# Effects of metformin on knee joint capsule fibrosis in a diabetic mouse model

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## Aims

The antidiabetic agent metformin inhibits fibrosis in various organs. This study aims to elucidate the effects of hyperglycaemia and metformin on knee joint capsule fibrosis in mice.

## Methods

Eight-week-old wild-type (WT) and type 2 diabetic (db/db) mice were divided into four groups without or with metformin treatment (WT met(-/+), Db met(-/+)). Mice received daily intraperitoneal administration of metformin and were killed at 12 and 14 weeks of age. Fibrosis morphology and its related genes and proteins were evaluated. Fibroblasts were extracted from the capsules of 14-week-old mice, and the expression of fibrosis-related genes in response to glucose and metformin was evaluated in vitro.

## Results

The expression of all fibrosis-related genes was higher in Db met(-) than in WT met(-) and was suppressed by metformin. Increased levels of fibrosis-related genes, posterior capsule thickness, and collagen density were observed in the capsules of db/db mice compared with those in WT mice; these effects were suppressed by metformin. Glucose addition increased fibrosis-related gene expression in both groups of mice in vitro. When glucose was added, metformin inhibited the expression of fibrosis-related genes other than cellular communication network factor 2 (*Ccn2*) in WT mouse cells.

## Conclusion

Hyperglycaemia promotes fibrosis in the mouse knee joint capsule, which is inhibited by metformin. These findings can help inform the development of novel strategies for treating knee joint capsule fibrosis.

## Article focus

- Effect of metformin on diabetes-induced fibrosis of knee joint capsule.

## Key messages

- The diabetic mouse model (db/db) has increased fibrosis-related genes and proteins in the knee joint capsule.
- Metformin inhibits hyperglycaemia-induced fibrosis of the knee joint capsule and the resulting fibroblasts.

## Strengths and limitations

- Fibrosis of the knee joint capsule is shown to be accelerated by hyperglycaemia in a diabetic mouse model, which is suppressed by metformin.
- Only male mice were used in this study.
- Further investigation of sex-dependent differences, including blood adiponectin concentrations, is needed.

## Introduction

Diabetes mellitus is characterized by hyperglycaemia and insulin resistance; it causes injury and dysfunction in several major organs in humans and other animals. One pathological response to tissue injury is the development of fibrosis with extracellular matrix (ECM) accumulation.[1] In patients

with diabetes, fibrosis contributes to liver dysfunction, cardiomyopathy, and retinopathy.[2-4]

Diabetes mellitus also causes diabetic hand syndrome, which is characterized by limited joint mobility (LJM) in the upper limbs, including trigger finger and Dupuytren's contracture, induced by fibrosis of the tendon sheath and palmar tendon membrane.[5] The main pathophysiology of LJM is finger joint contracture, characterized by a limited range of motion (ROM); however, the underlying mechanisms of joint contracture need to be elucidated.

Joint contracture compromises joint function and limits patients' daily activities. Joint contractures resulting from common treatments for musculoskeletal conditions cause significant discomfort to patients.[6] Physiotherapy and surgical release are widely used to prevent and treat joint contracture, with limited effectiveness;[7] therefore, better prevention and treatment methods are needed. Joint contracture is associated with arthrogenic (bone, capsule, synovium, and ligament) and myogenic (tendon and fascia) components. Fibrosis of the joint capsule, an arthrogenic component, is a primary cause of joint contracture.[8]

Type 2 diabetes db/db mice are a commonly used diabetic model derived from wild-type (WT) C57BL/6 J mice with a mutation in the leptin receptor gene, directly acting on the hypothalamus to suppress appetite and weight gain.[9] The generation of this model causes collagen accumulation and tissue fibrosis in the kidneys, heart, and liver.[10-12]

Metformin is one of the most widely used oral diabetes medications for type 2 diabetes, and is the first-line therapy for patients with this disease.[13] Metformin can inhibit fibrosis in the heart, lungs, and kidneys,[14-16] and is involved in adiponectin production and release in adipose tissue.[17] The anti-inflammatory and anti-fibrosis effects of adiponectin inhibit tissue fibrosis in the heart, kidney, and ligaments.[18-20] However, the effects of metformin on knee joint capsule fibrosis in patients with diabetes remain unclear.

The objectives of this study were to compare the knee joint capsule fibrosis status in WT and db/db mice and assess the effect of metformin on fibrosis in these mice. The results of this study will aid the development of new therapies for knee joint capsule fibrosis.

## Methods

### Experimental animals

Considering that oestrogen in female mice inhibits tissue fibrosis, this study used eight-week-old WT C57BL/6 J (22 g to 27 g) and db/db (41 g to 45 g) male mice (Clea, Japan).[21] Mice were housed in cages in a temperature-controlled room (20°C to 25°C) with a 12-hour light/dark cycle and fed standard rodent food and water ad libitum. The study was approved by our institution's Ethics Committee and followed the ARRIVE guidelines.

### Study design

WT and db/db mice were divided into four groups (n = 6/group): C57BL6J male mice without metformin (WT met(-) group) and with metformin (WT met(+) group), and db/db male mice without metformin (Db met(-) group) and with metformin (Db met(+) group). Animals without significant problems or complications were used. Metformin administration was initiated at week 0 (8-week-old mice); six mice in each met(+) group received metformin until week 4 (12-week-old mice) or until week 6 (14-week-old mice). In met(+) groups, metformin (100 mg/kg) (Wako Pure Chemicals, Japan) dissolved in saline was administered intraperitoneally daily (0.2 mL). Mice were euthanized by cervical dislocation under sevoflurane inhalation anaesthesia. The knee joint capsule of the right hindlimb was assigned for gene expression evaluation, while the capsule of the left hindlimb was assigned for histological evaluation and immunohistochemical staining (Figure 1). Visceral fat was collected from 12-week-old WT met(-/+) and Db met(-/+) mice, and adiponectin expression was assessed. The study design and the optimal animal age for metformin administration and metformin dosage followed previous studies.[22-25]

In vitro, fibroblasts were isolated from the right hindlimb knee joint capsules of eight 14-week-old WT and db/db mice. Fibroblasts were cultured in normal and high-glucose (HG) medium and divided into eight groups with/without metformin (Supplementary Table i), or eight groups with/without AdipoRon (AG-CR1-0154; Adipogen, USA) (Supplementary Table ii).

### Expression of fibrosis-related genes in knee joint capsules

After euthanasia, the right hindlimb was dissected at the hip level; the skin and muscles were removed from the disconnected right hindlimb to expose the knee joint capsule. The knee joint capsule tissues were collected.

Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, USA) using the acidic thiocyanate guanidinium phenol-chloroform method, and RNA integrity and purity were assessed by calculating the ratio of absorbance at 260 and 280 nm. Total RNA was reverse transcribed in a 20 μL reaction volume using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the CFX Connect system (Bio-Rad).

The expression of fibrosis-related genes, type I collagen-α1 (*Col1a1*), type Iα2 collagen (*Col1a2*), type IIIα1 collagen (*Col3a1*), actin α 2 (*Acta2*), cellular communication network factor 2 (*Ccn2*), and transforming growth factor-β1 (*TGFβ1*) were evaluated, with β-2 microglobulin (*B2m*) as a housekeeping gene. Primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research, USA) and listed in Supplementary Table iii.

### Histological evaluation

The left hindlimb was fixed in 4% paraformaldehyde (pH 7.4) at 4°C for approximately 36 hours. Samples were decalcified with K-CX (FALMA, Japan) for three hours at 20°C to 25°C and neutralized with 5% sodium sulphate solution (Wako Pure Chemicals). Samples were paraffin-embedded, and the knee angles were set to 60° in all groups. The samples were cut into 5 μm sagittal sections with a microtome.

Sections were cut from the medial side of the knee; serial sections were collected after the medial meniscus was separated anteriorly and posteriorly. Three sections were selected from every five sections of the first serial sectioning and stained with haematoxylin-eosin (HE). The posterior capsular thickness was measured behind the meniscus – the portion from the extension of the perpendicular line drawn from the apex of the meniscus to its trailing edge – using an

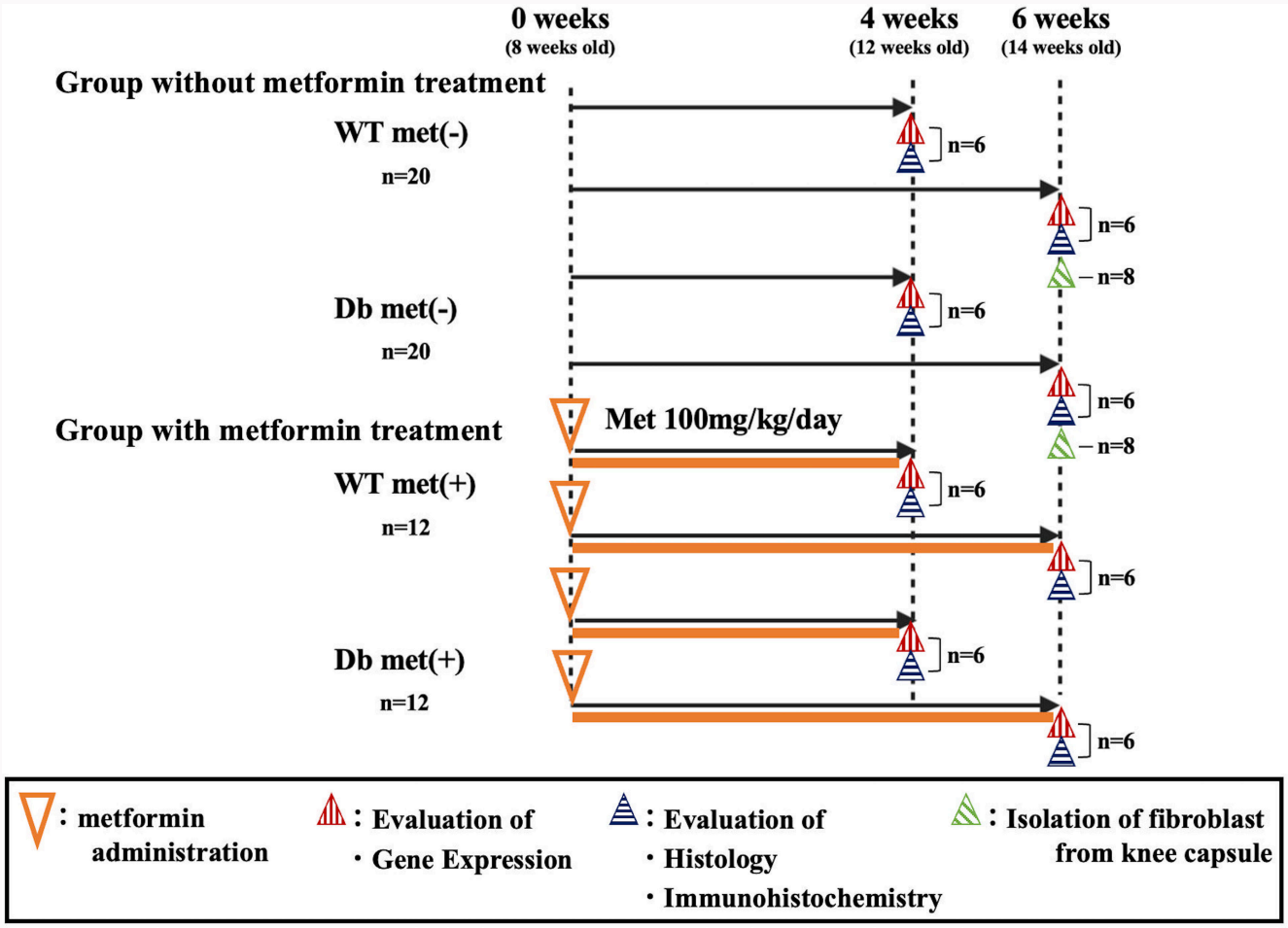


Fig. 1
Study flowchart. The experiment comprised four main groups categorized as wild-type or diabetic, with and without metformin administration. Metformin administration was initiated at week 0 (8-week-old mice); six animals in each group received metformin until week 4 (12-week-old mice) or week 6 (14-week-old mice). Mice from each group were killed at weeks 12 and 14 of age. Complementary DNA (cDNA) was synthesized from total RNA isolated from the knee joint capsule for genetic evaluation, and knee joints were paraffin-embedded for morphological and immunohistochemical evaluation. WT met(-), wild-type mice without metformin; Db met(-), type 2 diabetes mouse model without metformin; WT met(+), wild-type mice with metformin; and Db met(+), type 2 diabetes mouse model with metformin.

all-in-one fluorescence microscope BZ-X710 (Keyence, Japan). It was measured as the distance from the point in contact with the meniscus (i.e. anterior edge) to the last surface of the continuous collagen tissue (i.e. posterior edge). This analysis was performed using three sections per mouse (n = 6/group; Supplementary Figure aa).

The other three sections were stained with Picrosirius Red (PSR) (ab150681; Abcam, UK), and the ratio of the entire area of the posterior capsule to the area stained with PSR was assessed as the collagen fibre density (Supplementary Figure ab). Each area was quantitatively assessed using the ImageJ 1.46 r software package (National Institutes of Health, USA).

**Immunohistochemical staining**

Sections were washed with phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated with 3% $H_2O_2$/methanol for five minutes, and non-specific binding of immunoglobulins was blocked by overnight incubation with Protein Block Serum-Free (DAKO, USA). Slides were incubated with rabbit anti-mouse TGF-β1 polyclonal antibody (ab92486, Abcam), Acta2 polyclonal antibody (ab5694, Abcam), and Ccn2 polyclonal antibody (ab6992, Abcam) diluted with PBS by 250-fold, 100-fold, and 50-fold for 30 minutes at 20°C to 25°C. The slides were incubated with Histofine simply stained mouse MAX-PO(R) (Nichirei Bioscience Corporation, Japan) at 20°C to 25°C for 30 minutes. After washing with PBS, staining was performed using DAB IHC Substrate (GenWay Biotech, USA) for ten minutes. Contrast staining was performed using haematoxylin.

An all-in-one fluorescence microscope BZ-X710 was used to capture the posterior capsule of each slide at 400× magnification. The ImageJ 1.46 r software package was used to count the total cells, TGF-β1-positive, Acta2-positive, and Ccn2-positive cells in the posterior capsule, excluding vascular endothelial cells, adipocytes, and menisci. To enumerate the total cells, the threshold was set to 80 ± 5 pixels (px) for each image. To count the TGF-β1-positive, Acta2-positive, and Ccn2-positive cells, the threshold was set to 25 ± 5px. The percentage of TGF-β1-positive, Acta2-positive, or Ccn2-positive cells in the total cell count was calculated. Immunohistochemical analysis was performed in two different regions of each tissue section per mouse.

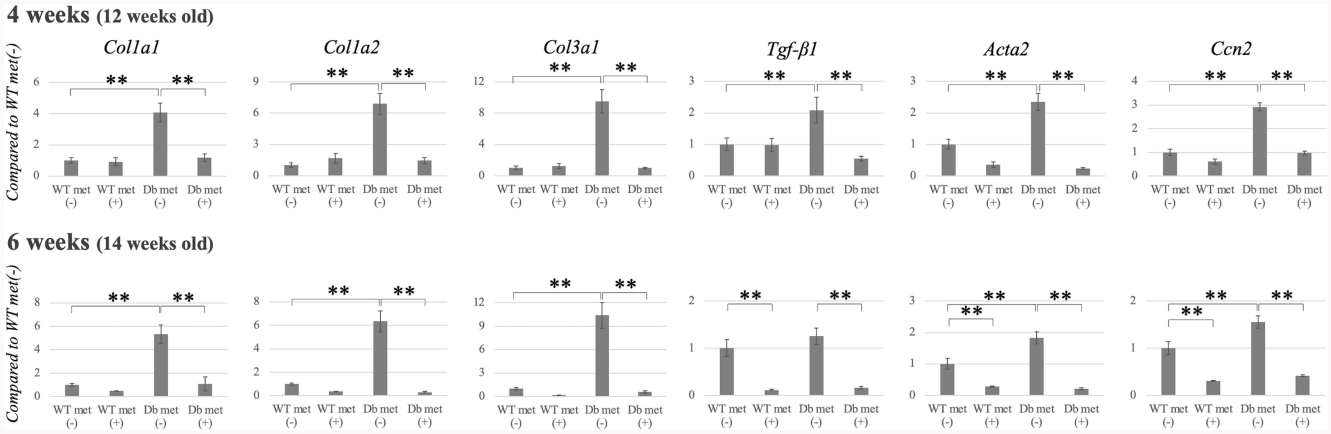


**Fig. 2**

Effect of metformin and hyperglycaemia on knee joint capsule fibrosis in a mouse model of type 2 diabetes mellitus (Db). The effect of metformin was analyzed by measuring the expression of fibrosis-related genes in the knee joint capsule, including IA1 collagen (*Col1a1*), IA2 collagen (*Col1a2*), IIIA1 collagen (*Col3a1*), transforming growth factor β1 (*TGF-β1*), actin α2 (*Acta2*), and cellular communication network factor 2 (*Ccn 2*); messenger RNA expression was normalized to β-2-microglobulin (*B2m*) expression levels. Data are expressed as mean and standard error of the mean (SEM). **p < 0.01; n = 6. T, wild-type.

### Expression of fibrosis-related genes in fibroblasts in knee joints

Knee joint capsules were harvested in the same manner as described in above. Eight knee joint capsule tissues were digested in collagenase (C0130; Sigma-Aldrich, USA) and heated at 37°C for two hours to isolate fibroblasts, which were then transferred to a Petri dish. The cells were cultured in minimal essential medium (12561-056; Thermo Fisher Scientific) containing Earle salt supplemented with 10% fetal bovine serum (S1780; Biowest, USA) and 1% antibiotic-antifungal agent (15240-062; Thermo Fisher Scientific) in an incubator with 5% $CO_2$ at 37°C. The medium was changed every three to four days. At 70% confluence, cells were seeded in normal and high-glucose medium (30 nmol/L) in six-well plates ($5 \times 10^5$ cells per well). After 72 hours, metformin (50 μM/L) was added, and total RNA was collected 24 hours later. AdipoRon (100 μM) was also added in the same manner. The concentrations of the high-glucose medium, metformin, and AdipoRon were based on previous reports.[20,24]

### Statistical analysis

All measurements are presented as the mean and standard error of the mean (SEM). Statistical significance was set at $p < 0.05$ or $p < 0.01$ (where noted). One-way analysis of variance (ANOVA) with Tukey's post-hoc analysis was used. Statistical analyses were performed using SPSS Statistics version 22 software (IBM, USA).

## Results

### Gene expression in the knee joint capsule

Excluding *TGF-β1* at six weeks, the expressions of fibrosis-related genes were increased in the Db met(-) group compared with the WT met(-) group (all $p < 0.05$, one-way ANOVA with Tukey's post-hoc analysis). The expression of all fibrosis-related genes in the Db met(+) group and of *TGF-β1*, *Acta2*, and *Ccn2* in the WT met(+) group were suppressed at six weeks compared with the WT met(-) group (all $p < 0.05$; Figure 2).

### Histological analysis

HE staining showed that the Db met(-) group had thicker posterior capsules than the WT met(-) group at weeks 4 and 6, and the Db met(+) group at week 6 (all $p < 0.05$, one-way ANOVA with Tukey's post-hoc analysis; Figure 3). PSR staining showed that the Db met(-) group had higher collagen density in the posterior capsule at weeks 4 and 6 than the WT met(-) and Db met(+) groups (all $p < 0.05$; Figure 3).

### Immunohistochemical analysis

The Db met(-) group had a higher percentage of ACTA2- and CCN2-positive cells than the WT met(-) group (all $p < 0.01$, one-way ANOVA with Tukey's post-hoc analysis; Figure 4) and Db met(+) group (all $p < 0.05$, one-way ANOVA with Tukey's post-hoc analysis; Figure 4) at weeks 4 and 6. The Db met(-) group also had a higher percentage of TGF-β1-positive cells than the Db met(+) group at week 6 ($p = 0.048$, one-way ANOVA with Tukey's post-hoc analysis; Figure 4).

### Effects of hyperglycaemia and metformin on mouse fibroblasts in vitro

The WT and Db met(-) HG groups expressed higher levels of fibrosis-related genes than the WT and Db met(-) groups (all $p < 0.05$, one-way ANOVA with Tukey's post-hoc analysis). The WT met(+) HG group expressed lower levels of *Col1a1*, *Col1a2*, *Col3a1*, *TGF-β1*, and *Acta2* than the WT met(-) HG group (all $p < 0.05$, one-way ANOVA with Tukey's post-hoc analysis). The Db met(+) and Db met(+) HG groups did not exhibit significant differences in the expression of these genes compared with the Db met(-) or Db met(-) HG groups (Figure 5).

### Effects of hyperglycaemia and AdipoRon on the visceral fat of mice

Metformin significantly increased adiponectin expression (Figure 6).

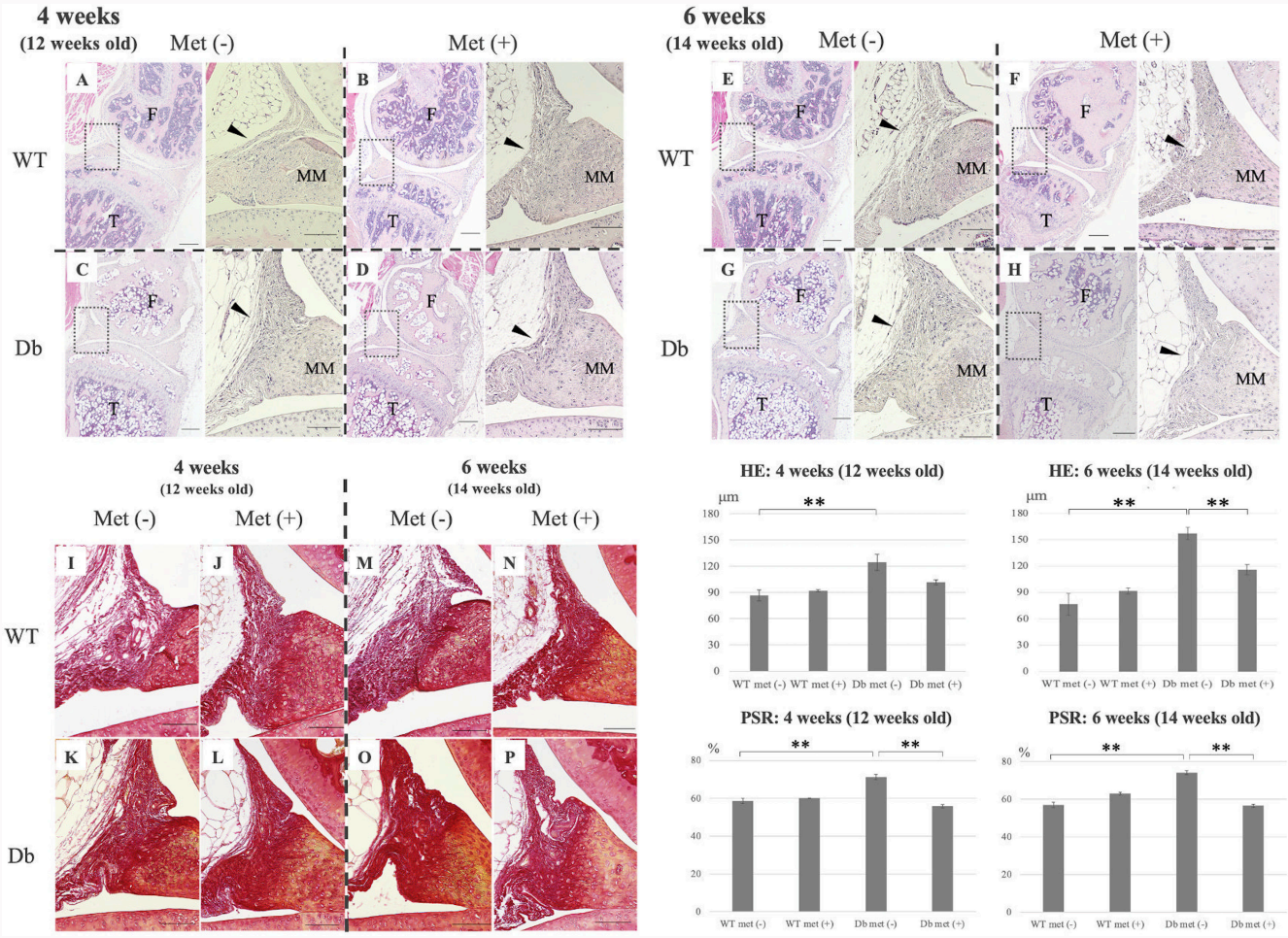


**Fig. 3**
Histological analysis of the effect of metformin on the knee joint capsule. Haematoxylin and eosin stained sections of knee joint capsule from each group after four and six weeks (low magnification, 20x). Arrows indicate posterior knee joint capsule. Each right-side image shows a higher magnification image (400x) of the dotted box of the corresponding left-side image (scale bar: 300 μm (right), 100 μm (left)). **p < 0.01; n = 6. At four weeks (12-weeks-old) A: WT met(-), B: WT met(+), C: Db met(-), D: Db met(+); at six weeks (14-weeks-old) E: WT met(-), F: WT met(+), G: Db met(-), H: Db met(+). Picrosirius Red-stained (PRS) sections (scale bar: 100 μm, magnification 400x) of posterior knee joint capsule for each group at four and six weeks of metformin treatment. The percentage of collagen fibres in the posterior capsule at four and six weeks was measured as the ratio of the total area stained red by PRS to the area of the posterior capsule. Data are expressed as mean and standard error. **p < 0.01; n = 6. At four weeks (12-weeks-old) I: WT met(-), J: WT met(+), K: Db met(-), L: Db met(+); at six weeks (14-weeks-old) M: WT met(-), N: WT met(+), O: Db met(-), P: Db met(+).

### Effects of hyperglycaemia and AdipoRon on mouse fibroblasts in vitro

The WT control HG groups expressed higher levels of *Col1a1*, *Col3a1*, *Acta2*, and *Ccn2* than the WT control groups (all p < 0.05). The Db con HG groups expressed higher levels of fibrosis-related genes than the Db con groups (all p < 0.05, one-way ANOVA with Tukey's post-hoc analysis). The WT and Db A100 HG group expressed lower levels of *Col1a1*, *Col1a2*, *Col3a1*, *Acta2*, and *Ccn2* than the WT and Db control HG groups (all p < 0.05, one-way ANOVA with Tukey's post-hoc analysis; Figure 7).

## Discussion

Hyperglycaemia-associated fibrosis results in ECM accumulation through pathways such as oxidative stress, neurohumoral signalling, inflammatory cascades, and growth factor cascades (e.g. TGF-β1/Smad3),[26] indirectly causing cell injury related to DNA damage and insulin resistance.[1] The mechanism of hyperglycaemia-induced fibrosis involves activating transcription factors associated with the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway, which in turn increases TGF-β1 and fibronectin synthesis, resulting in cell proliferation and substrate increase.[27,28] TGF-β1 is a critical cytokine in the fibrotic process as it promotes fibroblast-to-myofibroblast differentiation, myofibroblast proliferation, and collagen production,[29] playing a central role in organ fibrosis in diabetes.[30] TGF-β1 promotes fibroblast-to-myofibroblast differentiation under hyperglycaemia, increasing α-SMA expression.[31] TGF-β1 enhances Ccn2 expression in tissue fibrosis during hyperglycaemia,[31] which is a downstream mediator of TGF-β1 signalling in fibroblasts. As such, Ccn2 can mediate the fibrosis-promoting effects of TGF-β1.[29] This study showed increased expression of *Col1a1*, *Col1a2*, *Col3a1*, *Acta2*, and *Ccn2*, correlated with TGF-β1 upregulation, suggesting that the increased fibrosis of the knee joint capsule in db/db mice was due primarily to TGF-β1 upregulation. Glucose-induced upregulation of fibrosis-related genes in capsule fibroblasts of WT and db/db mice also

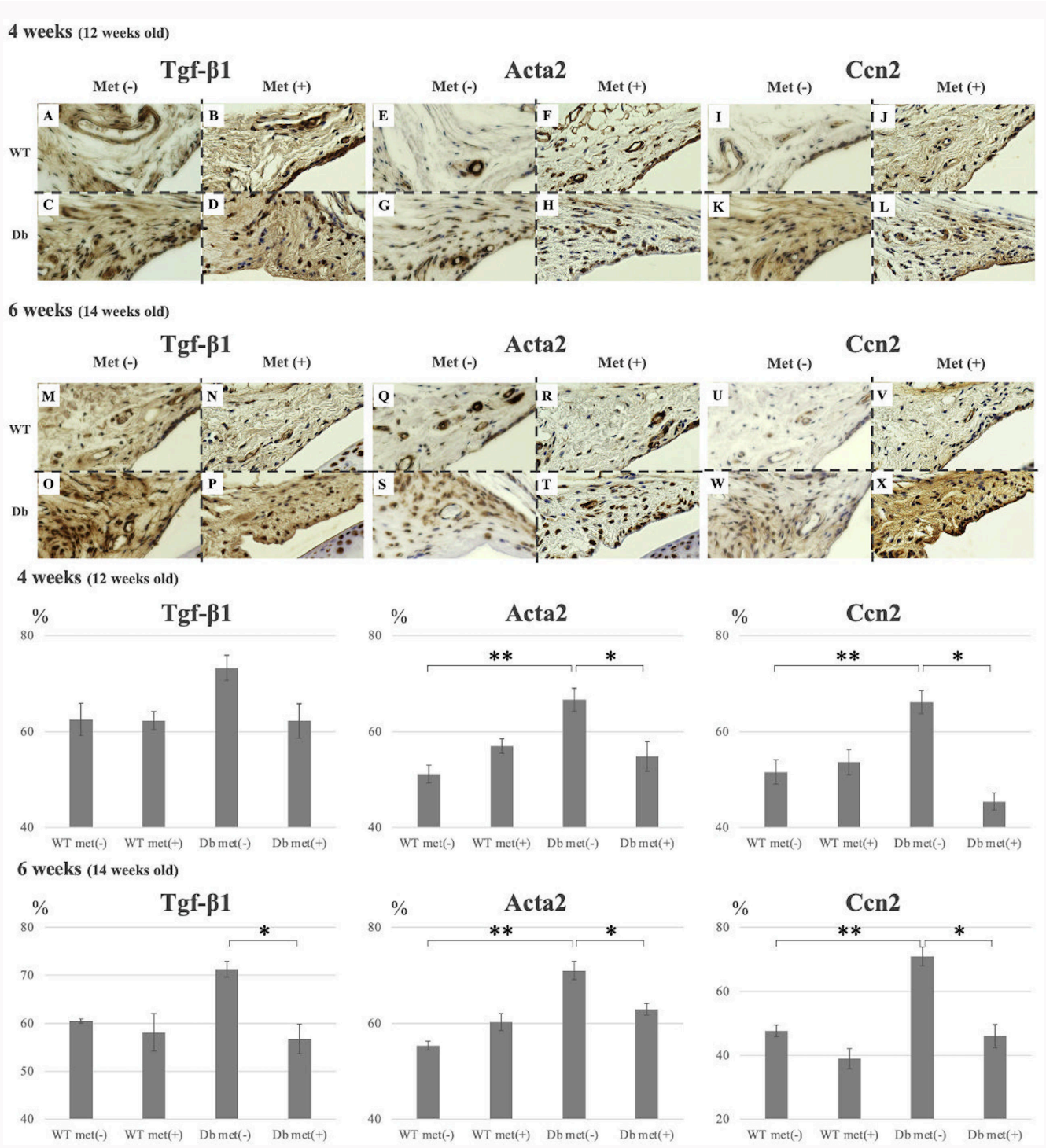


**Fig. 4**

Immunohistochemical analysis of the effect of metformin on the posterior knee joint capsule. The proportion of transforming growth factor β1 (TGFβ1)-positive-, actin α2 (Acta2)-positive-, and cellular communication network factor 2 (Ccn2)-positive cells relative to the total number of cells in the posterior capsule of each group are presented at four and six weeks of metformin treatment. The threshold of the total number of cells was set to 80 ± 10 pixels (px) for each image, and that of each type of positive cell was set to 25 ± 5 px. Data are expressed as mean and standard error of the mean (SEM). *p < 0.05, **p < 0.01; n = 6. At four weeks (12 weeks old), TGF-β1 A: wild-type (WT) met(-), B: WT met(+), C: diabetes mellitus (Db) met(-), D: Db met(+); Acta2 E: WT met(-), F: WT met(+), G: Db met(-), H: Db met(+); Ccn2 I: WT met(-), J: WT met(+), K: Db met(-), L: Db met(+); at six weeks (14 weeks old), TGF-β1 M: WT met(-), N: WT met(+), O: Db met(-), P: Db met(+); Acta2 Q: WT met(-), R: WT met(+), S: Db met(-), T: Db met(+); Ccn2 U: WT met(-), V: WT met(+), W: Db met(-), X: Db met(+).

suggested TGF-β1 involvement in hyperglycaemia-associated fibrosis.

Arthrogenic components are responsible for approximately 47% of joint contractures.[8] This study showed that db/db mice exhibited increased posterior capsule thickness and collagen density compared with WT mice, which was suppressed by metformin. Metformin did not further decrease the capsule thickness in WT mice. Previous studies reported that capsule fibrosis is enhanced by fixing the knee joint in

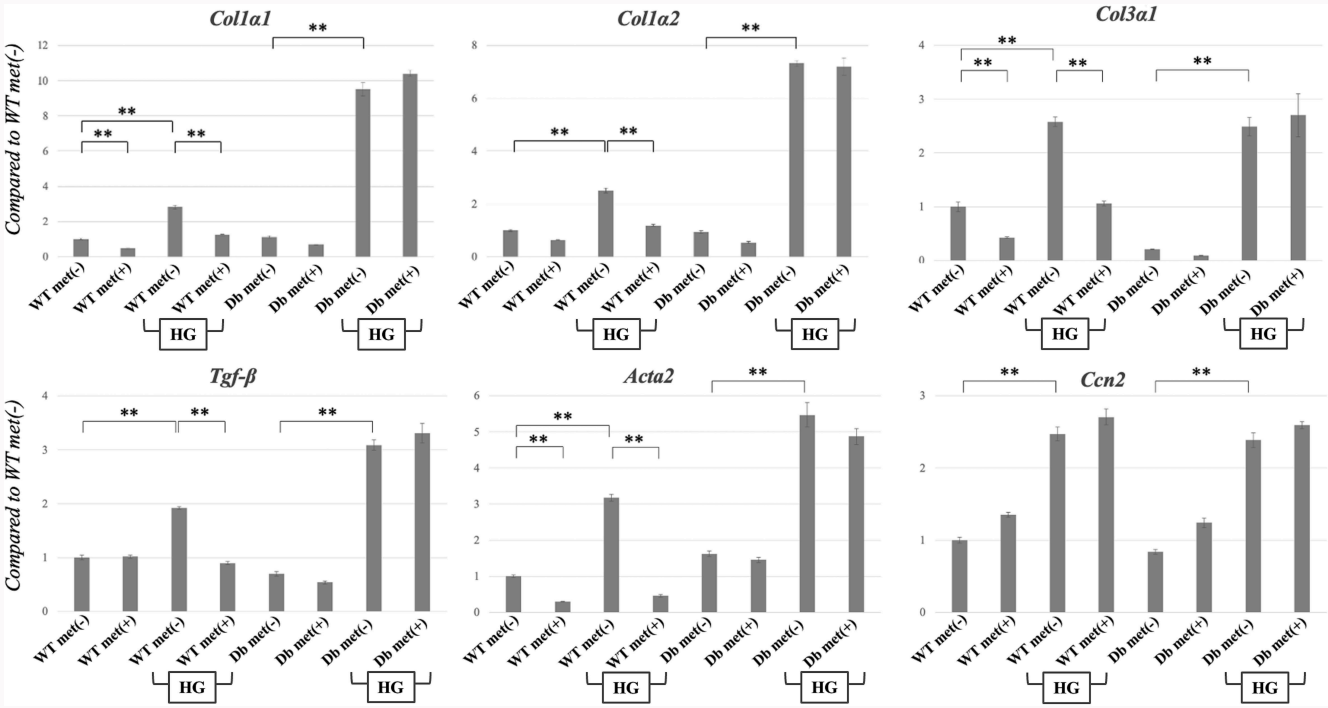


**Fig. 5**

Expression analysis of fibrosis-related genes. The analysis was performed in vitro to determine the effect of metformin on fibrosis in fibroblasts collected from knee joint capsules of wild-type (WT) and diabetic mice (Db) to determine the effect of hyperglycaemic conditions on fibrosis. Data are expressed as mean and standard error of the mean (SEM). **p < 0.01; n = 8. *Acta2*, actin α2; *Ccn2*, cellular communication network factor 2; *Col1a1*, IA1 collagen; *Col1a2*, IA2 collagen; *Col3a1*, IIIA1 collagen; HG, high-glucose; *TGF-β1*, transforming growth factor β1.

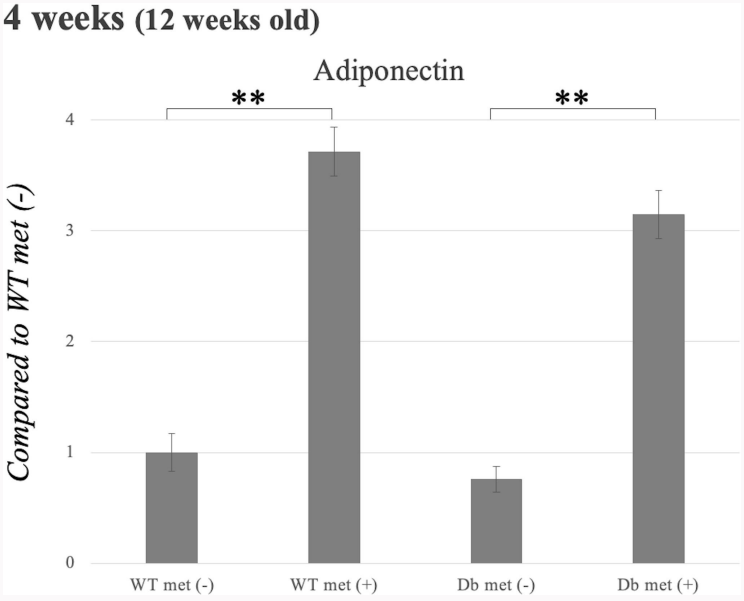


**Fig. 6**

In vivo expression analysis of adiponectin. The analysis was performed in vivo to determine the effect of metformin in visceral fats of wild-type (WT) and diabetic (Db) mice at four weeks of metformin treatment. Data are expressed as mean and standard error of the mean (SEM). *p < 0.05, **p < 0.01, one-way ANOVA with Tukey's post-hoc analysis; n = 6.

flexion in WT mice.[32,33] Thus, knee joint capsule fibrosis is similarly exacerbated in the diabetic mouse model.

Db/db mice are widely used as type 2 diabetic mice, with fibrosis being reported in various organs. Upregulation of the TGF-β/Smad3 signalling pathway contributes to tissue fibrosis,[10-12] similar to the current study. Meanwhile, although some studies have investigated knee joint contracture in diabetic patients and after knee arthroplasty in clinical practice, to our knowledge, no reports have evaluated the relationship with normal knee joints.[34,35] Thus, the observation of the current study that fibrosis occurs in the knee joint

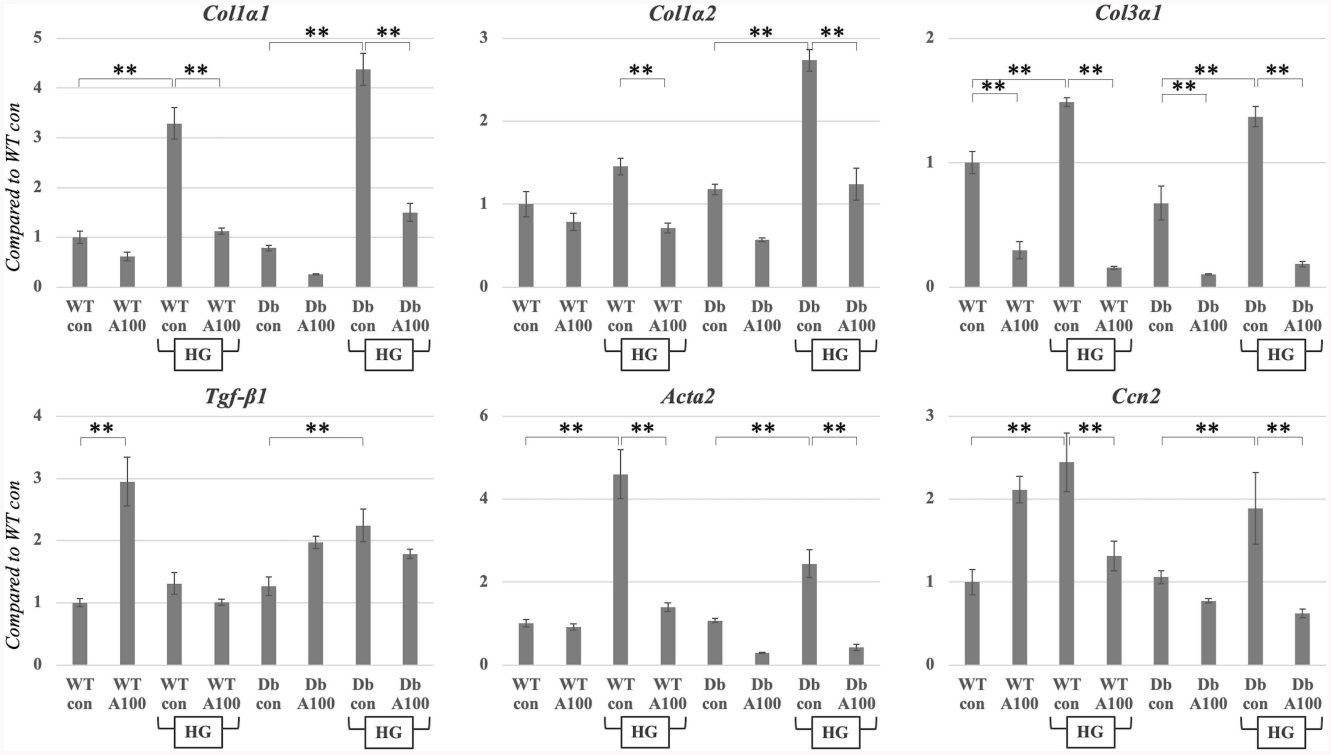


**Fig. 7**

In vitro expression analysis of fibrosis-related genes. The analysis was performed to determine the effect of AdipoRon on fibrosis in fibroblasts collected from knee joint capsules of wild-type (WT) and diabetic (Db) mice to determine the effect of hyperglycaemic conditions on fibrosis. Data are expressed as mean and standard error of the mean (SEM). **p < 0.01, one-way analysis of variance with Tukey's post-hoc analysis; n = 8. *Acta2*, actin α2; *Ccn2*, cellular communication network factor 2; *Col1a1*, IA1 collagen; *Col1a2*, IA2 collagen; *Col3a1*, IIIA1 collagen; HG, high-glucose; *TGF-β1*, transforming growth factor β1.

of diabetic mice provides important insights regarding the pathogenesis of knee joint contracture in diabetic patients.

Several studies have reported metformin administration to db/db mice at doses ranging from 100 to 250mg/kg.[23-25] The lowest concentration (100 mg/kg) effectively inhibits myocardial fibrosis; accordingly, 100 mg/kg was used in the current study, which better reflects clinical use in humans (20 to 40 mg/kg),[36] but was still relatively higher.

Metformin treatment increases the adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio by inhibiting complex I of the mitochondrial respiratory chain and ATP synthesis, activating the cellular metabolic stress sensor, AMP-activated protein kinase (AMPK),[37] which inhibits fibrosis by suppressing the expression of Smad3-dependent Ccn2, a factor involved in the TGF-β signalling.[16] AMPK activation also suppresses inflammation by ameliorating apoptosis in tendinopathy associated with obesity or insulin resistance.[38] Furthermore, it suppresses fibrosis by inhibiting TGF-β1 production, Smad3 nuclear translocation, and phosphorylation in diabetic nephropathy and cardiomyopathy.[14-16] Liang et al[39] showed that the expression of fibrosis-related factors, including type 3 collagen, was increased by glucose addition and decreased by metformin treatment in mouse renal tubular epithelial cells. Similarly, the current study showed that metformin suppressed the hyperglycaemia-induced upregulation of fibrosis-related genes in vivo/in vitro. Considering that metformin suppressed TGF-β1 expression, the direct suppression of TGF-β1 expression, rather than the Smad-mediated action of TGF-β signalling, is likely responsible for its suppressive effect on fibrosis.

In studies in which metformin was administered to seven- to ten-week-old diabetic model mice, daily intraperitoneal or oral administration for six weeks had an inhibitory effect on organ tissue fibrosis, whereas studies in which metformin administration was initiated after ten weeks showed only a tendency to inhibit fibrosis.[23-25] Therefore, in the current study, metformin administration was initiated in eight-week-old mice for six weeks. In addition, a four-week administration duration was added to clarify whether fibrosis inhibition can be achieved following a shorter treatment duration.

In vitro experiments using db/db mice did not suppress hyperglycaemia-induced upregulation of fibrosis-related genes. Since cells collected from knee joint capsules of 14-week-old (10-week-old or older) mice were used in this study, it is possible that the fibrosis-inhibitory effect was not achieved.

Adiponectin is an important adipokine with anti-inflammatory and antioxidant effects that improves remodelling and suppresses fibrosis in various pathological conditions, such as diabetic nephropathy and atrial fibrillation.[18-20] Metformin increases adiponectin secretion.[40] In patients with scleroderma and advanced fibrosis, the abundance of adiponectin and its receptor are reduced, suggesting that adiponectin is involved in progressive fibrosis in chronic fibrotic diseases.[41] Adiponectin is decreased in the adipose tissue around the palmar aponeurosis of patients with

Dupuytren's contracture, and it suppresses fibrosis of the palmar aponeurosis in a concentration-dependent manner.[20] The current study showed that the addition of adiponectin to db/db mice fibroblasts in vitro suppressed hyperglycaemia-induced upregulation of fibrosis-related genes, excluding TGF-β1, suggesting that adiponectin improves tissue remodelling; in contrast, metformin alone led to an irreversible effect. Thus, metformin-induced inhibition of fibrosis in the knee joint capsule in vivo might be caused by the combinational effect of TGF-β1 inhibition and adiponectin.

The prevalence of Dupuytren's contracture and LJM differs considerably depending on the presence or absence of diabetic retinopathy caused by long-term poor blood glycaemic control,[4,30] indicating that diabetes-induced fibrosis occurs progressively. Early therapeutic intervention in patients with diabetes is important to prevent musculoskeletal complications, suggesting that metformin stimulation of adiponectin production might also be broadly useful in preventing progressive fibrotic diseases.

This study has certain limitations. First, only male mice were used to eliminate the effect of oestrogen as previously reported.[21] However, sex differences have been reported in the effects elicited by metformin,[42] hence studies with female mice are needed. Second, in vitro experiments using fibroblasts collected from the knee joint capsule were conducted exclusively in 14-week-old mice. Future studies using fibroblasts from mice younger than ten weeks might clarify the effects of glucose and metformin on fibrosis at different ages. Third, this study evaluated adiponectin expression in visceral fat; therefore, only the endocrine effect of adiponectin on the knee joint was evaluated. Blood adiponectin concentration is derived from adipocytes,[43] and a decrease in blood concentration is associated with liver fibrosis.[44] An increase in blood concentration is associated with fibrosis suppression.[45] The underlying mechanism of adiponectin function is autocrine/paracrine and endocrine in various organs.[46] Metformin inhibition of fibrosis in the knee joint capsules of mice might be due to adiponectin upregulation in visceral fat and its endocrine effect. Fourth, this study was restricted to in vivo murine analyses to evaluate fibrosis of the knee joint capsule induced by metformin administration; hence, further investigation is needed to determine the effect on fibrosis of the knee joint in diabetic patients. Finally, we did not measure glucose concentrations in blood or culture medium. Although previous studies have reported that metformin administration reduces blood glucose concentration in diabetic model mice,[42] the results of the current study alone do not demonstrate whether the in vivo antifibrotic effect of metformin within the knee joint capsule is due to reduced blood glucose, or whether it is specific to metformin. Meanwhile, given that the glucose level does not reportedly change in the culture medium following the addition of metformin,[47] the in vitro antifibrotic effect induced by metformin cannot be due to a decrease in the culture glucose concentration but rather is specific to metformin.

In conclusion, compared with WT mice, db/db mice had higher levels of fibrosis-related genes and proteins, posterior capsule thickness, and collagen density in the knee joint capsule, which were inhibited by metformin treatment. In knee joint capsule fibroblasts, glucose addition upregulated fibrosis-related genes in WT and db/db mice, and metformin downregulated fibrosis-related genes only in WT mice; in contrast, adiponectin suppressed the expression of fibrosis-related genes, with the exception of *TGF-β1*, in all cases. The findings of this study provide insights to support the development of new strategies for improved treatment of knee joint capsule fibrosis.

## Supplementary material

Tables showing fibroblasts culture groups with or without metformin/AdipoRon, and the polymerase chain reaction and primer sequences. Figure showing the method for measuring the thickness and area of the posterior knee joint capsule. An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

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