Article

# Attenuation of Armanni–Ebstein lesions in a rat model of diabetes by a new anti-fibrotic, anti-inflammatory agent, FT011

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**Abstract**

*Aims/hypothesis* A key morphological feature of diabetic nephropathy is the accumulation and deposition of glycogen in renal tubular cells, known as Armanni–Ebstein lesions. While this observation has been consistently reported for many years, the molecular basis of these lesions remains unclear.

*Methods* Using biochemical and histochemical methods, we measured glycogen concentration, glycogen synthase and glycogen phosphorylase enzyme activities, and mRNA expression and protein levels of glycogenin in kidney lysates from control and transgenic (m*Ren-2*)27 rat models of diabetes that had been treated with and without a new anti-fibrotic agent, FT011.

*Results* Diabetic nephropathy was associated with increased glycogen content, increased glycogen synthase activity and decreased glycogen phosphorylase activity. Glycogenin, the key protein responsible for initiating the synthesis of each glycogen particle, had very high levels in the diabetic kidney together with increased mRNA expression compared with control kidneys. Treatment with FT011 did not change glycogen synthase or glycogen phosphorylase enzyme activities but prevented both glycogenin mRNA synthesis and accumulation of Armanni–Ebstein lesions in the diabetic kidney.

*Conclusions/interpretation* Armanni–Ebstein lesions found in diabetic nephropathy are due to aberrant glycogenin protein levels and mRNA expression, providing an explanation for the increased glycogen concentration found within the diabetic kidney. FT011 treatment in diabetic rats reduced glycogenin levels and, subsequently, renal glycogen concentration.

**Keywords** Armanni–Ebstein lesions, Diabetic kidney, Diabetic nephropathy, Glycogen, Glycogenin, Glycogen phosphorylase, Glycogen synthase

**Abbreviations**

| | |
|---|---|
| G6P | Glucose-6-phosphate |
| PAS | Periodic acid-Schiff |
| *Ren-2* | Transgenic (m*Ren-2*)27 |

## Introduction

Diabetes mellitus is associated with microvascular complications, including cardiomyopathy and nephropathy, and persistent hyperglycaemia. Diabetic nephropathy is associated with progressive kidney dysfunction and is the leading cause of end-stage renal disease.

A key morphological change associated with sustained hyperglycaemia in the diabetic kidney is the accumulation of glycogen [1], known as Armanni–Ebstein lesions [2]. Glycogen accumulation predominantly occurs in the cortical and outer medullary region of the diabetic kidney, primarily localised in the thick ascending limb of the loop of Henle and distal tubules [1]. The accumulation of glycogen in these cells has been suggested by several studies to compromise their viability and thus contribute to renal impairment in diabetic individuals [2]. Furthermore, a growing body of evidence suggests that glycogen nephrosis may be associated with the progression of diabetic nephropathy [1, 3].

Glycogen is a complex branched polymer of glucose that is found predominantly in liver and skeletal muscle but not in healthy kidneys due to its high metabolic turnover. Glycogen consists of a core protein, glycogenin, with covalent α-1,4-glycosidic linkages attached to

the polypeptide chain that are extended by the collaborative actions of glycogen synthase and glycogen-branching enzyme. Glycogen degradation is mediated by the concerted action of glycogen phosphorylase and glycogen-debranching enzyme [4].

We recently identified a new anti-fibrotic agent called FT011 that attenuated fibrosis in experimental diabetic cardiomyopathy and nephropathy [5, 6]. Investigation of the action of FT011 on the diabetic kidney resulted in the observation that Armanni–Ebstein lesions were absent. Here we show the molecular basis for Armanni–Ebstein lesions where they are due to increased production of glycogenin protein and mRNA expression and are attenuated by treatment with FT011.

**Methods**

Forty, 6-week-old female heterozygous transgenic (m*Ren-2*)27 (*Ren-2*) rats (St Vincent's Hospital Animal House, Melbourne, VIC, Australia) were assigned to receive either 55 mg/kg streptozotocin (Sigma-Aldrich, St Louis, MO, USA) diluted in 0.1 mol/l citrate buffer, pH 4.5, or citrate buffer alone (non-diabetic) by tail-vein injection following an overnight fast. Control and diabetic model animals were then randomised to two groups each (n=10), receiving treatment with either FT011 (200 mg $kg^{-1}$ $day^{-1}$; Fibrotech Therapeutics, Melbourne, VIC, Australia) or vehicle (1% carboxymethyl cellulose) by gavage for 16 weeks [5]. Blood glucose and blood pressure were measured as previously described [5]; the kidney tissues used in this study were taken from these animals.

Kidney tissues were homogenised and assessed for glycogen content [7], glycogen synthase activity [8] and glycogen phosphorylase activity [9]. Histochemical analysis of renal glycogen was determined by periodic acid-Schiff (PAS) staining [10]. For determination of

glycogenin protein levels, 0.4 μg phenylmethylsulfonyl fluoride (PMSF) -treated α-amylase (Sigma-Aldrich) was added to 50 μg tissue homogenate and incubated at room temperature for 20 min, prior to SDS-PAGE and subsequent western blot analysis. Rabbit anti-human glycogenin primary antibodies [7] were incubated overnight at 4°C and detected by swine anti-rabbit horseradish peroxidise (HRP) (Dako, Glostrup, Denmark) and subsequent enhanced chemiluminescence (ECL) [7]. Pan-actin was used as a loading control. To determine glycogenin mRNA (*Gyg1*) expression, quantitive real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Oligonucleotide sequences are available on request. The PCR protocol was set at 1×95°C for 10 min, 40×95°C for 30 s, 40×54°C for 1 min and 1×72°C for 30 s. Results were normalised to $C_t$ values of housekeeping gene, rat 18S mRNA.

Statistical analyses of the results were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Significance between groups was determined using one-way ANOVA with Tukey's post hoc test. A p value $< 0.05$ was considered to be statistically significant. Data were expressed as means ± SEM.

**Results**

Blood glucose was found to be significantly higher in diabetic and diabetic treated rats compared with control rats (33 ± 0.2 mmol/l and 30 ± 0.5 mmol/l, respectively, vs 5 ± 0.2 mmol/l; $p < 0.05$). No significant difference in blood glucose was found between treated and untreated diabetic rats and between treated and untreated controls. Similarly, systolic blood pressure was found to be significantly higher in untreated and treated diabetic rats compared with controls (184 ± 7 mmHg and 171 ± 5 mmHg, respectively, vs 150 ± 5 mmHg; $p < 0.05$). No significant difference in systolic blood pressure was found between

treated and untreated diabetic rats and between treated and untreated controls. These data have been previously described [5].

Blood glucose concentration (mmol/l) for control, diabetic, control + FT011 and diabetic + FT011 rats was 6.09, 32.16, 7.32 and 30.01, respectively ($n=9$, $p < 0.0001$, one-way ANOVA). Blood pressure (mmHg) for control, diabetic, control + FT011 and diabetic + FT011 rats was 159.9, 190.6, 151.1 and 163.9, respectively ($n=9$, $p < 0.05$, one-way ANOVA).

To confirm the presence of increased renal glycogen in the diabetic rats, glycogen was measured by both standard biochemical and PAS staining methods. Measurement of glycogen concentration determination confirmed that diabetic rats contained significantly higher levels of glycogen (Fig. 1a) compared with control animals, and PAS staining confirmed the presence of Armanni–Ebstein lesions in diabetic *Ren-2* rats (Fig. 1b). FT011-treated animals had decreased renal glycogen concentrations (Fig. 1a) and an attenuated number of Armanni–Ebstein lesions (Fig 1b).

Glycogen synthase and glycogen phosphorylase activities were measured to determine whether increased glycogen in the diabetic kidney was due to increased glycogen synthesis. Glycogen synthase activity was measured by an assay that calculated the ratio of activated to total glycogen synthase activity [8]. Glycogen synthase activity in the diabetic group was found to be higher than in the control group but was unchanged in animals treated with FT011 (Fig. 1c). Glycogen phosphorylase activity was found to be significantly lower in the diabetic group (Fig. 1d) but was unchanged in animals treated with FT011 (Fig. 1d).

Additionally, glycogenin protein levels were measured to determine whether they also contributed to the presence of Armanni–Ebstein lesions together with increased glycogen synthase (Fig. 1c) and decreased glycogen phosphorylase (Fig. 1d) activities. Kidney

lysates were incubated with α-amylase, an enzyme that digests the glycogen particle and exposes glycogenin found at the glycogen core [4], and assessed for glycogenin levels via western blotting. Protein levels of glycogenin were found to be 140-fold higher (Fig. 2a) in diabetic animals in comparison with controls. Diabetic tissue that was not pretreated with α-amylase did not display immunoreactivity for glycogenin (data not shown). Quantitive real-time PCR was performed to determine whether this correlated with a change in gene expression for glycogenin (*Gyg1*) (Fig. 2b). A significant increase in glycogenin mRNA expression was found in the diabetic group compared with the control group. Diabetic rats treated with FT011 had significantly decreased glycogenin protein levels (Fig 2a) and glycogenin mRNA expression comparable to the levels found in control animals (Fig. 2b).

**Discussion**

In the present study we have shown that in the *Ren-2* rat diabetic nephropathy is associated with increased glycogenin protein levels and increased glycogenin mRNA expression, together with increased glycogen synthase activity and decreased glycogen phosphorylase activity, which result in a significant increase in renal glycogen accumulation, known as Armanni–Ebstein lesions [2]. In addition, we have demonstrated that treatment with FT011 successfully attenuated renal glycogen concentration and this was associated with a significant decrease in glycogenin production. Attenuation of renal glycogen with FT011 treatment was independent of any change in blood glucose or blood pressure, suggesting that FT011 directly targets the renal glycogen pathway in the diabetic rat. Diabetes-induced glycogen accumulation in the tubular cells has previously been reported to be associated with fewer organelles, a reduced number of proximal tubule cell microvilli, and tubular cell

apoptosis [3]. But whether glycogen accumulation interferes with the physiological function of the tubular cells remains unknown.

New glycogen particles are initiated by the enzyme glycogenin, which covalently attaches a chain of glucose residues to a specific amino acid within its polypeptide chain [4]. Thus the 140-fold increase in glycogenin protein production we find in the diabetic rat correlates to a 140-fold increase in the number of glycogen particles. The new glycogen particle is further elongated by the activities of glycogen synthase and glycogen-branching enzyme. Glycogen synthase activity is increased in the diabetic kidney, which may be caused by either protein phosphatase-mediated dephosphorylation (leading to active glycogen synthase) or allosteric activation by glucose-6-phosphate (G6P) [4]. We hypothesise that the latter is most likely due to high intracellular G6P concentrations formed by the action of glucokinase in response to the high intracellular glucose concentration (data not shown). High G6P would also explain the reduced glycogen phosphorylase activity in the diabetic kidneys, since G6P allosterically inhibits glycogen phosphorylase [4].

Reasons for the increased renal glycogenin levels in diabetic nephropathy remain speculative. One study describes the characterisation of the glycogen promoter with the identification of several transcription factor binding sites, including a cAMP response element-binding site [11]. In skeletal muscle cells, glycogenin mRNA expression was inhibited with increasing concentrations of cAMP [11], suggesting that diabetic nephropathy may therefore be associated with an inhibition of upstream cAMP-related pathways. While this is an attractive hypothesis, it does not agree with other studies that show increased cAMP and activated downstream signalling molecules such as cAMP-dependent protein kinase in diabetic animals [12]. Treatment of diabetic animals with our new anti-fibrotic agent FT011 prevented the accumulation of Armanni–Ebstein lesions

(Fig. 1b) by specifically blocking diabetes-induced renal glycogenin mRNA expression, and therefore attenuating elevated glycogenin protein levels, and reduced the synthesis of new glycogen particles. Given that glycogen synthase and glycogen phosphorylase activities remained unchanged, it suggests that FT011 does not affect conversion of glucose to G6P.

In conclusion, we have shown that Armanni–Ebstein lesions in the diabetic kidney are caused by increased protein levels and mRNA expression of the glycogen initiator protein glycogenin. Furthermore, FT011, a new anti-fibrotic agent, prevents glycogen accumulation by reducing glycogenin mRNA translation.

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**Duality of interest**

DJK and DIS are co-founders and shareholders of Fibrotech Therapeutics, and DJK is also a director of Fibrotech Therapeutics.

**Contribution statement**

XL was involved in the acquisition, analysis and interpretation of data, drafting of the article and final approval of the version to be published. YZ was involved in the acquisition of data, revision of the article for important intellectual content and final approval of the version to be published. DJK and DIS made substantial contributions to the conception, design and interpretation of data, revision of the article for important intellectual content and final approval of the version to be published. XL measured glycogen, glycogen synthase/glycogen phosphorylase activities and glycogenin, and revised the manuscript. YZ was involved in the generation of the animal models, performing the PAS stain, and revised the manuscript. DJK and DIS made substantial contributions to the conception, design, interpretation of data and writing the manuscript.

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**Fig. 1** Glycogen accumulation is due to an increase in glycogen synthesis and a decrease in glycogen breakdown. (**a**) Quantitation of glycogen concentration in control ($n$=10), control treated with FT011 ($n$=10), diabetic ($n$=10) and diabetic treated with FT011 ($n$=10) groups. Glycogen concentration was based on the difference between baseline and total renal glucose concentration for each sample. Significance between groups was determined using one-way ANOVA. Columns are expressed as means±SEM (**$p$<0.01, ***$p$<0.001). (**b**) Representative photomicrographs showing ×400, and enlarged to ×800, sections of PAS-stained rat kidney sections from diabetic and diabetic treated with FT011 groups. Diabetic animals demonstrated marked glycogen deposition, known as Armanni–Ebstein lesions (arrows), in the lumen of tubular cells in diabetic rats. Treatment of diabetic rats with FT011 resulted in attenuation of glycogen deposition. (**c**) Quantitation of glycogen synthase activity ratio in control ($n$=10), control treated with FT011 ($n$=10), diabetic ($n$=10) and diabetic treated with FT011 ($n$=10) groups. The graph represents the ratio of glycogen synthase activity in the absence and presence of its allosteric activator glucose-6-phosphate. Significance between groups was determined using one-way ANOVA. Columns are expressed as means±SEM (*$p$<0.05). (**d**) Quantitation of relative glycogen phosphorylase activity in control ($n$=10), control treated with FT011 ($n$=10), diabetic ($n$=10) and diabetic treated with FT011 ($n$=10) groups. Significance between groups was determined using one-way ANOVA. Columns are expressed as means±SEM (**$p$<0.01)

**Fig. 2** Glycogenin is upregulated in diabetic nephropathy and attenuated by FT011. (**a**) Western blot (upper) for glycogenin immunoreactivity in kidney lysates following incubation with α-amylase, normalised to the protein loading control pan-actin. Densitometry quantitation (lower) of bands for glycogenin in control ($n$=9), control treated

with FT011 (*n*=9), diabetic (*n*=9) and diabetic treated with FT011 (*n*=9) groups. Densitometry values for each band are normalised with the densitometry value of pan-actin on its respective lane. Significance between groups was determined using one-way ANOVA. Columns are expressed as means±SEM (***$p<0.001$). (**b**) Quantitation of *Gyg1* expression in control (*n*=7), control treated with FT011 (*n*=8), diabetic (*n*=7) and diabetic treated with FT011 (*n*=8) groups. Values are normalised to $C_t$ values of housekeeping gene, rat 18S mRNA. Significance between groups was determined using one-way ANOVA. Columns are expressed as means±SEM (*$p<0.05$)

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Fig. 1

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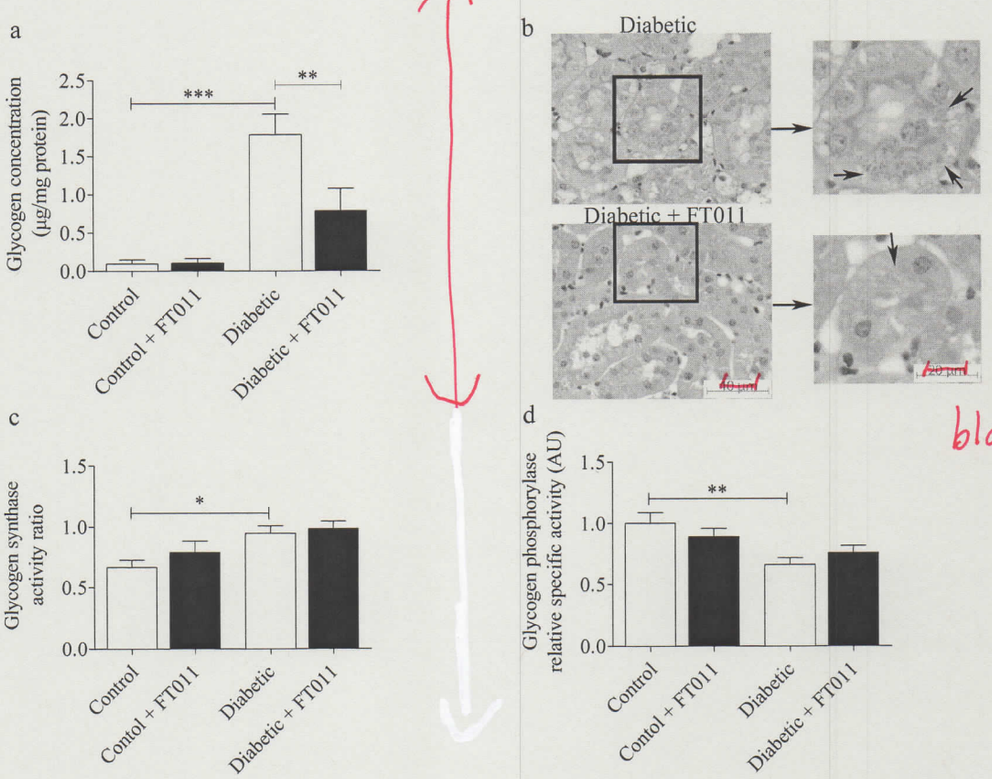


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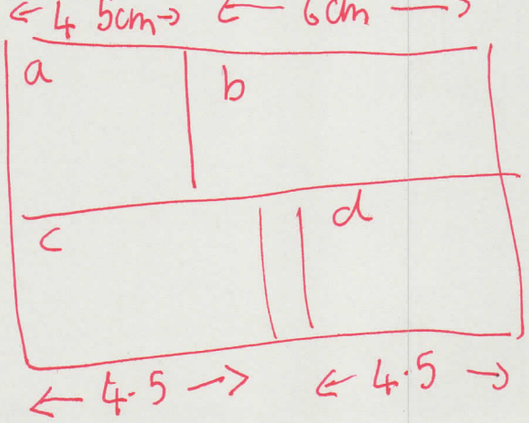

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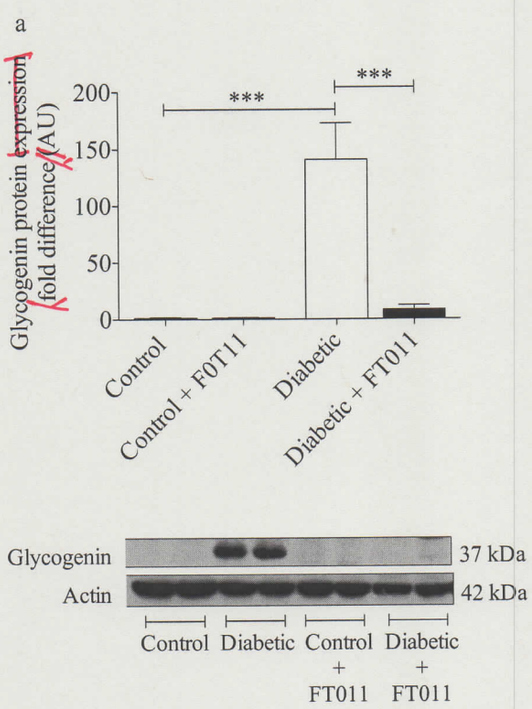
Fig. 2

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