance such as adipose tissue and skeletal muscle in patients with DM. TLR4 is associated with inflammation and catabolic response via regulation of matrix metalloproteases (MMPs). We hypothesized that *TLR4* and *MMP* expression may be increased in the synovial tissue (SYN) of KOA patients with diabetic pathology. We therefore investigated *TLR* and *MMP* expression in the SYN of KOA patients with and without high haemoglobin A1c concentrations.

Patients and methods: A total of 171 patients radiographically diagnosed with KOA were grouped based on their HbA1c concentration (HbA1c ≥ 6.5 and HbA1c < 6.5). We used real-time polymerase chain reaction to compare the expression of TLRs (*TLR2*, *TLR4*) and MMPs (*MMP2*, *MMP3*, *MMP9* and *MMP13*) in patients' SYN between the two groups. *MMP13* regulation by the TLR4 ligand, lipopolysaccharide (LPS), in SYN cells was examined in culture by stimulating SYN cells with LPS or vehicle (culture medium) for 24 h.

Results: The expression of *TLR4* and *MMP13* in the HbA1c ≥ 6.5 group was significantly elevated compared to that in the HbA1c < 6.5 group. In contrast, *TLR2*, *MMP2*, *MMP3* and *MMP9* expression levels were similar between the groups. *MMP13* mRNA and *MMP13* protein levels in SYN cells were significantly higher following stimulation with LPS compared to vehicle.

Conclusions: *TLR4* and *MMP13* expression were elevated in the synovium of osteoarthritis patients with high HbA1c concentrations. Our results may provide insight into the pathology of OA patients with DM.

Keywords: osteoarthritis, diabetes mellitus, TLR4, MMP13

Introduction

Several studies have reported an association between over-loading due to obesity and the development of knee osteoarthritis (KOA).¹⁻⁴ However, based on the epidemiological correlation between hand OA and overweight or obesity, systemic factors identified in the association with KOA may also be involved in general OA pathology.⁵⁻⁸ Some epidemiological studies have also suggested an association of diabetes mellitus (DM) and hyperglycemia with OA.⁹⁻¹³ However, the mechanism underlying such an association is not fully understood.

Toll-like receptors (TLRs) are transmembrane proteins with roles in DM pathology. Among the TLR subtypes, TLR2 and TLR4 play an important role in DM

Correspondence: Kentaro Uchida
Department of Orthopedic Surgery,
Kitasato University School of Medicine,
1-15-1 Minami-ku Kitasato, Sagami City,
Kanagawa 252-0374, Japan
Tel/Fax +81 042 778 9217
Email kuchida@med.kitasato-u.ac.jp

pathology in both experimental and clinical conditions.^{14–16} TLR4-mediated chronic inflammation is also associated with DM complications such as diabetic neuropathy, diabetic retinopathy, and diabetic nephropathy.¹⁷ Interestingly, TLR4 is also expressed in joint tissues and contributes to OA pathology via inflammation and inflammation-induced catabolism.^{18,19} TLR4 deficiency decreases disease severity, as evidenced by lower bone erosion, cartilage damage, and synovial cellular influx.²⁰ However, whether TLR4 levels are elevated in KOA patients with DM remains to be determined.

TLR4 signaling contributes to catabolic responses and results in elevated production of matrix metalloproteinase (MMP). Among the MMP subtypes, *MMP13* expression is most localized to connective tissue,²¹ where it degrades type II, IV and IX collagen in articular cartilage,²² and is a key enzyme targeting cartilage for degradation. TLR4 is linked to *MMP13* expression.^{22,23} TLR4 deficiency reduces *MMP13* expression in mice.²³ Interestingly, a recent study showed that glucose treatment of zebrafish induced diabetic conditions and increased *MMP13* expression.²⁴ We therefore hypothesized that MMP13 may be elevated in KOA patients under diabetic conditions.

Here, we compared *TLR* and *MMP* expression in synovial tissue (SYN) between KOA patients with and without high haemoglobin A1c concentrations.

Materials and methods

Patients

Power analysis using an alpha of 0.05, power of 0.80, and N2 (number of patients with HbA1c ≥ 6.5)/N1 (number of patients with HbA1c < 6.5) ratio of 0.155 was conducted in G*POWER3 to determine the optimal sample size. Power analysis revealed that 23 patients with HbA1c ≥ 6.5 and 148 patients with HbA1c < 6.5 were needed to detect a difference in TLR4 between HbA1c ≥ 6.5 and HbA1c < 6.5 groups.

We examined SYN from patients (38 men and 133 women) with KOA diagnosed by radiography who underwent total knee arthroplasty at our institution. KOA patients with a cancer diagnosis, trauma, rheumatoid arthritis or other collagen diseases were excluded from this study. SYN samples were harvested from the operated knee during the operation. A piece of each of the 171 SYN specimens was snap frozen in liquid nitrogen at -80°C prior to RNA extraction. Eight SYN specimens were used for cell culture.

This study protocol received approval by the Ethics Review Board of Kitasato University (reference number: KME0 B13-113). Written informed consent was obtained from all participants regarding participation in the study and the removal and use of their SYN one day prior to surgery. This study was conducted in accordance with the Declaration of Helsinki.

Quantitative polymerase chain reaction (qPCR) analysis

To evaluate the effect of the diabetic state on *TLR* and *MMP* expression, OA patients were grouped based on their HbA1c concentration (HbA1c ≥ 6.5 and HbA1c < 6.5).²⁵ Patients' clinical characteristics are summarized in Table 1 by group. Methods for total RNA extraction, cDNA synthesis and qPCR using SYBR Green are described elsewhere.²⁶ The following PCR primers were used for qPCR: *TLR2*-sense 5'-CCT GTG TGA CTC TCC ATC CC-3', *TLR2*-antisense 5'-GAC ATT CCG ACA CCG AGA GG-3 (product size: 71 bp); *TLR4*-sense 5'-CGA CAA CCT CCC CTT CTC AAC-3', *TLR4*-antisense 5'-AGA GGT GGC TTA GGC TCT GAT-3 (product size: 167 bp); *MMP2*-sense 5'-GTG AAG TAT GGG AAC GCC GA-3', *MMP2*-antisense 5'-AGA AGC CGT ACT TGC CAT CC-3 (product size: 154 bp); *MMP3*-sense 5'-GTG GAG TTC CTG ACG TTG GT-3', *MMP3*-antisense 5'-TGG AGT CAC CTC TTC CCA GA-3 (product size: 164 bp); *MMP9*-sense 5'-TTT GAG TCC GGT GGA CGA TG-3', *MMP9*-antisense 5'-GCT CCT CAA AGA CCG AGT CC-3 (product size: 197 bp); *MMP13*-sense 5'-TGA CTG AGA GGC TCC GAG AA-3', *MMP13*-antisense 5'-CAT CAG GAA CCC CGC ATC TT-3 (product size: 111 bp); *GAPDH*-sense 5'-TGT TGC CAT CAA TGA CCC CTT-3' and *GAPDH*-antisense 5'-CTC CAC GAC GTA CTC AGC G-3' (product size: 202 bp). *TLR* and *MMP* mRNA expression levels were normalized to *GAPDH* levels using the delta-delta Ct method. We

Table 1 Patients' clinical characteristics by HbA1c group

	HbA1c <6.5 (n=148)	HbA1c ≥ 6.5 (n=23)	P
Age (years)	73.9 \pm 0.7	74.0 \pm 1.7	0.944
Male/Female, n	34/114	4/19	0.787
KL (2/3/4), n	3/51/94	0/5/18	0.451
BMI (kg/m ²)	25.8 \pm 0.3	30.0 \pm 0.8*	<0.001

Notes: Data represent mean \pm standard error or n. *P<0.05 compared with HbA1c <6.5 group.

Abbreviations: KL, Kellgren/Lawrence grade; BMI, body mass index.

compared the expression of *TLR2*, *TLR4*, *MMP2*, *MMP3*, *MMP9* and *MMP13* in the SYN between the two HbA1c groups. Additionally, we grouped the patients based on the World Health Organization Body Mass Index (BMI) classification (Table 2) (normal, overweight, obese) and compared *TLR2*, *TLR4*, *MMP2*, *MMP3*, *MMP9* and *MMP13* expression among these groups. Relative expression was calculated using the mean of all control samples (samples from SYN from the HbA1c <6.5 group or α -MEM-treated SYN cells in vitro). To investigate potential gender effects, female and male KOA patients were grouped based on their HbA1c level and BMI and *TLR4* and *MMP13* levels were analyzed.

Cell culture

SYN cells derived from 8 patients were used to examine the effect of the TLR4 agonist, LPS, on *MMP13* mRNA expression and MMP13 protein production. Synovial cells were obtained following collagenase digestion of SYN as reported previously.²⁷ Isolated synovial cells were cultured in 6-well plates in α -minimal essential media (α -MEM) with 10% fetal bovine serum. One week later, the cell population of cultured SYN cells was determined using flow cytometric analysis as reported previously.²⁷ The cultured SYN cells were incubated with FITC-conjugated anti-CD45 (Biolegend, CA, USA) and allophycocyanin (APC)-conjugated anti-CD90 (Biolegend) antibodies for 45 min at 4 °C. After washing twice in PBS, 50,000 total events were acquired using FACSVerse™ (BD Biosciences, San Jose, CA, USA), and the data were analyzed using Flow Jo 10.0 (Tree Star, Ashland, OR). SYN cells were exposed to 1000 μ L of 0 (α -MEM), 100 or 1000 ng/mL LPS (Sigma, St. Louis, MO, USA) for 24 h. Subsequently, *MMP13* mRNA expression was examined using qPCR with the primers described above. MMP13 protein concentration in the cell supernatant was analyzed using a commercial ELISA kit (Human Pro-MMP-13

Quantikine ELISA Kit, R&D Systems, Inc., Minneapolis, MN, USA).

Statistical analysis

The SPSS 25.0 statistical package was used for statistical analysis. Continuous variables were analyzed using the Mann-Whitney U test and Tukey multiple comparisons test, and categorical variables were analyzed using Fisher's exact test. Statistical significance was defined by $P < 0.05$.

Results

Clinical characteristics of patients with HbA1c ≥ 6.5 and HbA1c <6.5

BMI among KOA patients with HbA1c ≥ 6.5 was significantly higher than that among KOA patients with HbA1c <6.5 (Table 1). Age, male/female ratio and Kellgren/Lawrence grade 2/3/4 ratio were similar between the groups (Table 1).

Expression of TLRs and MMPs among patients with HbA1c ≥ 6.5 and HbA1c <6.5

To determine whether TLR and MMP expression levels are increased in diabetic KOA patients, we examined TLRs and MMPs in the SYN of KOA patients with HbA1c ≥ 6.5 and HbA1c <6.5. *TLR2* expression was similar between patients with HbA1c ≥ 6.5 and HbA1c <6.5 ($P=0.078$; Figure 1A), while *TLR4* expression in the HbA1c ≥ 6.5 group was significantly higher than that in the HbA1c <6.5 group ($P=0.040$; Figure 1B). There were no differences in *MMP2*, *MMP3*, or *MMP9* expression between the groups (*MMP2*, $P=0.626$; *MMP3*, $P=0.876$; *MMP9*, $P=0.912$; Figure 1C–E). *MMP13* expression in the HbA1c ≥ 6.5 group was significantly higher than that in the HbA1c <6.5 group ($P < 0.001$; Figure 1F).

Effect of obesity on expression of TLRs and MMPs

Patients in the HbA1c ≥ 6.5 group had higher BMI than those in the HbA1c <6.5 group. We therefore investigated the effect of obesity on the expression of TLRs and MMPs. Patients in the obese group were significantly younger than those in the normal and overweight groups (Table 2). HbA1c levels in the obese group were significantly higher than those in the normal and overweight groups (Table 2). The Kellgren/Lawrence grade 2/3/4 ratio was similar among the groups (Table 2). Expression levels of *TLR2*,

Table 2 Patients' clinical characteristics by body mass index group

	Normal (n=69)	Overweight (n=72)	Obese (n=30)	P
Age (years)	76.0 \pm 0.9	73.9 \pm 0.8	69.3 \pm 1.6 ^{a,b}	<0.001
Male/Female, n	11/58	23/49	4/26	0.037
KL (2/3/4), n	2/19/48	1/28/43	0/9/21	0.591
HbA1c	5.8 \pm 0.0	6.0 \pm 0.1	6.3 \pm 0.1 ^{a,b}	<0.001

Notes: Data represent mean \pm standard error or n. ^a $P < 0.05$ compared with normal group. ^b $P < 0.05$ compared with overweight group.

Abbreviations: KL, Kellgren/Lawrence grade; BMI, body mass index.

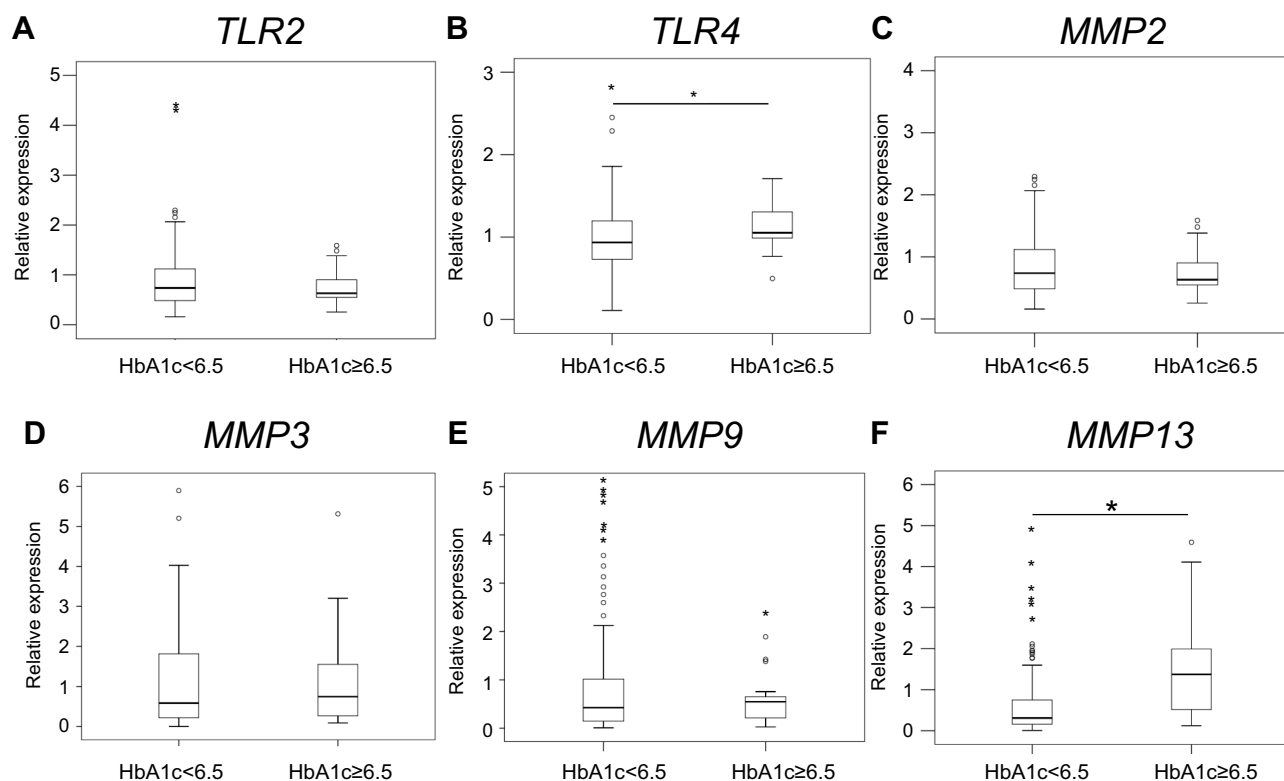


Figure 1 Effect of HbA1c concentration on TLR and MMP expression in the synovium. TLR2 (A), TLR4 (B), MMP2 (C), MMP3 (D), MMP9 (E) and MMP13 (F) expression in patients with HbA1c ≥ 6.5 and HbA1c < 6.5 . * $P < 0.05$ compared with HbA1c < 6.5 group.

TLR4, MMP2, MMP3, MMP9, and MMP13 were also similar among the normal, overweight, and obese groups (TLR2, $P=0.821$; TLR4, $P=0.075$; MMP2, $P=0.294$; MMP3, $P=0.636$; MMP9, $P=0.659$; MMP13, $P=0.853$; Figure 2).

Gender differences in expression of TLR4 and MMP13

Previous studies have suggested that there may be gender differences in pathological conditions in obese and diabetes patients.^{28–32} Therefore, we analyzed TLR4 and MMP13 expression between HbA1c ≥ 6.5 and HbA1c < 6.5 and among normal, overweight, and obese groups separately in male and female patients. Female patients but not male patients had higher BMI in the HbA1c ≥ 6.5 group than in the HbA1c < 6.5 group (Table 3). Among male OA patients, there was no difference in TLR4 or MMP13 between the HbA1c ≥ 6.5 and HbA1c < 6.5 group ($P=0.216$ and $P=0.341$, respectively; Figure 3A and B). TLR4 and MMP13 expression in female KOA patients with HbA1c ≥ 6.5 was significantly higher than that in female KOA patients with HbA1c < 6.5 ($P=0.021$ and $P < 0.001$, respectively; Figure 3C and D). Both male and female patients in the obese group were significantly younger than those in the normal group

(Table 4). HbA1c levels in female patients but not male patients were significantly higher in the obese group than in the normal and overweight groups (Table 4). There were no differences in TLR4 or MMP13 among the normal, overweight, and obese groups in male (TLR4, $P=0.888$; MMP13, $P=0.320$; Figure 4A and B) or female (TLR4, $P=0.096$, MMP13, $P=0.812$; Figure 4C and D) KOA patients.

Effect of TLR4 ligand, LPS, on MMP13 expression and MMP13 production

Flow cytometric analysis showed that almost all cells ($94.5 \pm 0.9\%$) among cultured SYN cells were CD45-CD90+ (synovial fibroblasts) (Figure 5A). TLR4 and MMP13 expression levels were elevated in the synovium of OA patients with high HbA1c concentrations. We therefore investigated whether a TLR4 agonist regulates MMP13 in synovial cells. Synovial cells stimulated with 100 or 1000 ng/mL LPS exhibited significant increases in MMP13 mRNA expression compared to vehicle-treated cells ($P=0.006$ and $P=0.007$, respectively; Figure 5B). MMP13 protein levels in the supernatant of synovial cells treated with 100 or 1000 ng/mL LPS were also

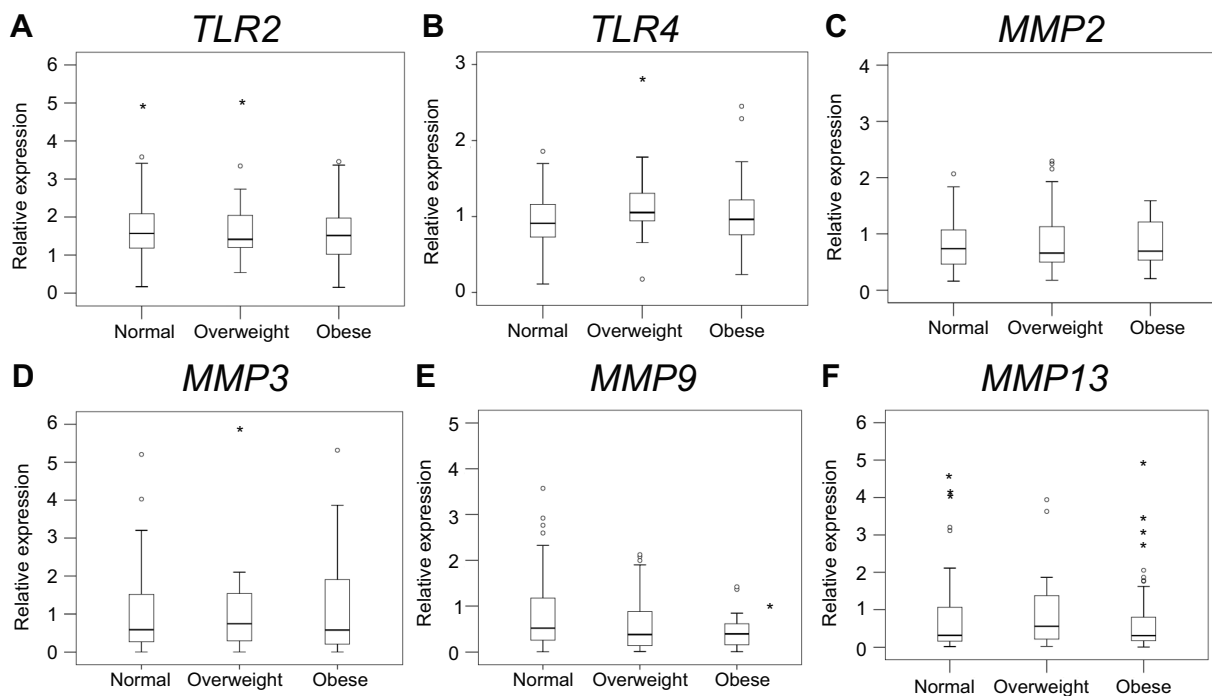


Figure 2 Effect of obesity on *TLR* and *MMP* expression in the synovium. *TLR2* (A), *TLR4* (B), *MMP2* (C), *MMP3* (D), *MMP9* (E) and *MMP13* (F) expression in normal, overweight, and obese groups.

Table 3 Patients' clinical characteristics by HbA1c group and gender

	Gender	HbA1c <6.5 (male, n=34 female, n=114)	HbA1c ≥6.5 (male, n=4; female, n=19)	P
Age (years)	Male	73.4±1.8	70.0±2.5	P=0.535
	Female	74.1±0.7	74.9±1.5	P=0.636
KL (2/3/4), n	Male	0/2/2	1/13/20	P=1.000
	Female	2/38/74	0/3/16	P=0.296
BMI	Male	26.6±3.2	28.3±2.6	P=0.309
	Female	25.5±0.4	30.4±1.0*	P<0.001

Notes: Data represent mean ± standard error or n. *P<0.05 compared with gender-matched HbA1c <6.5 group.

Abbreviations: KL, Kellgren/Lawrence grade; BMI, body mass index.

significantly higher than those of cells treated with vehicle ($P=0.021$ and $P=0.022$, respectively; Figure 5C).

Discussion

Studies have shown that *TLR2* and *TLR4* expression are raised in traditional target tissues of insulin resistance such as adipose tissue and skeletal muscle in DM patients.^{33,34} Consistent with these reports, we observed higher *TLR4* expression in the SYN of KOA patients with high HbA1c

concentrations. *TLR4* is associated with inflammatory and catabolic responses, and is up-regulated in inflamed SYN³⁵ and osteoarthritic lesions in OA patients.³⁶ Elevated *TLR4* expression in the SYN of KOA patients with high HbA1c concentrations may therefore be associated with OA pathology.

Clinical studies have reported elevated *MMP13* expression in patients with articular cartilage destruction,^{37,38} indicating that raised *MMP13* levels may contribute to cartilage degradation. A previous study showed that transgenic mice overexpressing *Mmp13* exhibit OA phenotype, including cartilage degradation. *TLR4* signaling is associated with catabolic response via *MMP13*.³⁹ *TLR4* ligands stimulate *MMP13* in human chondrocytes^{40,41} and synovial fibroblasts.⁴² Here, *MMP13* expression was increased in the SYN of KOA patients with high HbA1c concentrations. In addition, the *TLR4* agonist, LPS, stimulated *MMP13* expression and *MMP13* production. Several endogenous *TLR4* ligands that may exacerbate diabetic conditions have been reported, including advanced glycation end products,⁴¹ macroglobulin⁴³ and amyloid- β .⁴⁴ However, it is unclear which endogenous ligands contribute to the elevation of *MMP13* in KOA patients with high HbA1c concentrations. Nevertheless, elevation of *TLR4* and *MMP13* expression in the SYN of KOA patients

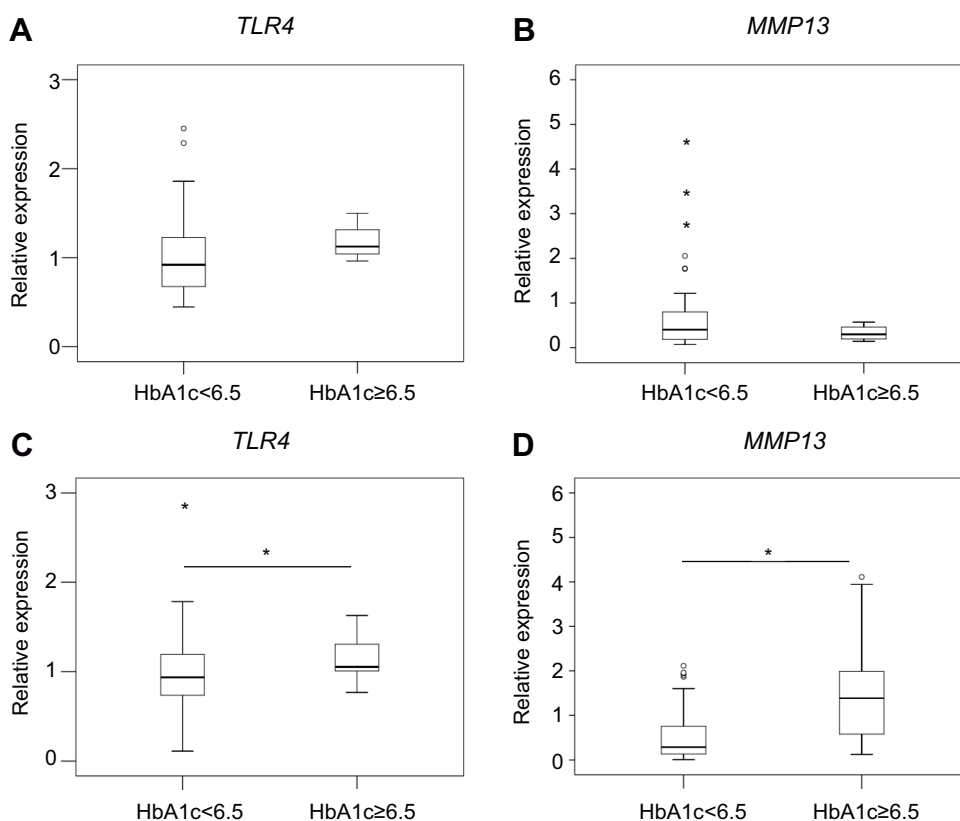


Figure 3 Effect of HbA1c on *TLR4* and *MMP13* expression in the synovium of male and female patients. (A-B). *TLR4* (A) and *MMP13* (B) expression in male patients with HbA1c ≥ 6.5 and HbA1c < 6.5 . C-D. *TLR4* (C) and *MMP13* (D) expression in female patients with HbA1c ≥ 6.5 and HbA1c < 6.5 . * $P < 0.05$ compared with gender-matched HbA1c < 6.5 group.

Table 4 Patients' clinical characteristics by body mass index group and gender

	Gender	Normal male, n=10 female, n=58	Overweight male, n=24 female, n=49	Obese male, n=4 female, n=26	P
Age (years)	Male	74.2 \pm 2.9	74.9 \pm 1.7	59.0 \pm 7.0 ^{a,b}	$P=0.011$
	Female	76.7 \pm 0.9	73.0 \pm 1.3 ^a	70.8 \pm 1.3 ^{a,b}	$P < 0.001$
KL (2/3/4), n	Male	0/2/8	1/12/11	0/1/3	$P=0.352$
	Female	2/16/40	0/17/32	0/8/18	$P=0.758$
HbA1c ≥ 6.5	Male	5.8 \pm 0.1	5.9 \pm 0.1	6.0 \pm 0.3	$P=0.820$
	Female	5.8 \pm 0.0	6.0 \pm 0.1	6.3 \pm 0.1 ^{a,b}	$P < 0.001$

Notes: ^a $P < 0.05$ compared with gender-matched normal group. ^b $P < 0.05$ compared with gender-matched overweight group.

with high HbA1c concentrations may explain some of the epidemiological findings showing that DM is associated with OA. Previous epidemiological studies have reported that DM patients have high prevalence of overweight/obesity.⁴⁵⁻⁴⁷ Fatani et al reported that 41% of a DM patient population was obese.⁴⁶ Hedley et al reported that 70% of DM patients were obese.⁴⁷ In our study, patients with HbA1c ≥ 6.5 had higher BMI than those with HbA1c < 6.5 , and 56% of OA patients with HbA1c ≥ 6.5 were obese. However, *TLR4* and *MMP13* expression were

comparable among normal, overweight, and obese patients. Therefore, our results suggest that higher *TLR4* and *MMP13* expression may be reflective of diabetic conditions but not obesity.

Several studies have reported gender differences in type 2 DM^{28,32} and obese patients.²⁹⁻³¹ For example, the effect of type 2 DM on the risk of coronary heart disease is greater in females than males.²⁸ Fat distribution with menopause is the main contributor to obesity in females.^{30,31} In our study, there were no significant differences in *TLR4* or *MMP13*

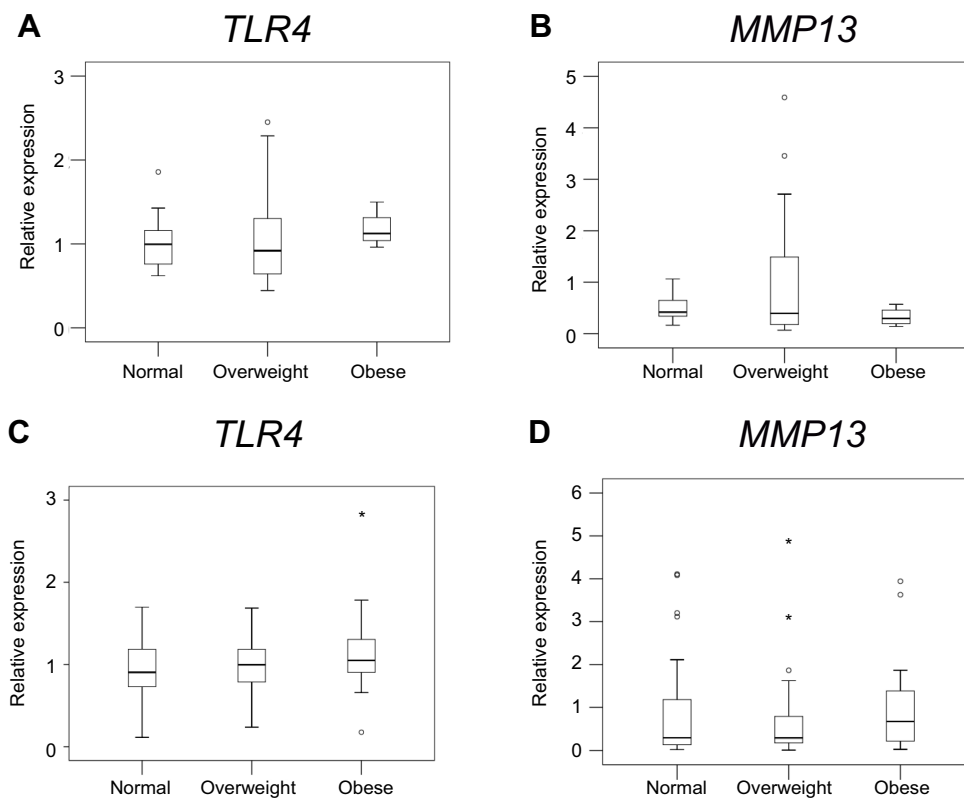


Figure 4 Effect of obesity on *TLR* and *MMP* expression in the synovium of male and female patients. (A-B). *TLR4* (A) and *MMP13* (B) expression in male patients in the normal, overweight, and obese groups. C-D. *TLR4* (C) and *MMP13* (D) expression in female patients in the normal, overweight, and obese groups.

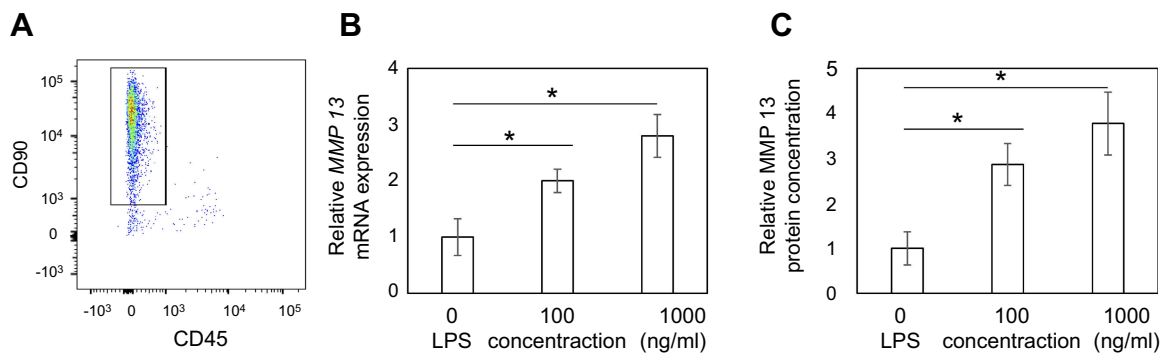


Figure 5 Effect of the *TLR4* agonist lipopolysaccharide on *MMP13* expression and *MMP13* production. (A). Flow cytometric analysis of cultured synovial tissue-derived cells. X-axis indicates CD45, y-axis indicates CD90. (B-C). Effect of the *TLR4* agonist lipopolysaccharide on *MMP13* expression and *MMP13* production. Relative *MMP13* expression (B) and *MMP13* protein concentration in the culture supernatant (C) of synovial cells treated with or without lipopolysaccharide were examined by quantitative PCR and enzyme-linked immunosorbent assay, respectively. Data indicate mean \pm standard error (n=8). * P <0.05.

expression among the normal, overweight, and obese groups in either males or females. In contrast, *TLR4* and *MMP13* expression in female KOA patients with HbA1c ≥ 6.5 was significantly higher than that in female KOA patients with HbA1c < 6.5 . No such difference was observed between male KOA patients with HbA1c ≥ 6.5 and HbA1c < 6.5 . Our results may indicate gender differences in KOA patients with diabetes. However, our study lacked sufficient

numbers of male patients for statistical analysis. Further investigations are needed to reveal the potential gender differences in KOA pathology with diabetes.

Several limitations of the present study warrant mention. First, the present study lacked oral glucose tolerance test (OGTT) data. Further characterization with OGTT data may more accurately reflect *TLR4* and *MMP13* expression in diabetic conditions. Second, the inclusion

of a non-KOA population is needed to confirm whether *TLR4* and *MMP13* expression are increased in diabetic individuals and if this directly contributes to KOA progression. Measurement of MMP levels in the serum of diabetic patients with and without OA and non-diabetic controls with and without OA is needed to support our conclusion. Third, elucidation of the mechanism by which *TLR4* and *MMP13* contributes to KOA pathology is needed. Fourth, we assessed the mRNA expression of TLRs and MMPs in SYN. Further investigation, such as a protein profiling study, is needed to tie the gene expression profile results together. Fifth, a positive control using a human synovial fibroblast or chondrocyte cell line was lacking in the in vitro study. Finally, whether there is also an increase in *MMP/TLR* expression in cartilage remains to be determined.

In summary, *TLR4* and *MMP13* were elevated in the synovium of osteoarthritis patients with high HbA1c concentrations. Our results may provide insights into the pathology of KOA patients with DM.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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