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ABOUT COVER

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REVIEW

Diabetes-induced changes in cardiac voltage-gated ion channels

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Abstract

Diabetes mellitus affects the heart through various mechanisms such as microvascular defects, metabolic abnormalities, autonomic dysfunction and incompatible immune response. Furthermore, it can also cause functional and structural changes in the myocardium by a disease known as diabetic cardiomyopathy (DCM) in the absence of coronary artery disease. As DCM progresses it causes electrical remodeling of the heart, left ventricular dysfunction and heart failure. Electrophysiological changes in the diabetic heart contribute significantly to the incidence of arrhythmias and sudden cardiac death in diabetes mellitus patients. In recent studies, significant changes in repolarizing K^+ currents, Na^+ currents and L-type Ca^{2+} currents along with impaired Ca^{2+} homeostasis and defective contractile function have been identified in the diabetic heart. In addition, insulin levels and other trophic factors change significantly to maintain the ionic channel expression in diabetic patients. There are many diagnostic tools and management options for DCM, but it is difficult to detect its development and to effectively prevent its progress. In this review, diabetes-associated alterations in voltage-sensitive cardiac ion channels are comprehensively assessed to understand their potential role in the pathophysiology and pathogenesis of DCM.

Key Words: Diabetes; Action potential; Cardiac ion channels; L-type Ca^{2+} channels; Potassium channels; Sodium channels

Core Tip: Diabetes mellitus is a multisystemic disease that affects many organs. It causes diabetic cardiomyopathy (DCM) in the heart which is a distinctive pathology that occurs independent of vascular complications. In DCM, altered action potential morphology and contractile dysfunction are mostly associated with defective cardiac ion channels such as voltage-gated K^+, Na^+ and Ca^{2+} channels. Therefore, with therapeutic agents specific to cardiac ion channels, both arrhythmogenic events and

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other functional problems can be mitigated in the diabetic heart.

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INTRODUCTION

Diabetes mellitus (DM) is a complex and heterogeneous chronic metabolic disease caused by high blood sugar levels. Diabetic heart disease is a growing and serious public health risk which affects more than 350 million people worldwide[1]. Considering the fact that these figures refer to the year 2011, it is unfortunate to note that the numbers will increase much more in the coming years. DM is divided into four different etiological categories: Type 1, type 2, gestational DM and other specific types. Type 1 DM results from T cell-mediated autoimmune destruction of pancreatic cells which leads to insulin deficiency[2] and it mostly occurs in young people, usually up to the age of 30. Type 2 DM is characterized by both insulin resistance and the failure of pancreatic cells. Other specific types of DM are caused either by other pathological diseases of the pancreas due probably to single genetic mutations or drugs. Gestational diabetes, on the other hand, develops during pregnancy.

The mortality rate associated with DM due to cardiovascular disease is 65%. It is, therefore, considered a risk equivalent to coronary heart disease and generally affects the heart in three ways: Cardiac autonomic neuropathy, coronary artery disease (CAD) and diabetic cardiomyopathy (DCM)[3]. DCM is characterized by abnormal myocardial structure and reduced contractile performance even in the absence of other risk factors such as CAD, hypertension and significant valvular heart disease in individuals with DM. It was first described in the postmortem pathological findings of 4 diabetic patients who showed heart failure (HF) symptoms without coronary artery or valvular heart disease in 1972, and it was later confirmed in diabetic women with a 5-fold higher incidence of HF in the Framingham Heart Study in 1974[4,5]. DCM was described in 2013 as a clinical condition of ventricular dysfunction in patients with DM in the absence of coronary atherosclerosis and hypertension, in collaboration with the American College of Cardiology Foundation, the American Heart Association, the European Society of Cardiology and the European Association for Diabetes Research[6,7].

In the early stages of DM, significant changes occur in myocardial function and structure due to DCM, and these changes include left ventricular hypertrophy, increased fibrous tissue and cell signal abnormalities. These pathological changes cause cardiac contractile and diastolic dysfunction associated with ventricular fibrosis and hypertrophy which are the earliest pathophysiological complications in DCM[8,9]. Mechanisms underlying these changes include hyperglycemia, systemic and cardiac insulin resistance, increased free fatty acid levels, systemic and tissue inflammation, oxidative stress, renin-angiotensin-aldosterone system and activation of the sympathetic nervous system[10]. On the other hand, systolic dysfunction develops in the later stages of the disease and may be caused by diastolic dysfunction and decreased cardiac compliance resulting from the progression of DCM[8-10]. Furthermore, when systolic dysfunction occurs, the cardiac output gradually decreases with the severity of the disease and thus leads to HF. Consistent with this, The Framingham Heart Study showed that the frequency of HF was five times higher in diabetic women and two times higher in diabetic men than in age-matched control subjects[11]. HF leads to a low quality of life in individuals and makes it quite difficult to treat DM by simply changing the pharmacokinetics of anti-diabetic drugs. Therefore, diagnosing these patients faster and treating them earlier is extremely important. This review focuses on the role of voltage-sensitive ion channels in the electrophysiological disturbance of the diabetic heart and thus provides refined evidence that enables the understanding of the molecular mechanisms underlying the pathogenesis of DCM which may help to develop diagnostic methods and treatment strategies.

ELECTROPHYSIOLOGICAL CHANGES IN THE DIABETIC HEART

In diabetic patients, the incidence of cardiac arrhythmia is higher, as well as ventricular fibrillation and sudden death, and significant changes mostly associated with the repolarization of ventricles are observed in the electrocardiogram (ECG). Diabetic patients have higher heart rates, lower ECG potential amplitudes and more T-wave inversions than normal individuals. In addition, DM leads to sudden cardiac deaths that may be associated with an increase in the QT interval[12,13]. In type 1 DM, prolonged QTc interval and increased QTc dispersion have been observed[14,15]. In follow-up using Holter ECG monitoring, the occurrence of ventricular late potentials in patients with type 1 DM is observed more frequently than in healthy people and is more common in patients with type 2 DM[4,5]. This increased QTc interval is thought to be associated with an increased risk of mortality, like non-diabetic subjects with QTc prolongation[16,17]. Studies performed in twins have shown that QTc is longer in type 1 diabetic than non-diabetic subjects which implies that QTc prolongation is caused by diabetes rather than genetic factors[18]. The changes in the ECG are mostly associated with the prolonged cardiac action potential (AP) which is mostly ascribed to diabetes-induced alterations in repolarizing potassium currents (Figure 1)[19-21]. On the other hand, experimental studies have shown that these changes in repolarizing currents of cardiac myocytes can be different depending on the species of animal used, the type and duration of diabetes (Table 1)[22-25].

DIABETES-INDUCED ALTERATIONS IN CARDIAC ACTION POTENTIAL

DCM is often associated with impaired contraction and ECG abnormalities. The changes in the ECG that have been attributed to prolonged cardiac AP duration arise due to a decrease in repolarizing potassium currents caused by diabetes (Figure 1)[19-21].

The set off and regular spread of cardiac electrical stimulation depends on the formation of a normal cardiac AP throughout the myocardium. Depolarization and repolarization of AP are mediated by multiple inward and outward currents passing through specific membrane ion channels. The initial depolarization phase is generated by the inward Na^+ current (I_{Na}), mainly through voltage-sensitive sodium channels (Nav1.5), in the form of a rapid upstroke. In the subsequent early repolarization and plateau phases, the transient outward K^+ current and the inward L-type Ca^{2+} current (I_{CaL}) are prevalent, respectively. During this process, the Ca^{2+} ions entering through L-type Ca^{2+} channels (LTCC) induce a large amount of Ca^{2+} release from the sarcoplasmic reticulum (SR), thereby activating the excitation-contraction coupling. The repolarization, which ultimately returns the membrane to its resting potential, is mainly driven by the outward current through the voltage-gated K^+ channels (Kv)[26]. K^+ channel activity is the main determinant of AP duration as it limits depolarization time and the refractory period as well as the time period of Ca^{2+} -mediated contraction. There are numerous and diverse types of K^+ channels, each with specific kinetic and voltage dependent properties. They have specific roles in different repolarization stages of cardiac AP such that they determine the repolarization time and repolarization reserve as well as maintaining the resting membrane potential. The repolarization reserve refers to the partially overlapping function of these currents, namely rapid delayed rectifier K^+ currents (I_{Kr}), slow delayed rectifier K^+ currents (I_{Ks}) and inward rectifier K^+ currents (I_{K1})[27]. Repolarization kinetics of these K^+ currents is highly variable depending on the region of the heart and the species studied. This reflects the difference in the expression and density of different K^+ channel subtypes. Experimental studies have shown that different repolarizing currents decrease depending on the type of animal used to induce type 1 DM[28-30]. In the human heart ventricle, the main repolarizing currents are fast transient-outward K^+ current ($I_{to,f}$), slow transient-outward K^+ current ($I_{to,s}$), I_{Kr}, I_{Ks} and steady-state K^+ current (I_{ss}), while they are $I_{to,f}$, ultra-rapid delayed rectifier K^+ current (I_{Kur}) and I_{Ks} current in the atrium. All these features suggest that the investigation of the K^+ channels is important for understanding the mechanisms underlying cardiac dysfunction and arrhythmias caused by DCM and this can be a useful pharmacological target for the development of therapeutic agents.

Table 1 Overview of diabetes mellitus-induced alterations in cardiac K⁺ currents

Diabetes Mellitus Type	Duration	Transient outward K⁺ currents		Delayed rectifier K⁺ currents		Inward rectifier K⁺ currents	Ref.
		I_{to}	I_{ss}	I_{Kr}	I_{Ks}	I_{K1}	
Type 1	≤ 4 wk	↓	↓	?	↓	↔	[20,45,49,50,54,72,73,143-145]
	4-8 wk	↓	↓	↓↔	↓	↔	[19,21,29,40-43]
	> 8 wk	↓	↔	↓↔	↓	↓↔	[51,52,69]
Type 2		↓	↓	?	↓	↔	[30,39,49]

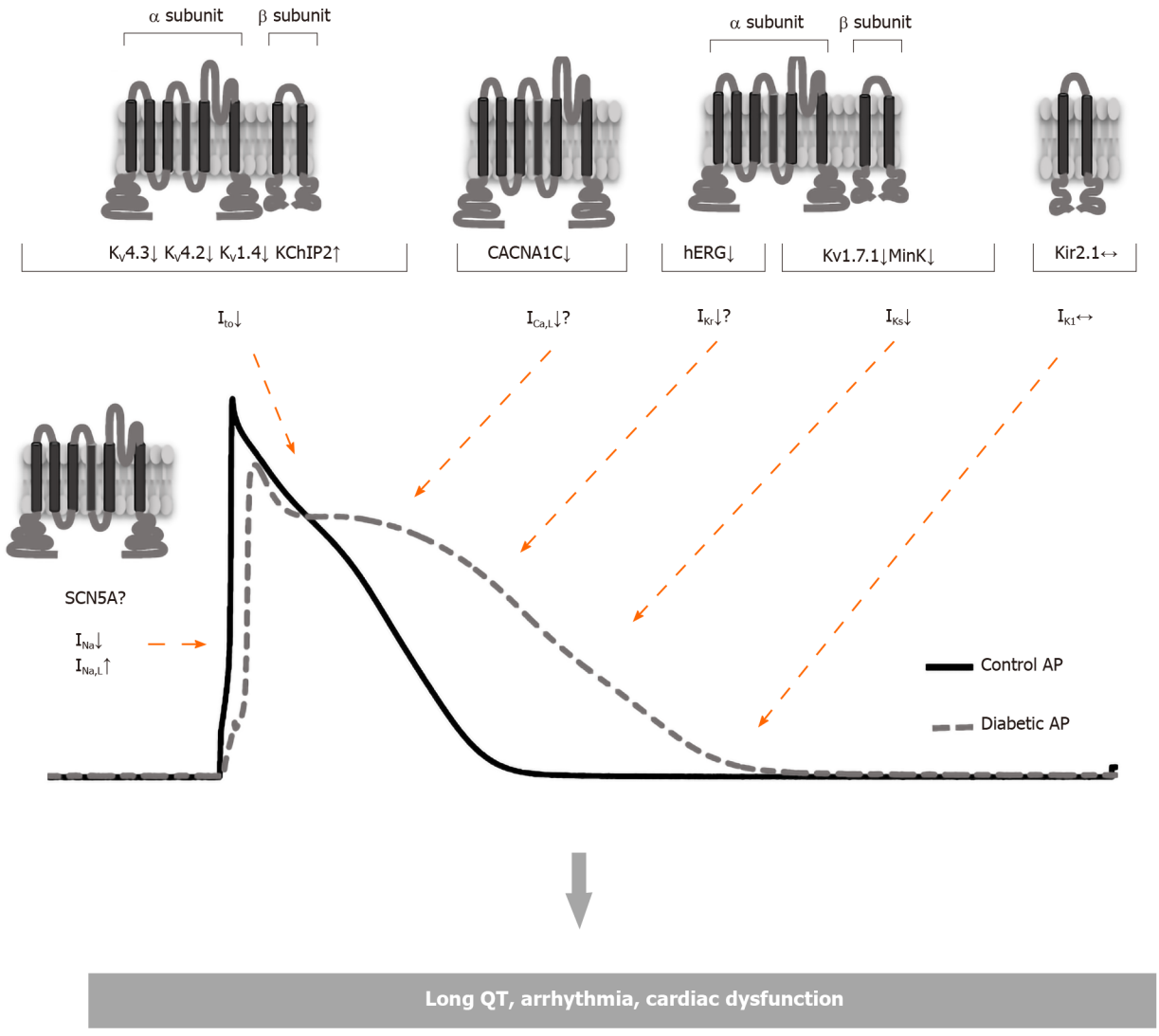

Figure 1 Pathological alterations in voltage-gated cardiac ion channels that contribute to the action potential of the ventricular myocytes due to diabetes mellitus.

POTASSIUM CURRENTS IN DIABETIC CARDIOMYOCYTES

K⁺ channels represent the most functional and diverse types of cardiac ion channels[31-34]. They tightly regulate the cardiac repolarization, thereby providing a stable and consistent AP signal. Different K⁺ channel types may have overlapping functions that provide some degree of functional redundancy and thereby contribute to the repolarization reserve[27,35]. All of the α-subunits of different K⁺ channel types have a pore-forming region that has a selective permeability to the K⁺ ion. This can be associated with a particular structural motif and allows K⁺ movement from the plasma

membrane under the effect of an electrochemical gradient. In addition, there are ligand binding sites that can change the channel conformation and gating mechanisms in response to membrane depolarization.

Transient outward potassium current (I_{to})

In diabetic patients, the incidence of cardiac arrhythmia, ventricular fibrillation and sudden cardiac death is higher, and most of them have significant changes in ECG recordings due most probably to abnormal AP repolarization. Accordingly, in myocytes isolated from diabetic hearts, I_{to} is the mainly affected repolarizing current.

I_{to} is basically responsible for the early repolarization phase of the AP. Two subtypes of I_{to} are defined; one is blocked by 4-aminopyridine (4-AP) and not dependent on Ca^{2+} (I_{to1}), while the other is not blocked by 4-AP but modulated by Ca^{2+} (I_{to2})[34]. Cardiac regions with shorter AP duration, such as the epicardium, right ventricle, and septum, have higher transient outward K^+ channel expression. Due to their discrete characteristics, I_{to1} currents are subdivided into $I_{to,f}$ and $I_{to,s}$ components. $I_{to,f}$ and $I_{to,s}$ currents are both present in the ventricles, however, $I_{to,f}$ is the dominant current expressed in the atrium[36]. Although the $I_{to,s}$ currents have not so long inactivation time, their classification as "slow" is only relative to $I_{to,f}$. Nevertheless, both $I_{to,f}$ and $I_{to,s}$ channels are activated and inactivated rapidly compared to other K^+ channels.

Many studies have been conducted in rats to elucidate the cellular mechanisms of diabetes-induced repolarization abnormalities[19,37-43]. In these studies, it has been shown that the I_{to} amplitude reduction which is responsible for the prolongation of AP repolarization in diabetic rats is associated with downregulation of the expression of Kv4.3 and Kv4.2 channel proteins[39-42,44]. However, an increase in the protein expression of Kv1.4, which is responsible for the regulation of $I_{to,s}$ currents has been reported[29,44,45]. In the case of a depressed I_{to} channel, protein expression may change in the opposite direction and thus the upregulation of Kv1.4 and KChIP2 may be associated with a decrease in Kv4.3 expression (Figure 1). Consistently, in the Kv4.3 gating model supported by Patel *et al*[46], KChIP2 isoforms have suggested an acceleration in the recovery from inactivation and promotion of the open-state inactivation with slower closed-state inactivation. As a result, the upregulated KChIP2 causes slower inactivation in depolarized potentials and enhances the re-opening of the I_{to} channels during membrane repolarization. This eventually increases the repolarizing force in the plateau phase which may contribute to late repolarization[46]. However, in rat myocardium, ventricular repolarization includes different mechanisms to dogs, humans, and other mammals as it lacks a pronounced plateau phase and has a short AP duration. Therefore, these results obtained in rats have relatively limited value in understanding the repolarization abnormalities observed in diabetic myocardium. Expression of ion channel proteins has also been widely investigated to elicit the molecular mechanisms underlying electrophysiological changes by generating an experimental type 1 DM model in animal species with a pattern of cardiac repolarization and ionic currents that are more similar to those in the human heart. In dog cardiomyocytes, both I_{to} reduction and downregulation of Kv4.3 protein (the dominant subunit forming the pore in dog and human ventricular myocytes) are consistent with the data obtained in rats[29]. As a result, I_{to} currents and expression of those proteins involved in channel regulation are consistent in rats and dogs. Contrary to these findings obtained in other studies, no significant change in I_{to} current was observed in rabbits. The reason for this discrepancy can be explained by the fact that the rabbit I_{to} current has a different molecular basis. In rabbits, I_{to} is mediated mostly by Kv1.4 channels, but not Kv4.3 channels as in rats, dogs and humans[47]. Nevertheless, different results have also been obtained in animal models in which the experimental type 2 DM model was induced by different methods. In myocytes isolated from db/db mice, a leptin receptor mutant showing type 2 DM symptoms, K^+ currents have been shown to decrease and AP duration is prolonged[48]. On the other hand, there was no change in K^+ currents and AP duration measured in the type 2 DM rat model induced by feeding on a diet enriched with fructose for 6-10 wk[49]. Thus, it can be concluded that ionic currents and the expression of protein channel domains that precipitate the prolongation of AP duration observed in diabetes vary according to the animal species studied, the diabetes model created and duration of the diabetic condition.

Although studies have demonstrated that DM causes a significant decrease in $I_{to,f}$ and $I_{to,s}$ current amplitudes, neither the voltage dependence of the inactivation nor the time dependency of the reactivation has changed[20,30,50,51]. However, contrary to the results generally obtained, in long-term diabetes (24-30 wk) significant changes in inactivation and reactivation kinetics of I_{to} have also been reported in rat cardiac myocytes[52]. Therefore, it seems likely that different results in channel kinetics will be

seen depending on the duration of the diabetic state.

Two hypotheses have been proposed regarding the effects of type 1 DM on potassium currents in the heart muscle. The first hypothesis involves insulin deficiency as it affects the gene expression of a large number of proteins, including potassium channel proteins[53]. Incubation of diabetic cardiac myocytes with insulin for 6 h restored the $I_{to,f}$ to control values and this effect was prevented by protein synthase inhibitors[54]. In a study using a cardiomyocyte-restricted insulin receptor knockout (CIRKO, cardiac-specific insulin receptor knockout) mouse model, impaired insulin signaling resulted in a decrease in mRNA and protein expression of K^+ channels prominent in ventricular repolarization. Specifically, in isolated left ventricular CIRKO myocytes, Kv4.2 and KChiP2 expression decreased, consistent with a decrease in $I_{to,f}$ amplitude. The alleviated $I_{to,f}$ in turn resulted in a prolonged ventricular AP and prolonged QT interval in surface ECG[30]. These results support the idea that the lack of insulin signal in the heart is sufficient to cause repolarization abnormalities described in other diabetic animal models. The second hypothesis assumes that the cause of decreased cardiac $I_{to,f}$ in DM is defective glucose metabolism. This hypothesis was supported by the reversal of potassium currents in diabetic cardiomyocytes to normal levels after 6 h of incubation with metabolic enhancers such as L-carnitine, glutathione, or pyruvate[38,51,55].

Activation of the renin-angiotensin system has also been demonstrated in insulin-dependent diabetic rats, and I_{to} has been reduced with increased angiotensin II (Ang II) levels[56]. Inhibition of the production or action of Ang II has been found to reverse the decreased I_{to} in both type 1 and type 2 DM. In ventricular myocytes of streptozotocin (STZ)-induced type 1 DM rats, decreased I_{to} and I_{ss} currents have been shown to be significantly increased after incubation with the Ang II receptor blockers saralasin or valsartan[48]. Incubation of ventricular myocytes isolated from the mutant db/db mice with valsartan (> 6 h) has been shown to reverse the reduced I_{to} and I_{ss} currents[48]. These results confirm that cardiac myocytes contain a local renin-angiotensin system that is activated in diabetes. These effects of Ang II can be explained by the fact that it has a large number of various cellular effects mediated by protein kinase A, protein kinase C and tyrosine kinases that may lead to inhibition of some ionic channels. It is suggested that the changes caused by the chronic release of Ang II on ionic currents and AP can be eliminated by blocking the formation or effect of Ang II. Consistently this implies that Ang II receptor blockage or angiotensin-converting enzyme inhibition can protect against cardiac arrhythmias that may occur in DCM. However, more studies are needed to explain these elaborate findings unequivocally.

Delayed rectifier potassium current

Delayed rectifiers, along with other ion channels, mainly determine the waveform as well as the AP duration and thus play critical roles in heart physiology and pathophysiology (Figure 1). Disruption of the normal functions of the delayed rectifier channels makes the heart more sensitive to abnormal electrical activity and prone to arrhythmia. This class of K^+ channels includes I_{Ks}, I_{Kr} and atrial specific I_{Kur} channels.

Like I_{to}, I_{Kur} is also effective in the early repolarization phase of the AP. This current quickly activates in less than 10 milliseconds at plateau voltage ranges and slowly disappears through the AP repolarization period[57-59]. I_{Kur} current is the dominant delayed rectifier current for atria, and therefore shorter AP duration is seen in the atrial myocytes compared to ventricles[36,57,59,60]. In regions where I_{Kur} currents are observed, ion channels are not evenly distributed on the myocyte membrane and are mostly localized in the intercalated discs[32]. This specific localization of I_{Kur} in the atrium makes it an interesting target for atrial selective treatment, so that inhibition of I_{Kur} prolongs the AP duration of atrial myocytes, this prolongation is not observed in the ventricles[32].

On the other hand, I_{Kr} currents are critical for phase 3 repolarization of AP. It shows a relatively rapid activation with depolarization, however, the rate of inactivation is about 10 times higher than the rate of activation. This ensures that these channels are relatively non-conductive during the 1st and 2nd stages of cardiac AP[61-63]. So even though this current is called delayed rectifier, it also shows an inward rectification property at positive potentials[24,62,63]. However, as the membrane potential reaches 0 mV with the end of phases 1 and 2, I_{Kr} is activated once again, but deactivation is much slower during this phase. This causes a large outward flow of K^+ ions during phase 3 repolarization[27,64]. I_{Kr} is found in both the atrium and ventricles of humans but is expressed at higher levels in the left atrium and ventricular endocardium[36].

Cardiac repolarization is also affected by the I_{Ks} current which is slowly activated at potentials around -20 mV. Unlike I_{Kr}, I_{Ks} is almost completely inactive in phase 2 repolarization and significantly affects phase 3 repolarization of cardiac AP[27,65,66]. This

feature of I_{Ks} is especially important in relatively longer atrial and ventricular APs. It is also important for the reactive shortening of AP duration during a physiological increase in heart rate. Namely, a significant increase in the heart rate leads to a decrease in the inactivation time of I_{Ks} current which results in higher I_{Ks} and a steeper decrease in the repolarization phase of AP[67,68]. Blocking I_{Ks} current causes a prolongation in AP duration at particularly increased heart rates[68]. Inhibition of I_{Ks} current can also increase the reactivation of voltage-sensitive Ca^{2+} channels, thereby increasing the risk of arrhythmic events[27]. All cardiac cell types have I_{Ks}, but their expression is significantly reduced in the middle of the myocardial wall; this explains the longer AP duration in this region[36].

Studies performed using various animal models have reported a decreased I_{Ks} current in diabetic dog and rabbit hearts[28,29]. However, there are controversial findings regarding the effects of diabetes on I_{Kr} current because it has been reported to decrease in diabetic rabbits[21] or not to change in diabetic rabbits, dogs, and mice[28,29,51]. A significant reduction in I_{Kr} current and hERG expression along with a prolonged QTc interval have been demonstrated in alloxan-induced diabetic rabbits[21,69,70]. In these reports, the DM-induced changes are apparent after an 11-wk period, whereas QTc prolongation is less pronounced in rabbits at the end of the 3-wk diabetes period, and no changes were observed in the I_{Kr} current[28]. These results show that DM-induced changes in different ionic currents of cardiac myocytes may develop at different time points of the disease. However, neither activation nor deactivation kinetics of I_{Kr} current have been changed in diabetic cardiac myocytes[28]. In addition, it has been shown that the suppression of I_{Ks} current along with a moderate extension of the QTc interval occurs at an early stage, such as the third week of DM. However, after the eighth week of alloxan-induced diabetes, no change in I_{Kr} current has been observed, whereas I_{Ks} current was suppressed in dog myocytes[29]. In addition to the fact that the regional differences in AP duration and left ventricular ionic currents are important in the reduction of the repolarization reserve in diabetes, the severity of diabetes is also prominent in the extent of these changes[37,71-73]. Therefore, more experimental and clinical data are needed to clarify this issue.

It is surprising that the decrease in the density of the I_{Ks} and the expression of the regulatory β-subunit channel protein, MinK, is associated with increased expression of the pore-forming α-subunit Kv1.7.1[74]. On the other hand, there may be more direct interactions between Kv1.7.1 and hERG, which are α subunits of I_{Ks} and I_{Kr} currents, respectively, and Kv1.7.1 can modulate not only the distribution but also the biophysical properties of hERG. Indeed, Kv1.7.1 overexpression has been shown to elicit a dramatic increase in hERG current density[74]. Therefore, it can be considered that the downregulation of MinK is the primary result of diabetes, and the concomitant upregulation of Kv1.7.1 may be a secondary compensatory process that can partially oppose the downregulation of MinK arising due to diabetes. Besides, it has been demonstrated that hERG is negatively modulated by hyperglycemia, tumor necrosis factor, ceramide and reactive oxygen species which are cellular metabolites accumulated in diabetic tissues[75,76]. It has also been reported that insulin metabolism affects the hERG expression as well as I_{Kr}/hERG function and it is quite possible for insulin to modulate different ion channels through separate mechanisms[21]. These results in total suggest that I_{Kr}/hERG is a potential target for the treatment of cardiac arrhythmias in diabetic patients.

Deschênes *et al*[77] reported that when Kv4.3 was co-expressed with MinK, the current density was five times higher than that of Kv4.3 expressed alone and that the inactivation and reactivation kinetics of Kv4.3 slowed down through MinK. Therefore, modifying MinK by diabetes can at least partially explain the diminution of I_{to} density in the dog myocytes. It is also worth noting that insulin administration can completely prevent the diabetes-induced reduction of I_{Ks} current (and associated changes in the expression of channel proteins), but only a limited protective effect on I_{to}. The reason for this discrepancy remains uncertain, but it should be noted that patients with type 1 DM may have an increased proarrhythmic risk even when they are treated regularly with insulin.

Inward rectifier K^+ current (I_{K1})

Inward rectifier K^+ current is active in a narrow membrane potential range. The rectifying property results in a marked decrease in I_{K1} conductivity in positive depolarized membrane potentials and an increase in I_{K1} current in negative membrane potentials. As a result, this has the effect of stabilizing the resting membrane potential close to the K^+ equilibrium potential[64]. The channel mediating the I_{K1} current does not show voltage-dependent activation and does not have a voltage sensor. However, the I_{K1} current modulation associated with the movement of Mg^{2+} and polyamines

provides indirect sensitivity of the channel to voltage[78-81]. Since the channel is inhibited by Mg^{2+} and polyamines at membrane potentials more positive than 20 mV, I_{K1} channel has no conductivity between phase 0 and phase 2 of the AP. When the potential returns to more negative values (typically around -40 mV), the blockade mediated by Mg^{2+} and polyamines on I_{K1} channel conductivity is relieved, and this contributes to the phase 3 repolarization of cardiac AP[80]. I_{K1} current is present in both atria and ventricles and therefore plays an important role in determining resting membrane potentials. Channels that transmit I_{K1} current are more expressed in the ventricles, making the ventricles less sensitive to the pacemaker effect[36].

As mentioned, I_{K1} current has been one of the most widely studied K^+ currents in DCM due to its importance in stabilizing the membrane potential and its contribution to AP duration (Figure 1). In these studies where different animal species (mouse, rat, rabbit, and dog) were used and different diabetes periods were applied (3 wk, 4 wk, 8 wk, 10 wk) changes in I_{K1} were extensively examined. However, neither the I_{K1} current amplitude[19,20,38,49,50] nor the expression of Kir2.1, the main component of the I_{K1} channel, changed in the diabetic heart[69,82]. Therefore, these findings confirming the absence of a shift in resting membrane potential of diabetic cardiomyocytes imply that I_{K1} current is less likely to contribute to DM-induced AP prolongation as well.

VOLTAGE-GATED Ca^{2+} CHANNELS IN DIABETIC CARDIOMYOCYTES

Intracellular Ca^{2+} dysregulation is a well-defined complication in DCM and it has been demonstrated in both type 1 and type 2 DM (Figure 1 and Table 2)[83-86]. Although the cellular mechanisms underlying this impaired Ca^{2+} handling have not been fully explained, a significant decrease in SR Ca^{2+} content associated with reduced SERCA2 expression/activity, decreased phospholamban phosphorylation and increased ryanodine receptor (RyR) Ca^{2+}-leak have been widely reported in type 1 and type 2 diabetic heart myocytes, and as a result the diastolic Ca^{2+} concentration increased and the amplitude and decay rate of the Ca^{2+} transients significantly decreased[40,84,87-91]. Also, there was a decrease in Na^+-Ca^{2+} exchanger (NCX) expression in type 1 diabetic myocardium[92]. In addition to these findings, the role of LTCC in the impaired Ca^{+2} handling in DCM has not been fully clarified.

Basically, bulk Ca^{2+} release from SR into the cytosol is mediated by activation of RyR which is triggered by inward Ca^{2+} current through LTCC. This mechanism is described as Ca^{2+}-induced Ca^{2+} release and it is a critical event for excitation-contraction coupling in cardiac myocytes[93,94]. LTCC which acts as a trigger for excitation-contraction coupling is an ion channel family with four different members. Of these, $Ca_V1.2$ is the main Ca^{2+} channel expressed in cardiomyocytes which has four homologous domains and expressed by CACNA1C or α1C[95]. Each of these domains is characterized by six transmembrane α-helix structures. This channel has a current-voltage relationship that activates at the potential value of -40 mV, gives the maximum amplitude at potentials between 0-10 mV, and reverses at +60 to +70 mV[96,97].

Many studies have examined LTCC in DCM. However, contradictory findings have been reported about the activity of LTCC in these studies (Table 2). Some of them have demonstrated an unchanged current-voltage relationship of LTCC in DCM[29,50,98-102]. In these studies, diabetes duration is generally less than 10 wk (4-8 wk)[50,99,101,102]. The lack of significant change in L-type Ca^{2+} current (I_{CaL}) despite the reduced Ca^{2+} transient amplitude and slowed rate of removal indicates that the coupling between the LTCC and RyR is impaired in DCM[99,101]. Accordingly, Lacombe *et al*[101] showed a reduced gain in the Ca^{2+}-induced Ca^{2+} release mechanism of diabetic myocytes due most probably to altered LTCC-RyR coupling. Dysregulation of intracellular Ca^{2+} handling in the diabetic heart might also be mediated by alterations in NCX, SERCA2, PLB and RyR expression or phosphorylation[98].

However, some other studies have reported significant changes in the amplitude of I_{CaL} current[52,89,103-108]. This discrepancy regarding the activation of I_{CaL} current might have arisen due to two main factors: the diabetes model used in the study and the duration of the diabetic state. For type 2 DM, transgenic animal models have been mostly used and, in these studies generally, I_{CaL} current has been found to decrease in ventricular myocytes[89,105-107]. However, in the type 1 DM models induced by chemical agents, the experimental period varies between 4 and 12 wk and this may at least partially explain the difference observed in I_{CaL} current densities[109]. Nevertheless, some studies using a similar duration of diabetes have also demonstrated different I_{CaL} current amplitudes in ventricular myocytes. In general, it is most likely that there is a decrease in the amplitude of I_{CaL} current of the ventricular myocytes in the STZ-

Table 2 Changes in L-type Ca^{2+} and Na^{+} currents in diabetic heart myocytes

Diabetes Mellitus Type	Duration	Ca^{2+} currents	Ref.	Na^{+} currents		Ref.
		I_{CaL}		I_{Na}	$I_{Na,L}$	
Type 1	≤ 4 wk	↔	[50]	↔	?	[130]
	4-8 wk	↓↔	[29,99,101-103,108]	↓	?	[130,132]
	> 8 wk	↓↔	[52,98]	↔↓	?	[69]
Type 1 transgenic		↓	[104]	?	↑	[135]
Type 2		↓	[89,105-108]	?	↑	[135]

induced DM model after ten or more weeks of diabetes duration[52,103]. Similar to that of the type 2 DM model, the density of I_{CaL} current decreased in cardiac myocytes of transgenic animals with type 1 DM[104].

In the studies where I_{CaL} current amplitude was found to be decreased in diabetic heart myocytes compared to that of control, it was approximately 15%-30% lower in the negative membrane potentials range, and this difference maintains up to +20 mV[89,104,106,108]. This reduction in I_{CaL} current may be due to the activation/inactivation kinetics of the channels, the expression of the channel proteins, or the change in the single-channel conductance. Pereira *et al*[89] measured the single-channel current in diabetic myocytes to test whether it is the likely explanation for the reduced I_{CaL} current and they did not find a significant difference compared to control myocytes. Thus, it was concluded that the activity of the single-channel current is not responsible for the decreased macroscopic I_{CaL} currents in the diabetic heart. Instead, it can be ascribed to the altered channel kinetics or reduced expression of channel proteins due to diabetes.

Considering that I_{CaL} current reaches its maximum value between 0-10 mV membrane potentials, the DCM-related decrease in current amplitude may have occurred due to the altered channel kinetics. As a matter of fact, the potential value required for half of the channels to be open (V_h) has shifted to more positive values in diabetic myocytes. This may explain why fewer LTCC channels are opened at lower potentials and why the measured current is lower. There was no significant difference observed in the half-inactivation potential ($V_{1/2}$) where half of the channels are closed and recovered from inactivation in diabetic myocytes[89,104,106,110].

Another explanation for the reduced I_{CaL} current in DM is the change in channel protein expression. As mentioned earlier, $Ca_V1.2$ is the main Ca^{2+} channel type expressed in the heart, and studies have shown that expression of the a1C subunit of $Ca_V1.2$ decreases in type 1 and type 2 diabetic hearts[89,104]. Therefore, it is likely that the decrease in I_{CaL} is due to both the change in LTCC activation and the change in channel expression[111].

The physiological mechanisms underlying this decrease in I_{CaL} current in diabetic cardiac myocytes could be the phosphatidylinositol 3-kinase (PI3K)/Akt pathway[112-114]. Consistently, activation of the PI3K/Akt pathway, which is a potent modulator of I_{CaL} currents, is downregulated due to diabetes and this decrease triggered the reduction of I_{CaL} in diabetic myocytes[115]. It is known that insulin or insulin growth factor (IGF-1) mediated activation of PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3), and thus PIP3 synthesis stimulates Akt (protein kinase B)[112]. Recent studies have shown that the PI3K/PIP3/Akt pathway performs its mediator effect by providing phosphorylation of the $Ca_V\beta2$ subunit of LTCC[116,117]. However, PI3Ks are a large molecular family in which PI3Kα is in the class I group and it is one of the prominent mediators in the activation of LTCC in cardiac myocytes. Consistently, I_{CaL} current density has been shown to decrease in PI3Kα-null cells due to the downregulation of LTCC[118]. It has also been demonstrated that the activation of Akt with PIP3 infusion can reverse the decrease in I_{CaL} current and that $Ca_V\beta2$ phosphorylation protects the $Ca_V1.2$ pore-forming subunit from proteolytic degradation[116].

Another important point is that PI3Kα and PI3Kβ both take part in the T-tubule network. Since LTCC is primarily located in the T-tubule, I_{CaL} current density decreases due to T-tubule disorganization in PI3Kα and PI3Kβ deficiency[119]. As mentioned earlier, the subunits of PI3Ks is an important point that needs to be considered during intervention since I_{CaL} density may decrease in treatments that are not specific to

PI3Kα[112,120-122]. PI3Ks can also reduce the response to β-AR stimulation through associated kinases. Particularly, PI3-δ, which is in the class I B PI3K group, shows its effect through the G proteins. Accordingly, in a study of transgenic PI3K$^{-/-}$ animals, isoproterenol application to ventricular myocytes increased I_{CaL} and intracellular Ca^{2+} transients more than control myocytes, and this was claimed to lead to HF[121,123].

In conclusion, conflicting findings in I_{CaL} current densities and $Ca_V1.2$ expression in DCM may be related to the type of diabetes model and its duration. In studies using transgenic animals for both type 1 and type 2 DM models, have been shown to cause a marked reduction in I_{CaL} current density and $Ca_V1.2$ expression. The difference observed in the diabetes model induced with STZ injection may be related to the duration of diabetes and the amount of STZ administered. As previously emphasized, I_{CaL} current has been shown to decrease significantly in diabetes periods of more than 10 wk, while findings varied in studies with shorter diabetes duration. This may be due to multiple physiological mechanisms acting on I_{CaL} current, whose activity is changed by the duration of the diabetic condition. Currently, the most likely mechanism suggested to be responsible for the reduced I_{CaL} current in DCM is the altered PI3K/PIP3/Akt pathway due to insulin or IGF-1 decrease. However, current findings need to be supported using comparable models for both types of diabetes, and it is also important to clearly determine whether cellular signaling mechanisms underlying the pathogenesis of these types of disease are similar and how changes in these molecular pathways affect I_{CaL} current depending on the duration of the disease.

VOLTAGE-GATED Na^+ CHANNELS IN DIABETIC CARDIOMYOCYTES

Na^+ ion plays a vital role in many cellular mechanisms such as the upstroke phase of AP (voltage-dependent Na channels, Na_V), Ca^{2+} cycling (NCX), metabolic processes (Na^+-glucose cotransporter) and regulation of the intracellular pH (Na^+-HCO_3^- cotransporter, Na^+/H^+ exchanger) in cardiomyocytes[93,124,125]. However, the main scope of this review is the Na_V channels that ensure the fast depolarization phase of cardiac AP. So far, nine different Na_V types have been identified ($Na_V1.1$ to $Na_V1.9$, respectively)[126], and the major Na_V type expressed in cardiomyocytes is $Na_V1.5$ encoded by the SCN5A gene[127]. This channel has four homologous domains (D1-D4) and each domain consists of six transmembrane segments (S1-S6)[128,129].

Although the intracellular Na^+ concentration has been shown to increase dramatically in diabetic cardiomyocytes, few studies have examined the changes in the structure and activation of Na_V channels[125]. Earlier studies have suggested that there is no significant change in Na_V channels associated with DCM, while recent studies have shown altered I_{Na} current in diabetic cardiac myocytes (Table 2)[69,130-132]. These conflicting results regarding the amplitude of I_{Na} current may be related to the duration of diabetes, as in Ca^{2+} channels. As a matter of fact, Bilginoglu *et al*[130] reported that there was no change in I_{Na} current of ventricular myocytes at the end of the 4-wk diabetes period, whereas there was a significant decrease at 7-8 wk. In addition, a leftward shift has been observed in both activation and inactivation curves of Na_V channels in diabetic myocytes[130]. The observation of similar findings in metabolic syndrome, in which insulin resistance is increased, suggests that these effects may be mediated directly or indirectly by insulin signaling[133,134]. Stabler *et al*[131] have also observed a significant decrease in the amplitude of I_{Na} current in diabetic rabbit ventricles, while the channel kinetics did not change.

There is also a significant change in late Na^+ currents ($I_{Na,L}$), which have recently been reported to be responsible for many cardiologic pathologies including DCM[135-137]. Although the amplitudes of these currents are up to only 1% of conventional voltage-dependent fast Na^+ currents, it is thought that the long-term activation of Na^+ channels can trigger pathological changes in AP[136,138].

$I_{Na,L}$ current has been shown to increase significantly in DCM and is therefore suggested to increase the likelihood of arrhythmia by causing prolongation of AP[135]. Although many different treatments and interventions (ranolazine, mexiletine, PIP3, *etc*) to inhibit $I_{Na,L}$ current have been shown to reverse the prolonged AP duration, it is difficult to attribute the prolongation of AP solely to $I_{Na,L}$ current due to the prominent role of K^+ and Ca^{2+} currents in AP morphology[113,132,135,139]. Most importantly the role of K^+ currents in AP prolongation has been extensively investigated in diabetic hearts for a long time even though the number of studies showing the effect of $I_{Na,L}$ current on AP duration is relatively limited. Therefore, this finding should be carefully examined and confirmed by new studies in both type 1 and type 2 DM models.

CONCLUSION

DM is one of the most common chronic diseases worldwide and is mostly associated with serious cardiovascular complications that significantly increase the risk of mortality in diabetic patients. The abnormalities observed in the ECG and cardiac function of diabetic patients are mostly related to alterations in the voltage-gated ion channels that are critical determinants of the duration and morphology of cardiac AP. At the cellular level, prolongation of AP and defective contractile function typically arise due to a combination of reduced K^+ currents, irregularities in Na^+ currents and changes in Ca^{2+} currents along with impaired intracellular Ca^{2+} handling in diabetic cardiomyocytes. DM can affect not only the amplitude but also kinetics of the cardiac ion channels by modifying the biophysical behavior and/or the expression levels of the channel-forming proteins. Disruption of protein expression or alteration of biophysical properties of ion channels or both can contribute to the reduced currents caused by DM in diabetic cardiomyocytes. However, observing that there is often no change in the inactivation or reactivation kinetics of the cardiac ion channels in diabetic cardiomyocytes suggests that an abnormality in protein expression is more likely.

As a result, under pathological conditions such as DM, depressed K^+ currents may cause abnormal prolongation in AP duration due to insufficient repolarization and therefore lead to the development of early and late afterdepolarizations[140-142]. Therefore, it is likely that decreased K^+ currents in diabetic myocardium will reduce the repolarization reserve and increase the risk of arrhythmias. Nevertheless, as stated earlier, cardiac Na^+ and Ca^{2+} channels also have important effects that cannot be neglected in diabetic cardiac pathologies and should be taken into account in order to understand the pathogenesis of DCM.

Future perspectives

The effect of DM on the electrical conduction of the myocardium and the development of cardiac arrhythmias is becoming more evident. Due to its complex and multifactorial nature, the relationship between diabetes and cardiac arrhythmias is not yet fully understood. Hence, understanding the precise ionic mechanisms of APD/QT prolongation in DM is of great importance to develop more distinctive approaches for the prevention and treatment of electrical disturbance in diabetic patients.

Remodeling of the expression of K^+, Na^+ and Ca^{2+} channels in various physiological and pathological conditions is a complex phenomenon that can alter both the morphology of cardiac AP and contractile function of the heart. In diabetic patients, voltage-gated ion channels play a vital role in cardiac AP repolarization, and expectedly they are potential targets for the development of specific treatments to prevent cardiac arrhythmia and DCM-associated ventricular dysfunction. Using drugs particularly effective on ion channels and optimizing the effectiveness of their therapeutic action on the arrhythmogenic trend will minimize the potential cardiac and extracardiac toxicity problems. However, due to their complex mechanisms, more experimental and clinical research is needed to fully elucidate the relationship between diabetes and arrhythmias and to develop new therapeutic strategies.

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ORIGINAL ARTICLE

Basic Study

Metformin regulates inflammation and fibrosis in diabetic kidney disease through TNC/TLR4/NF-κB/miR-155-5p inflammatory loop

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Abstract

BACKGROUND

Type 2 diabetes mellitus (T2DM) is significantly increasing worldwide, and the incidence of its complications is also on the rise. One of the main complications of T2DM is diabetic kidney disease (DKD). The glomerular filtration rate (GFR) and urinary albumin creatinine ratio (UACR) increase in the early stage. As the disease progresses, UACR continue to rise and GFR begins to decline until end-stage renal disease appears. At the same time, DKD will also increase the incidence and mortality of cardiovascular and cerebrovascular diseases. At present, the pathogenesis of DKD is not very clear. Therefore, exploration of the pathogenesis of DKD to find a treatment approach, so as to delay the development of DKD, is essential to improve the prognosis of DKD.

AIM

To detect the expression of tenascin-C (TNC) in the serum of T2DM patients, observe the content of TNC in the glomerulus of DKD rats, and detect the expression of TNC on inflammatory and fibrotic factors in rat mesangial cells (RMCs) cultured under high glucose condition, in order to explore the specific molecular mechanism of TNC in DKD and bring a new direction for the treatment

of DKD.

METHODS

The expression level of TNC in the serum of diabetic patients was detected by enzyme-linked immunosorbent assay (ELISA), the protein expression level of TNC in the glomerular area of DKD rats was detected by immunohistochemistry, and the expression level of TNC in the rat serum was detected by ELISA. Rat glomerular mesangial cells were cultured. Following high glucose stimulation, the expression levels of related proteins and mRNA were detected by Western blot and polymerase chain reaction, respectively.

RESULTS

ELISA results revealed an increase in the serum TNC level in patients with T2DM. Increasing UACR and hypertension significantly increased the expression of TNC ($P < 0.05$). TNC expression was positively correlated with glycosylated haemoglobin (HbA1c) level, body mass index, systolic blood pressure, and UACR ($P < 0.05$). Immunohistochemical staining showed that TNC expression in the glomeruli of rats with streptozotocin-induced diabetes was significantly increased compared with normal controls ($P < 0.05$). Compared with normal rats, serum level of TNC in diabetic rats was significantly increased ($P < 0.05$), which was positively correlated with urea nitrogen and urinary creatinine ($P < 0.05$). The levels of TNC, Toll-like receptor-4 (TLR4), phosphorylated nuclear factor-κB p65 protein (Ser536) (p-NF-κB p65), and miR-155-5p were increased in RMCs treated with high glucose ($P < 0.05$). The level of TNC protein peaked 24 h after high glucose stimulation ($P < 0.05$). After TNC knockdown, the levels of TLR4, p-NF-κB p65, miR-155-5p, connective tissue growth factor (CTGF), and fibronectin (FN) were decreased, revealing that TNC regulated miR-155-5p expression through the TLR4/NF-κB p65 pathway, thereby regulating inflammation (NF-κB p65) and fibrosis (CTGF and FN) in individuals with DKD. In addition, metformin treatment may relive the processes of inflammation and fibrosis in individuals with DKD by reducing the levels of the TNC, p-NF-κB p65, CTGF, and FN proteins.

CONCLUSION

TNC can promote the occurrence and development of DKD. Interfering with the TNC/TLR4/NF-κB p65/miR-155-5p pathway may become a new target for DKD treatment.

Key Words: Tenascin-C; miR-155-5p; Metformin; Type 2 diabetes mellitus; Diabetic kidney disease; Toll-like receptor 4

Core Tip: We investigated the correlation between serum tenascin-C (TNC) levels and urine albumin clearance ratios in a total of 380 patients with type 2 diabetes mellitus, TNC can regulate miR-155-5p through Toll-like receptor 4/nuclear factor κB signaling in rat mesangial cells under hyperglycaemia condition, and metformin can inhibit inflammation and fibrosis in diabetic kidney disease through TNC.

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INTRODUCTION

Diabetes has now developed into the most common metabolic disease and affects the

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health of approximately 6.4% of the global population. Among patients with diabetes, diabetic kidney disease (DKD) is one of the most important causes of poor prognosis[1,2]. Approximately 40% of patients with type 2 diabetic mellitus (T2DM) will eventually progress to DKD. The combined actions of high glucose levels, oxidative stress, inflammatory chemokines, and multiple signalling pathways lead to endothelial cell injury, haemodynamic changes, and increased microvascular permeability, all of which may recruit inflammatory cells to the site of the diseased kidney, leading to the occurrence and development of DKD[3]. Therefore, studies of the mechanism of DKD are particularly important for developing new treatments, particularly treatments specific for DKD[4].

Tenascins are a family of extracellular matrix (ECM) glycoproteins comprised of tenascin-C (TNC), tenascin-X, tenascin-R, and tenascin-W[5]. Tenascin-R is mainly expressed in the central nervous system, tenascin-W is mainly expressed in stem cells, and tenascin-X is mainly expressed in loose connective tissue[6]. TNC is the most critical tenascin. TNC is expressed at very low levels in normal adult tissues, and its levels are only increased during embryonic development, in tumours, during injury repair and in inflammation[7]. TNC is the main regulator of early fibrosis, and an increase in TNC expression may reflect the development of fibrosis as a useful biomarker of matrix reconstruction[8]. Ishizaki *et al*[9] identified TNC as a potentially important indicator of disease severity. In patients with various chronic kidney diseases, plasma TNC levels are increased and negatively correlate with the glomerular filtration rate; additionally, urine TNC levels are negatively correlated with creatinine clearance[10]. In patients with DKD, TNC is widely expressed in renal tissues and positively correlated with ECM expansion[11]. However, the precise pathway by which TNC affects the development of DKD has not been reported.

Toll-like receptors (TLRs) are type I membrane glycoproteins that can identify pathogen-related molecular patterns, such as lipopolysaccharide (LPS), and endogenous damage-associated molecular pattern molecules, such as high mobility group box 1 (HMGB1)[12]. HMGB1 is an endogenous activator of oxidative stress in individuals with DKD[13]. After high-glucose treatment, TLR4 activates HMGB1 and further promotes the development of tubulointerstitial inflammation in individuals with DKD[14]. In addition, TLR4 stimulates the generation of IL-1 receptor associated kinase (IRAK) and tumor necrosis factor receptor associated factor (TRAF) through myeloid differentiation factor 88 (MyD88) and finally produces nuclear factor-κB (NF-κB), which promotes the occurrence and development of DKD[15].

MicroRNAs are small non-coding RNAs of 21-25 nucleotides in length that regulate the development of diseases by regulating the expression of target genes[16]. As shown in the study by Guay *et al*[17], miR-155 in exosomes released by T lymphocytes is converted into its activated form to promote the apoptosis of insulin beta cells, which leads to the occurrence of type 1 diabetes. Beltrami *et al*[18] also observed increased expression of miR-155 in the urine of patients with DKD. Although studies have shown the presence of miR-155 in individuals with DKD, the pathway by which miR-155 modulates the development of DKD has not been reported.

As a first-line drug for the treatment of diabetes, metformin has been widely explored for the treatment of DKD. In recent years, metformin has been shown to inhibit the occurrence and development of inflammation through NF-κB[19]. However, the mechanism by which it relieves inflammation through NF-κB and delays the development of DKD requires further investigation.

MATERIALS AND METHODS

Human studies

Subjects: Three hundred and eighty patients who were newly diagnosed with T2DM at our hospital from January 2018 to December 2018 were selected as an experimental group. Members of a healthy normal control group (n = 60) were selected from The Physical Examination Center of The First Affiliated Hospital of China Medical University. The exclusion criteria were as follows: (1) Patients under the age of 18 years old or who experienced acute complications of diabetes, such as hyperosmolar coma or diabetic ketoacidosis, within the last 3 mo; (2) Patients with cardiovascular, cerebrovascular, liver, kidney, infectious, autoimmune, or other systemic diseases or malignant tumours; (3) Patients with a recent infection, patients who underwent surgery or experienced trauma, patients under other stressful conditions, patients who were pregnant or breast feeding, and patients in other special physical states; and (4) Patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor

blockers over the last 3 mo.

Patients with T2DM were divided into a normal albuminuria group [n = 70, urinary albumin creatinine ratio (UACR) < 30 mg/g], microalbuminuria group (n = 105, UACR of 30-300 mg/g), microalbuminuria with hypertension group (n = 75, UACR of 30-300 mg/g, with hypertension), macroalbuminuria group (n = 65, UACR > 300 mg/g), and macroalbuminuria with hypertension group (n = 65, UACR > 300 mg/g, with hypertension).

Data collection: Patient age, height, weight, systolic blood pressure (SBP), and diastolic blood pressure were collected. Fasting plasma glucose, fasting plasma insulin, glycosylated hemoglobin (HbA1c), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), total serum cholesterol, triglyceride, and uric acid levels were determined. Five millilitres of morning urine was collected, and urinary albumin and creatinine levels were detected. Body mass index (BMI) and UACR were calculated. Homeostasis model assessment of insulin resistance levels were calculated to evaluate insulin resistance.

Enzyme-linked immunosorbent assay: Five millilitres of venous blood was used for the enzyme-linked immunosorbent assay (ELISA). The blood was incubated at room temperature for 30 min, an anticoagulant was added, and the blood was centrifuged for 15 min (3000 r/min, 4 °C). The supernatant was collected and stored at -80 °C in aliquots. An ELISA was used to determine serum TNC levels (SEB975Hu, Cloud-Clon Corp) among patients within the group (variable < 8%, variance < 10%).

Animal experiments

Animal feeding: Eight-week-old male Sprague-Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were fed in a standard specific pathogen-free laboratory on a 12 h light/dark cycle, housed in groups of three rats/cage, and provided free access to food and water. All experiments were approved by The Institutional Animal Care and Use Committee (IACUC) of The China Medical University Animal Experiment Department (Approval No. 2017112).

We used a well-established low-dose streptozotocin (STZ, S0130, Sigma-Aldrich, United States)-induced diabetic rat model. The rats were fasted for 12-16 h and injected with a low dose of STZ (35 mg/kg, dissolved in cold 0.1 mol/L citrate buffer pH 4.5). A One Touch Ultra glucometer and test paper (Johnson & Johnson, United States) were used to measure the blood glucose levels of diabetic rats in blood samples collected from the tail vein when the condition of diabetes was stable at 72 h after the STZ injection. Rats with significant hyperglycaemia (blood glucose > 16.7 mmol/L) accompanied by polydipsia were considered the diabetic rat model.

Histological analysis: Renal cortices were fixed with polyoxymethylene and embedded in paraffin. The sections (3 um) were stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). The pathological changes were observed using a Leica microscope.

Immunohistochemistry: The deparaffinized and rehydrated sections (3 um) of paraffin-embedded renal cortices were subjected to heat-mediated antigen retrieval and incubated with 3% H_2O_2 for 10 min. Sections were then incubated overnight at 4 °C with primary antibody against TNC (CST, 12221, 1:400), and the pathology score of micrographs was blindly evaluated by more than two experienced pathologists. At least ten images from each kidney section were counted.

ELISA and biochemical index determination: Five millilitres of venous blood of rats was used for ELISA. The blood was incubated at room temperature for 30 min, an anticoagulant was added, and the blood was centrifuged for 15 min (3000 r/min, 4 °C). The supernatant was collected and stored at -80 °C in aliquots. An ELISA was used to determine serum TNC levels (SEB975Ra, Cloud-Clon Corp) among rats within the group. The levels of urea nitrogen (C013-2-1, Nanjing Jiancheng Bioengineering Institute) and creatinine (C011-2-1, Nanjing Jiancheng Bioengineering Institute) in serum of rats were measured with commercial kits.

Cell-based experiments

Reagents and antibodies: The antibodies used in this study were rabbit polyclonal antibodies against fibronectin (FN) (sc-8422, purchased from Santa), connective tissue growth factor (CTGF) (sc-365970, purchased from Santa), TNC (12221, purchased from CST), TLR4 (sc-30002, purchased from Santa), NF-κB p65 (8242, purchased from CST), p-p65 (3033, purchased from CST), and GAPDH (Zhongshan Jinqiao). In addition,

miR-155-5p was detected using a kit and primer purchased from Gema (Suzhou, China).

Cell culture: Rat mesangial cells (RMCs) were purchased from the Wuhan Cell Bank (Wuhan Collection Center, China). The cells were cultured in minimal essential medium (Life Technologies, Carlsbad, CA, United States) supplemented with 10% foetal bovine serum (Abgent, San Diego, CA, United States) at a density of 5 × 10^5 cells/2 mL in a 6-well plate at 37 °C in an atmosphere containing 5% CO_2 and 95% air. Cells were pre-treated with serum-free medium overnight before subsequent experiments.

High-glucose screening experiment. After RMCs had grown to a confluence of approximately 50%, the cells were selected and divided into a normal glucose group, high mannitol osmotic control group (osmotic pressure control group), and hyperglycaemic group and cultured as described below: (1) The normal glucose control group (NG, 5.5 mmol/L glucose) was cultured for 24 h, and cells were then collected; (2) The high mannitol osmotic control group (5.5 mmol/L glucose + 24.5 mmol/L mannitol; the osmotic pressure was the same as the high glucose group) was cultured for 24 h, and cells were then collected; and (3) The high glucose group (30 mmol/L glucose) was cultured for 24 h, and cells were then collected. The collected cells and supernatants were stored in a freezer at -80 °C. Real-time polymerase chain reaction (PCR) was conducted to detect the expression of miR-155-5p in RMCs, Western blot was used to detect the levels of the TNC, TLR4, p-p65, NF-κB p65, CTGF, and FN proteins in RMCs, and ELISA was used to detect the levels of TNC expression in supernatants.

Transient transfection. Small interfering RNAs (siRNAs) targeting TNC, TLR4, and miR-155-5p were designed and synthesized by Gema (Shanghai, China). All of the sequences are listed in Table 1. Lipofectamine 2000 (Life Technologies, Carlsbad, CA, United States) was placed into four plates and used for transient transfection according to the manufacturer's instructions. The cells were plated in 6-well plates, and the siRNAs were then added. After transfection for 6 h, the media were replaced with normal- or high-glucose medium.

TNC screening experiment. The siRNAs designed by Gema were screened. Appropriate fragments for subsequent experiments were selected according to their transfection efficiencies.

Signaling pathway experiment. TNC blocking peptide (3628BP-50, Biovision) was used to block TNC. TN-C blocking peptide (0.5 mg/mL) was incubated with RMCs at 37 °C for 1 h and then high glucose stimulation was performed. Western blot was subsequently performed as above. RMCs were treated with 2.5 μg/mL recombinant TNC (r-TNC; CC065, Sigma). All RMCs were treated for 24 h and collected for subsequent experiments.

Metformin treatment. Metformin powder was purchased from Beyotime (s1741). A metformin stock solution was prepared and diluted to 1, 5, 10, 20, and 50 μmol/L metformin solutions. RMCs were divided into MET (5.5 mmol/L glucose + 10 μmol/L metformin), 24H (30 mmol/L glucose), H+1 (30 mmol/L glucose + 1 μmol/L metformin), H+5 (30 mmol/L glucose + 5 μmol/L metformin), H+10 (30 mmol/L glucose + 10 μmol/L metformin), H+20 (30 mmol/L glucose + 20 μmol/L metformin), and H+50 (30 mmol/L glucose + 50 μmol/L metformin) groups. All RMCs were treated for 24 h and collected for subsequent experiments.

MiRNA extraction and detection of miR-155-5p expression using real-time quantitative PCR

The miRNAs were isolated using a miRcute miRNA extraction kit (Gema, Shanghai, China) according to the manufacturer's instructions. Five microliters of miRNAs were used for reverse transcription using the miRcute miRNA first strand cDNA synthesis kit (Gema, Shanghai, China). A miRcute miRNA qPCR kit (SYBR Green; Gema, Shanghai, China) was used for PCR with 2.5 μL of cDNA templates and the following protocol: 40 cycles of 94 °C for 2 min, 94 °C for 20 s, and 60 °C for 34 s. The expression in each sample was standardized to the corresponding U6 expression level. The experimental data were analysed using the $2^{-\Delta\Delta Ct}$ method. Each sample was tested three times. Experiments were repeated independently six times under the same experimental conditions.

Protein extraction and Western blot analysis

Radioimmunoprecipitation buffer containing phenylmethanesulfonyl fluoride as a protease inhibitor and phosphatase inhibitors was used for cell lysis. The protein concentration was detected with a BCA kit (Beyotime Institute of Biotechnology,

Table 1 Sequences of siRNAs used for transfection

Gene	Sequence	
TNC-siRNA	siRNA-TNC-T1 sense	5′-GCA UCU GUG AUG AUG ACU ATT-3′
	Anti-sense	5′-UAG UCA UCA UCA CAG AUG CTT-3′
	siRNA-TNC-T2 sense	5′-GCU GAG AAG GGC AGA CAU ATT-3′
	Anti-sense	5′-UAU GUC UGC CCU UCU CAG CTT-3′
	siRNA-TNC-T3 sense	5′-GCA UUU GUG AGG AUG GUU UTT-3′
	Anti-sense	5′-AAA CCA UCC UCA CAA AUG CTT-3′
	NC-siRNA-T	5′-UUC UCC GAA CGU GUC ACG UTT-3′
	Anti-sense	5′-ACG UGA CAC GUU CGG AGA ATT-3′
TLR4-siRNA	siRNA-TLR4	5′-CGA GCU GGU AAA GAA UUU ATT-3′
	Anti-sense	5′-UAA AUU CUU UAC CAG CUC GTT-3′
	NC-siRNA-TLR4 sense	5′-UUC UCC GAA CGU GUC ACG UTT-3′
	Anti-sense	5′-ACG UGA CAC GUU CGG AGA ATT-3′
miR-155-5p inhibitor	Inhibitor	5′-ACC CCU AUC ACA AUU AGC AUU AA-3′
	Inhibitor-NC	5′-CAG UAC UUU UGU GUA GUA CAA-3′

TNC: Tenascin-C; TLR4: Toll-like receptor-4; NC: Normal control.

China), and a standard curve was generated with bovine serum albumin (BSA). Samples were mixed with 5 × loading buffer and heated in a water bath at 100 °C for 5 min. Equivalent amounts of proteins (50 μg for each sample) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an 8%-12% separating gel and then transferred to a methanol-activated polyvinylidene fluoride membrane using a constant current of 200 mA. BSA was diluted with Tris-buffered saline-Tween 20 (pH 7.6). The membrane was incubated with a 5% BSA solution at room temperature for 2 h and then incubated with the primary antibody at 4 °C overnight. Antibodies against TNC, TLR4, NF-κB p65, p-NF-κB p65 (Ser536), FN, and CTGF were diluted 1:500, while the antibody against GAPDH was diluted 1:1000. After the incubation with the primary antibody and washing, the membrane was sequentially incubated with anti-rabbit and anti-mouse IgG secondary antibodies (1:500). Then, horseradish peroxidase was detected with enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology, China), and a MicroChemi 4.2 Bio-imaging system was used to detect luminescence. Gelpro32 software was used to analyse the grey values of the bands. The densities of phosphorylated proteins were compared with the corresponding total protein densities to detect protein activation. The experiment was repeated independently six times under the same experimental conditions.

Statistical analysis

Analysis of ELISA data: The obtained data were statistically analysed using SPSS statistical analysis software (version 19.0, IBM Corp., United States). The normality of the distribution of each variable was assessed, and normally distributed measured data are reported as the mean ± SE, while measured data with a non-normal distribution are reported as medians (interquartile intervals). For inter-group comparisons of normally distributed measured data, one-way ANOVA was used to determine the significance of differences in the indicators among three or more groups, and the LSD method was used for pairwise comparisons between groups. Indicator data that did not conform to a normal distribution were statistically analysed after logarithmic transformation. The correlation between TNC levels and the UACR was analysed with a ridge regression model. $P < 0.05$ indicated statistical significance.

Cytological data analysis: The obtained data were statistically analysed using SPSS statistical analysis software (version 19.0, IBM Corp., United States). All data were obtained from six independent experiments and are reported as the mean ± SE. ANOVA was used to analyze differences among three or more groups, and the LSD

method was used to analyse normally distributed data. All P values were double-tailed, where $P < 0.05$ indicated a statistically significant difference and $P < 0.001$ indicated a highly statistically significant difference.

RESULTS

Serum TNC levels in patients with T2DM are significantly increased, and the expression levels of TNC are positively correlated with diabetes-related indicators

In the present study, the serum TNC level in patients with T2DM was measured using an ELISA. With an increasing UACR, the TNC level was significantly increased, and the TNC level was further increased in the hypertensive group compared to the control group ($P < 0.05$) (Table 2). According to the statistical analysis, serum TNC levels in patients with T2DM were positively correlated with HbA1c levels, BMI, SBP, and UACR (Table 3). The microalbuminuria and macroalbuminuria groups presented significantly higher levels of TNC than the normal albuminuria group ($P < 0.05$) (Table 2). The serum TNC level in the macroalbuminuria group was obviously higher than that in the microalbuminuria group ($P < 0.05$) (Table 2).

The prevention and treatment of DKD primarily include the control of blood glucose levels and the use of antihypertensive drugs, and an increase in blood pressure will further increase the rate of urinary albumin excretion[20-22]. Therefore, we assayed the hypertensive group to observe the effect of hypertension on the serum TNC level. Serum TNC levels in the microalbuminuria hypertension and macroalbuminuria hypertension groups were significantly higher than those in the microalbuminuria and macroalbuminuria groups, respectively ($P < 0.05$) (Table 2). Moreover, the serum TNC level in the macroalbuminuria hypertension group was significantly higher than that in the microalbuminuria hypertension group ($P < 0.05$) (Table 2).

The serum TNC level was used as the dependent variable, while the HbA1c level, BMI, SBP, and UACR were used as independent variables for the collinearity analysis. The tolerance values revealed by the statistical analysis of collinearity were all less than 0.05, and the variance inflation factor values were all greater than 30, indicating a collinearity between the dependent variable and the independent variables and suggesting that these variables could be included in the ridge regression analysis.

A ridge regression analysis was conducted with the serum TNC level as the dependent variable and the HbA1c level, BMI, SBP, and UACR as independent variables. The following ridge regression equation was obtained: $Y_{lgTNC} = 0.0001 + 0.405X_1 + 0.110X_2 + 0.341X_3 + 0.131X_4$ ($P < 0.05$, $X_1 = lgHbA_{1c,}$ $X_2 =$ BMI, $X_3 =$ SBP, and $X_4 =$ lgUACR). From this equation, the HbA1c, BMI, SBP, and UACR coefficients are positive and $P < 0.05$, indicating the significance of this equation; namely, the TNC level was positively correlated with the HbA1c level, BMI, SBP and UACR. As the HbA1c level, BMI, SBP and UACR increased, the TNC level also increased (Figure 1).

The expression level of TNC is significantly increased in the kidney tissue and serum of diabetic rats, and is positively correlated with impaired renal function

Each kidney section had at least ten images, and the pathology score of micrographs was blindly evaluated by more than two experienced pathologists. The HE and PAS staining results showed an increase in mesangial dilation and ECM deposition in DKD rats. Further immunohistochemical staining was performed using kidney tissues from rats with DKD. We stained the glomeruli of DKD rats and observed a significant increase in the expression of TNC in the glomeruli of DKD rats compared to the normal rat kidney tissue ($P < 0.05$) (Figure 2), which suggested that TNC may be involved in the occurrence and development of DKD.

In clinical practice, serum creatinine is the most commonly used indicator to determine whether the kidney is damaged. When the glomerular damage is severe and the filtration rate decreases, the serum creatinine increases, which can accurately reflect the degree of kidney damage. As the main component of low-molecular-weight nitrogen-containing substances excreted by the kidneys, urea nitrogen is of great significance for the diagnosis of the course and prognosis of renal diseases. After the diagnosis of diabetes, a kidney examination found that serum creatinine and urea nitrogen increased, indicating that the patient's kidneys were damaged. Therefore, we detected the expression of TNC, serum creatinine, and urea nitrogen in the serum of diabetic rats and normal rats. We found that the expression levels of serum TNC, urea nitrogen, and creatinine in diabetic rats were significantly higher than those of the

Table 2 Comparison of clinical characteristics and biochemical data among groups [mean ± SEM, M (quartile)]

Group	*n*	Age (yr)	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	UA (μmol/L)	HbA1C (%)	TNC (pg/mL)	UACR (mg/g)	SBP (mmHg)	DBP (mmHg)	HOMA-IR
A	60	50 ± 1.29	1.17 (0.92-1.48)	4.58 ± 0.11	2.80 ± 0.11	1.44 ± 0.05	279.54 ± 8.43	5.40 (5.25-5.70)	15.20 (14.08-15.95)	9.92 (7.58-11.23)	119 ± 1.55	72 ± 1.29	1.02 ± 0.08
B	70	52 ± 1.79	0.91 (0.71-1.59)	5.13 ± 0.15	3.40 ± 0.14	1.29 ± 0.04	265.77 ± 7.08	7.30 (6.05-8.70)[a]	15.07 (12.10-17.95)	16.76 (13.58-24.26)[a]	118 ± 1.79	74 ± 0.84	2.53 ± 0.12[a]
C	105	56 ± 1.07	2.07 (1.32-3.01)[a,b]	4.62 ± 0.07	2.88 ± 0.06	1.12 ± 0.03[a]	340.24 ± 8.17[a,b]	8.40 (7.50-9.80)[a]	17.07 (14.70-19.88)[a,b]	44.85 (33.91-59.59)[a,b]	126 ± 0.68	77 ± 0.68	4.68 ± 0.26[a,b]
D	75	57 ± 1.5	1.84 (1.55-3.87)[a,b]	4.49 ± 0.13	2.89 ± 0.11	1.00 ± 0.02[a,b]	348.87 ± 10.83[a,b]	7.30 (6.60-8.50)[a]	18.96 (14.70-21.40)[a,b,c]	56.17 (45.80-159.74)[a,b,c]	152 ± 1.04[a,b,c]	92 ± 1.5[a,b,c]	4.85 ± 0.34[a,b]
E	65	52 ± 0.99	2.08 (1.76-2.88)[a,b]	5.61 ± 0.16[a,c,d]	3.68 ± 0.13[a,c,d]	1.21 ± 0.03	381.11 ± 10.47[a,b]	7.80 (7.00-9.25)[a]	18.71 (16.40-19.95)[a,b,c]	871.58 (390.94-871.58)[a,b,c,d]	127 ± 1.36	79 ± 0.99	5.65 ± 0.45[a,b]
F	65	59 ± 1.36	1.99 (1.38-4.07)[a,b]	4.84 ± 0.15	2.84 ± 0.12[e]	1.11 ± 0.04[a]	358.38 ± 8.27[a,b]	9.10 (7.10-9.70)[a]	21.16 (16.92-21.35)[a,b,c,d,e]	1094.62 (790.00-5246.40)[a,b,c,d,e]	160 ± 1.49[a,b,c,e]	96 ± 1.86[a,b,c,e]	5.75 ± 0.4[a,b]

[a]$P < 0.05$ compared with group A.
[b]$P < 0.05$ compared with group B.
[c]$P < 0.05$ compared with group C.
[d]$P < 0.05$ compared with group D.
[e]$P < 0.05$ compared with group E. 1 mmHg = 0.133 kPa; A: Healthy group; B: Normal albuminuria group; C: Microalbuminuria group; D: Microalbuminuria combined with hypertension group; E: Macroalbuminuria group; F: Macroalbuminuria combined with hypertension group. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; UACR: Urinary albumin to creatinine ratio; HOMA-IR: Homeostasis model assessment-insulin resistance; HOMA-IR: Homeostasis model assessment of insulin resistance, = FRG × FINS / 22.5; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; TC: Total cholesterol; TG: Triglycerides; UA: Uric acid; TNC: Tenascin-C.

normal group ($P < 0.05$) (Table 4). Pearson correlation analysis showed that TNC levels were positively correlated with creatinine and urea nitrogen ($r = 0.796$ and 0.958, respectively, $P < 0.01$).

High-glucose stimulation increases the levels of TNC protein in RMCs

The mechanism underlying the correlation between TNC levels and the UACR has not been reported. Cell-based experiments were conducted to further explore this mechanism. We used RMCs (HBZY-1 cells) for the experiment designed to analyse the effect of high-glucose stimulation on TNC expression. RMCs were stimulated with normal glucose (5.5 mmol/L glucose) or hypertonic glucose (5.5 mmol/L glucose + 24.5 mmol/L mannitol) for 72 h and with high glucose (30 mmol/L glucose) for 24, 48, and 72 h. Western blot analysis showed that high-glucose stimulation increased the level of TNC protein, which peaked at 24 h ($P < 0.05$) (Figure 3). Therefore, we applied high-glucose stimulation for 24 h in subsequent experiments. In addition, 24.5 mmol/L mannitol was added to the normal glucose group each time to control for

Table 3 Correlation between tenascin-C levels and urinary albumin excretion rate

Index	lgTNC		
	β	*t*	*P* value
Constant	0.0001	-31.965	0.001
$lgHbA_{1c}$	0.405	15.893	0.001
BMI	0.110	4.090	0.002
SBP	0.341	13.379	0.002
lgUACR	0.131	4.621	0.001

BMI: Body mass index; SBP: Systolic blood pressure; 1 mmHg = 0.133 kPa; UACR: Urinary albumin to creatinine ratio; TNC: Tenascin-C.

Table 4 Comparison of expression of tenascin-C, creatinine, and urea nitrogen in serum of rats [mean ± SD, M (quartile)]

Group	*n*	TNC (ng/mL)	Creatinine (mmol/L)	Urea nitrogen (mmol/L)
N	6	40.85 ± 2.99	37.27 ± 6.41	6.02 ± 1.57
D	6	59.25 ± 3.46[a]	129.08 ± 39.88[a]	22.48 ± 2.71

[a]*P* < 0.05 compared with group. N, *n* = 6 for each group. N: Normal rat serum; D: Diabetic rat serum; TNC: Tenascin-C.

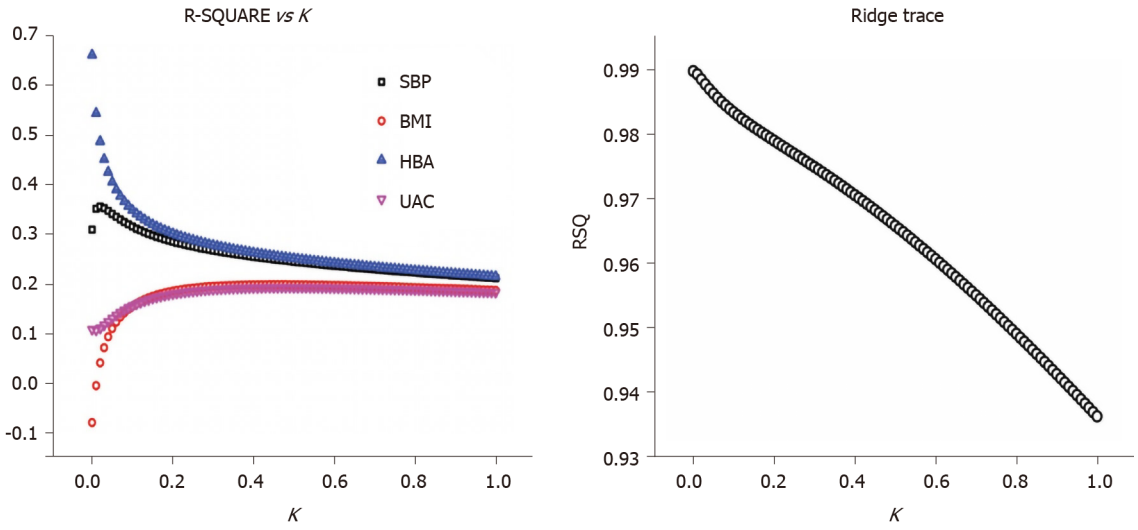


Figure 1 Relationship between the determination coefficient (R^2) and *K* value, as shown by the ridge regression analysis. RSQ: R-SQUARE; SBP: Systolic blood pressure; BMI: Body mass index; HBA: Glycosylated hemoglobin; UAC: Urinary albumin creatinine ratio.

permeability, and the addition of mannitol did not significantly increase the level of TNC protein (Figure 3). Thus, the increase in the levels of the TNC protein in RMCs treated with high glucose was not caused by an alteration in the osmotic pressure.

Moreover, the TNC expression level in the high glucose treated RMC supernatant was significantly higher than that in the normal glucose group ($P < 0.05$) (Table 5).

High-glucose stimulation of RMCs increases TLR4 expression through TNC

As shown in previous studies, TNC and TLR4 jointly promote the inflammatory response through a loop in macrophages[6,23-25], monocytes[26], dendritic cells[27], bone marrow stem cells[26,28], adipocytes[29,30], astrocytes[31], and liver cancer cells[32]. RMCs were separately cultured under normal glucose, hypertonic, and hyperglycaemic conditions to explore the role of TLR4 in RMCs cultured with high glucose concentrations. Compared with RMCs cultured with normal glucose levels, high-glucose-cultured RMCs exhibited significantly increased levels of TLR4 protein ($P < 0.05$) (Figure 3). However, a significant difference in the level of TLR4 protein was not

Table 5 Comparison of expression of tenascin-C in the supernatant of rat mesangial cell culture [mean ± SD, M (quartile)]

Group	*n*	TNC (ng/mL)
Ns	6	57.43 ± 5.94
Hs	6	70.13 ± 2.84[a]

[a]$P < 0.05$ compared with group Ns, $n = 6$ for each group. Ns: Normal condition supernatant (NG, 5.5 mmol/L glucose). Hs: High-glucose condition supernatant (HG, 30 mmol/L glucose).

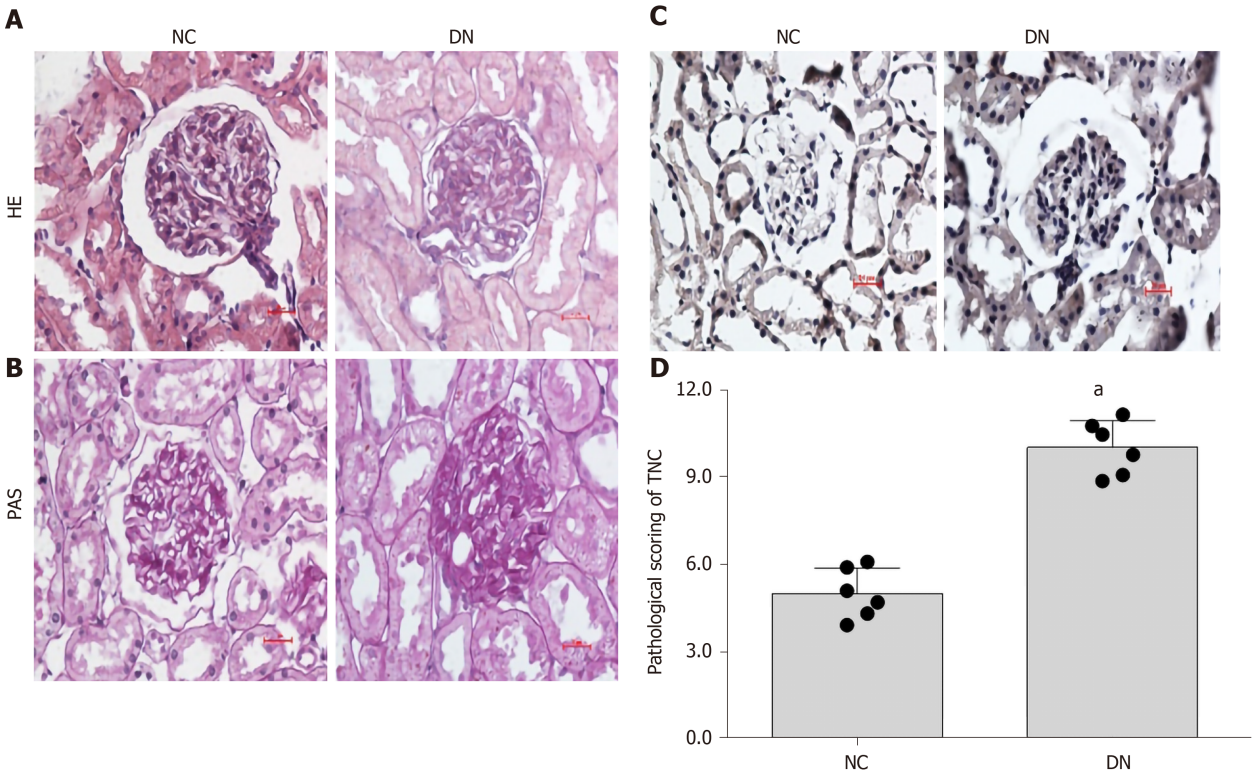


Figure 2 Histological changes and tenascin-C expression in the kidneys of diabetic rats. A: Hematoxylin-eosin staining; B: Periodic acid-Schiff staining (400 ×); C: Immunohistochemical staining; D: Pathological scoring of tenascin-C (TNC). Scale bar: 20 μm. Representative immunohistochemical images and immunohistochemical scores for TNC in renal section are shown. [a]$P < 0.05$ compared with the NC control group. NC, $n = 6$, DN, $n = 6$. NC: Normal rat renal section; DN: Diabetic rat renal section; HE: Hematoxylin-eosin; PAS: Periodic acid-Schiff; TNC: Tenascin-C.

observed between RMCs cultured under normal-glucose and hyperosmotic conditions (Figure 3), excluding an effect of osmotic pressure.

We used siRNA technology to silence the expression of TNC and further confirm its effect on TLR4 expression in RMCs cultured with high glucose concentrations. First, three different siRNAs for TNC (siRNA-TNC-T1, siRNA-TNC-T2, and siRNA-TNC-T3) were used to silence TNC expression, and non-specific siRNA (NC-siRNA-T) was used as a control (Table 1). Cells were transfected for 6 h and cultured with normal glucose concentrations for 24 h. Compared with the normal control (N) group, transfection with the three different siRNAs targeting TNC reduced the expression of TNC ($P < 0.05$) (Figure 4). Among the three siRNAs, siRNA-TNC-T2 displayed the highest transfection efficiency ($P < 0.05$) (Figure 4). Therefore, siRNA-TNC-T2 was used in subsequent experiments. A significant difference in TNC expression was not observed between the N and NC-siRNA-T groups ($P > 0.05$) (Figure 4). Therefore, the effect of the transfection on the experimental results was eliminated. Next, siRNA-TNC-T2 was used to silence the expression of the *TNC* gene, and NC-siRNA-T was used as a blank control. After transfection for 6 h, RMCs were cultured with normal glucose or high glucose concentrations for 24 h. Compared with RMCs cultured with normal glucose concentrations, those cultured with high glucose concentrations exhibited significantly increased TLR4 expression ($P < 0.05$) (Figure 5). However, this effect was blocked by siRNA-TNC-T2 ($P < 0.05$) (Figure 5). In addition, the expression of TLR4 in cells cultured with normal glucose concentrations was not affected by

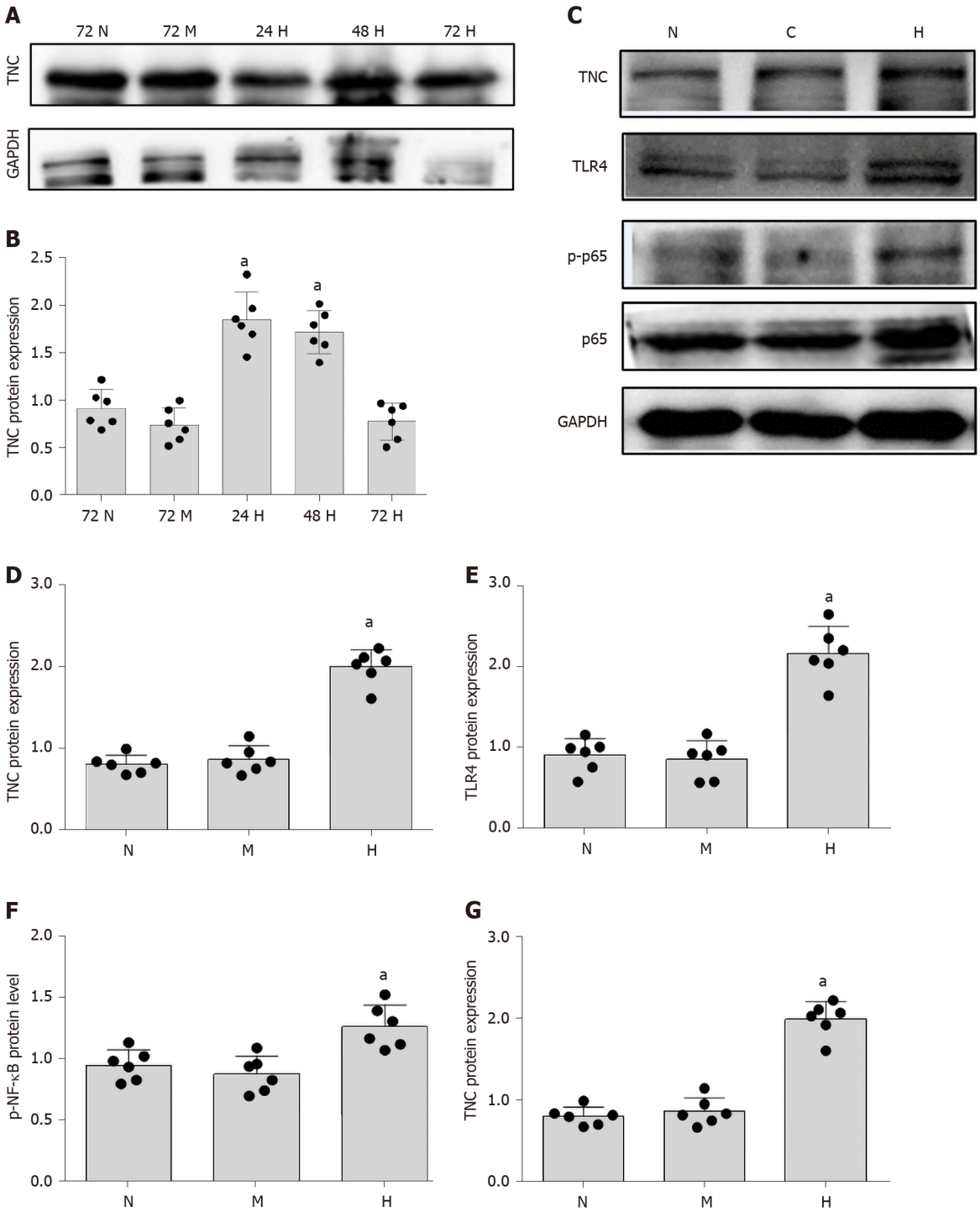


Figure 3 Changes in the levels of the tenascin-C and toll-like receptor-4 proteins and nuclear factor-κB p65 protein (Ser536) phosphorylation in cells stimulated with high glucose. A and B: Protein bands and protein expression of tenascin-C (TNC). Rat mesangial cells (RMCs) were cultured under normal (NG, 5.5 mmol/L glucose) and hypertonic (HM, 5.5 mmol/L glucose + 24.5 mmol/L mannitol) conditions for 72 h, or high-glucose (HG, 30 mmol/L glucose) conditions for 24, 48, and 72 h. $^{a}P < 0.05$ compared with the 72 h control group; C-G: Protein bands and protein expression of TNC, Toll-like receptor-4, and phosphorylated nuclear factor-κB p65 protein (Ser536). RMCs were cultured under normal (NG, 5.5 mmol/L glucose), hypertonic (HM, 5.5 mmol/L glucose + 24.5 mmol/L mannitol), and high-glucose (HG, 30 mmol/L glucose) conditions for 24 h. $^{a}P < 0.05$ compared with the N control group. Protein levels were detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TNC: Tenascin-C; TLR4: Toll-like receptor-4; p-NF-κB p65: Phosphorylated nuclear factor-κB p65 protein (Ser536); N: Normal glucose; M: Hypertonic; H: High-glucose.

siRNA-TNC-T2 ($P > 0.05$) (Figure 5).

Therefore, the inhibition of TNC expression decreases the level of TLR4 in high-glucose-cultured RMCs.

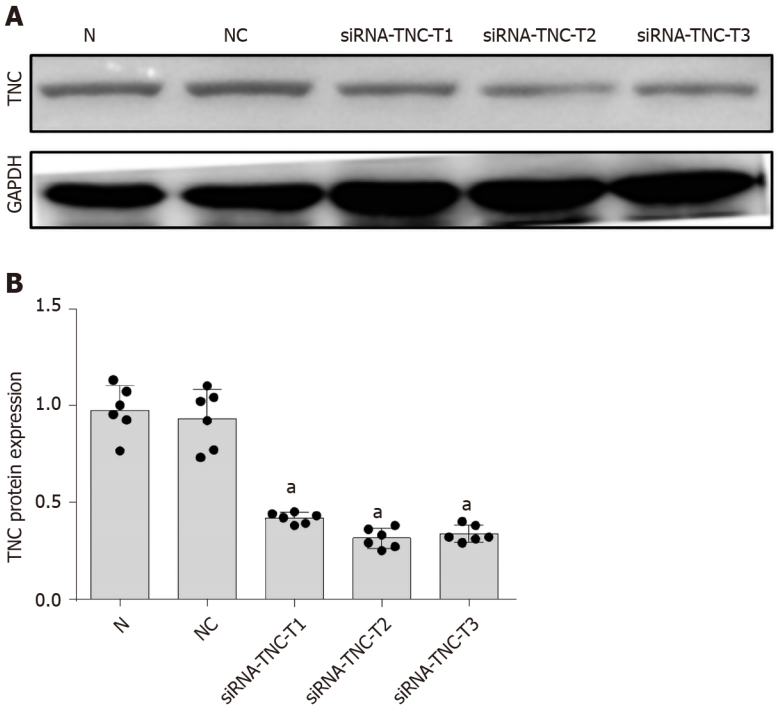


Figure 4 Screening siRNAs to silence the expression of tenascin-C protein. A: Protein bands; B: Protein expression of tenascin-C (TNC). Rat mesangial cells (RMCs) were transfected with siRNA-TNC-T1, siRNA-TNC-T2, siRNA-TNC-T3, and siRNA-NC. RMCs were cultured in normal glucose (NG, 5.5 mmol/L glucose) medium for 24 h. $^{a}P < 0.05$ compared with untransfected RMCs cultured with normal glucose concentrations. The level of the tenascin-C protein was detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TNC: Tenascin-C; NC: Negative control; N: Normal glucose.

TNC expressed in high-glucose-cultured RMCs induces the expression of NF-κB through TLR4

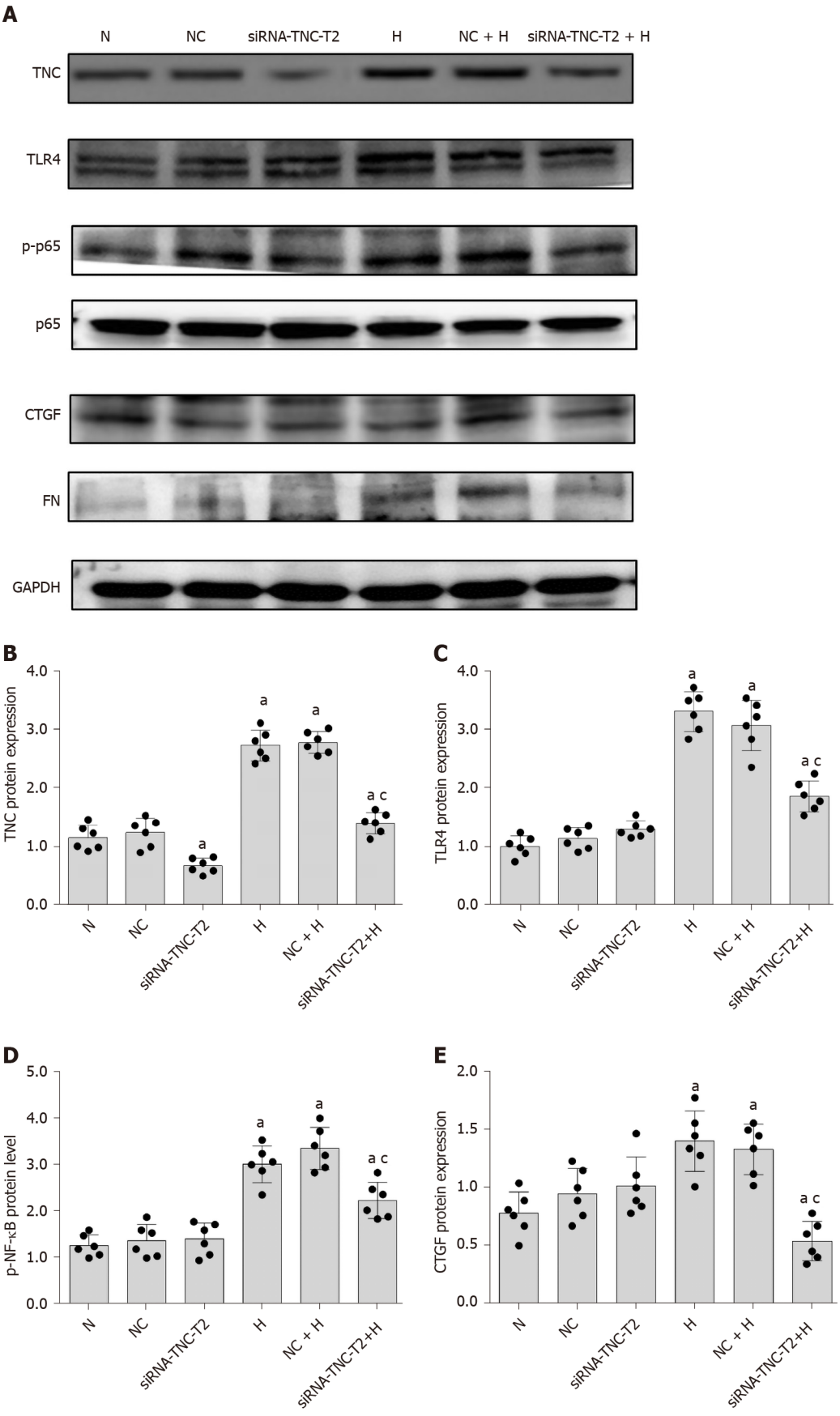
The inhibition of NF-κB alters the expression of TNC in primary human monocytes[28]. After TNC stimulation, the expression of the *TLR4* mRNA is significantly increased in Huh 7.0 (hepatocellular carcinoma) cells, and NF-κB fluorescence in the nucleus is increased[1]. In subsequent experiments, we decided to verify the effect of TNC on TLR4/NF-κB in high-glucose-cultured RMCs.

First, we cultured RMCs under normal-glucose, hyperosmotic, and hyperglycaemic conditions. Compared with the levels measured after culture with normal glucose concentrations, NF-κB p65 phosphorylation was significantly increased after culture with high glucose concentrations for 24 h ($P < 0.05$) (Figure 3). However, a significant difference in the level of NF-κB p65 phosphorylation was not observed between cells cultured under normal-glucose and hyperosmotic conditions ($P > 0.05$) (Figure 3). Therefore, we eliminated the potential effect of osmotic pressure on the experimental results.

Based on the results from previous studies by our research group, siRNA-TLR4 was used to silence the expression of the *TLR4* gene (Table 1), and a non-specific siRNA (NC-siRNA-TL) was used as a control. RMCs were transfected with the siRNAs for 6 h and cultured with normal glucose or high glucose concentrations for 24 h. Notably, siRNA-TLR4 significantly reduced the level of NF-κB p65 phosphorylation upon high-glucose stimulation ($P < 0.05$) (Figure 6) but had no effect on NF-κB p65 phosphorylation in cells cultured with normal glucose concentrations. In addition, the silencing of TLR4 expression inhibited not only NF-κB p65 phosphorylation but also the expression of the upstream protein TNC ($P < 0.05$) (Figure 6). Based on these results, TLR4 silencing decreases the activation of the NF-κB p65 signalling pathway induced by high glucose concentrations. Furthermore, transfection with siRNA-TNC-T2 not only inhibited the expression of the TLR4 protein ($P < 0.05$) (Figure 5) but also decreased the phosphorylation of NF-κB p65 in high-glucose-cultured RMCs ($P < 0.05$) (Figure 5).

High glucose induces the expression of miR-155-5p in RMCs

Pre-miR-155 adopts two mature forms, miR-155-3p and miR-155-5p, of which miR-155-5p is the most abundant[33]. Most studies of DKD have focused on miR-155-5p.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C
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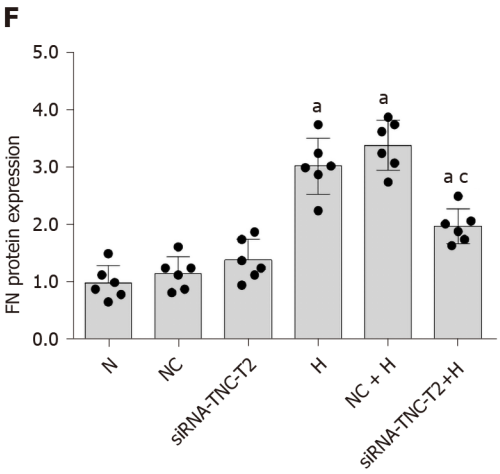


Figure 5 Silencing of tenascin-C protein expression inhibits the expression of Toll-like receptor-4 and fibrosis factors (connective tissue growth factor and fibronectin), as well as the phosphorylation of p65. A: Protein bands; B-F: Protein expression of tenascin-C (TNC), Toll-like receptor-4 (TLR4), phosphorylated nuclear factor-κB p65 (Ser536) (p-NF-κB p65), connective tissue growth factor (CTGF), and fibronectin (FN). Rat mesangial cells (RMCs) were transfected with siRNA-TNC-T2 for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h (N, 5.5 mmol/L glucose). RMCs were transfected with siRNA-TNC-T2 and siRNA-NC for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations. TNC, TLR4, p-NF-κB p65, NF-κB p65, CTGF, and FN levels were all detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TNC: Tenascin-C; TLR4: Toll-like receptor-4; p-p65: Phosphorylated nuclear factor-κB p65 (Ser536); p65: Nuclear factor-κB p65; CTGF: Connective tissue growth factor; FN: Fibronectin; N: Normal control; NC: Negative control; H: High glucose.

Therefore, in this experiment, we investigated the effect of high glucose concentrations on miR-155-5p levels in RMCs. RMCs were treated with high glucose or normal glucose concentrations (as a control) for 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h. Real-time PCR results revealed an increase in the expression of miR-155-5p in cells cultured with high-glucose medium; its expression peaked at 4 h and was 24.03-fold higher than miR-155-5p expression in cells cultured in normal-glucose medium ($P < 0.05$) (Figure 7).

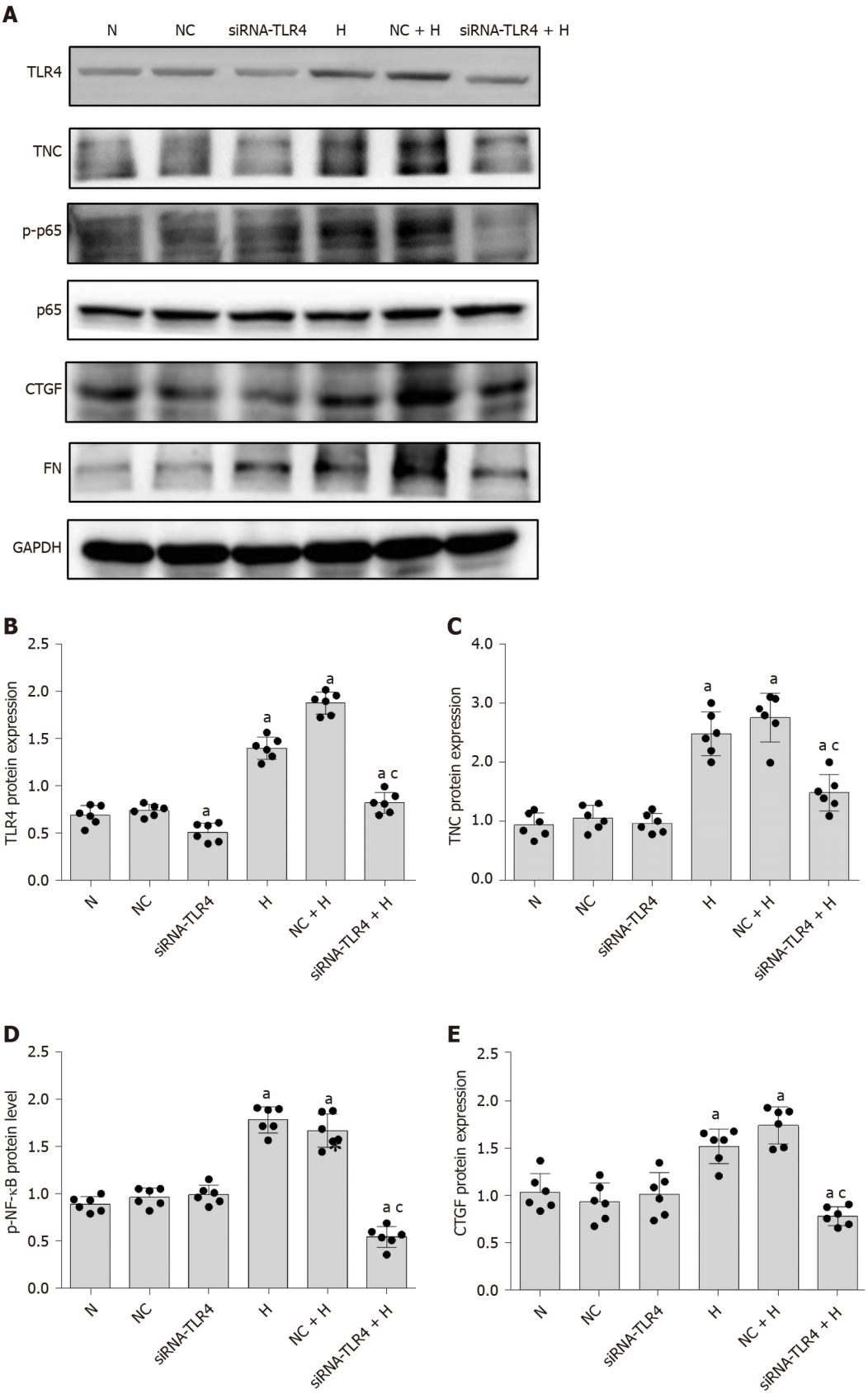
TNC regulates the expression of miR-155-5p in high-glucose-cultured RMCs through the TLR4/NF-κB pathway

In bone marrow-derived macrophages, LPS stimulation significantly increases the expression of miR-155, which is significantly inhibited in *TNC* knockout (TNKO) mice[33]. In addition, in a liver fibrosis study, increased expression of miR-155 was observed in Kupffer cells, along with increased expression of TLR4 protein[34,35]. Furthermore, an NF-κB inhibitor inhibits the expression of miR-155 in RAW264.7 macrophages[36]. Thus, TNC may regulate the expression of miR-155 through the TLR4/NF-κB pathway, but the specific mechanism still requires experimental verification.

In the present study, high-glucose stimulation significantly increased the expression of miR-155-5p in RMCs by 5.38-fold ($P < 0.05$; Figure 8). We transfected cells with siRNA-TLR4 to silence the expression of the *TLR4* gene and further confirm the role of TLR4 in increased miR-155-5p levels in RMCs cultured under high-glucose conditions. Real-time PCR showed a significant decrease in the expression of miR-155-5p after TLR4 silencing, and miR-155-5p was expressed at 2.29-fold lower levels than in cells cultured under high-glucose conditions ($P < 0.05$; Figure 8). Furthermore, transfection of RMCs with siRNA-TNC-T2 not only inhibited the activity of TLR4 and phosphorylation of NF-κB p65 ($P < 0.05$; Figure 5) but also downregulated the expression of miR-155-5p by 2.12-fold ($P < 0.05$; Figure 9) in high-glucose-cultured RMCs.

TNC regulates the expression of inflammatory and fibrogenic factors in high-glucose-treated RMCs through the TLR4/NF-κB/miR-155-5p pathway

In the unilateral ureteral obstruction (UUO) model, the level of TNC protein was significantly increased in day 7, while PCR showed that the level of the *TNC* mRNA started to increase on day 1 after UUO, indicating that TNC was produced before the development of renal fibrosis. The stimulation of rat stromal fibroblasts (NRK-49F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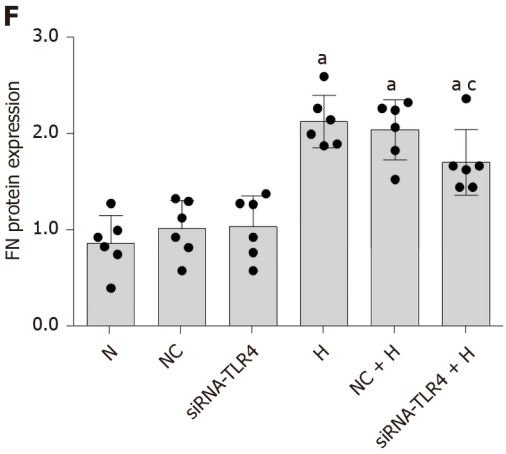


Figure 6 Silencing Toll-like receptor-4 protein expression inhibits the expression of tenascin-C and fibrosis factors (connective tissue growth factor and fibronectin), as well as nuclear factor-κB p65 phosphorylation. A: Protein bands; B-F: Protein expression of tenascin-C (TNC), Toll-like receptor-4 (TLR4), phosphorylated nuclear factor-κB p65 (Ser536) (p-NF-κB p65), connective tissue growth factor (CTGF), and fibronectin (FN). Rat mesangial cells (RMCs) were transfected with siRNA-TLR4 for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h (NG, 5.5 mmol/L glucose), (HG, 30 mmol/L glucose). RMCs were transfected with siRNA-TLR4 and siRNA-NC for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations. TNC, TLR4, p-NF-κB p65, NF-κB p65, CTGF, and FN levels were detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TNC: Tenascin-C; TLR4: Toll-like receptor-4; p-p65: Phosphorylated nuclear factor-κB p65 (Ser536); p65: Nuclear factor-κB p65; CTGF: Connective tissue growth factor; FN: Fibronectin; N: Normal control; NC: Negative control; H: High glucose.

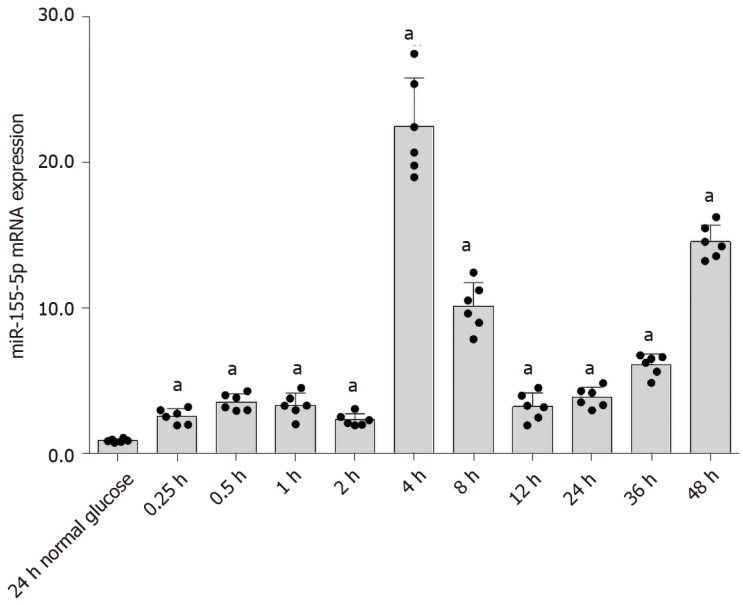


Figure 7 Expression of miR-155-5p induced by high glucose treatment for different durations. Rat mesangial cells (RMCs) cultured under normal-glucose (NG, 5.5 mmol/L glucose) and high-glucose (HG, 30 mmol/L glucose) conditions were treated with high glucose concentrations for 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h. The expression of miR-155-5p increased beginning at 0.25 h and peaked after 4 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations. The expression of miR-155-5p was quantified using real-time polymerase chain reaction. The results were normalized to the expression of the U6 mRNA and are presented as the mean ± SD of six independent experiments.

cells) with the recombinant human TNC protein significantly increased the number of NRK-49F cells, suggesting that TNC promotes the proliferation of renal fibroblasts. TNC, a secreted protein that is part of the ECM, is involved in formation of the ECM microenvironment. Overexpression of TNC promotes the expansion of fibroblasts. After decellularization of the UUO kidney, TNC still promoted the proliferation and expansion of fibroblasts, indicating that TNC was concentrated in the matrix. A TNC-specific siRNA was injected into UUO mice, and TNKO significantly reduced the renal expression of the FN and alpha smooth muscle actin proteins, as well as fibrotic injury,

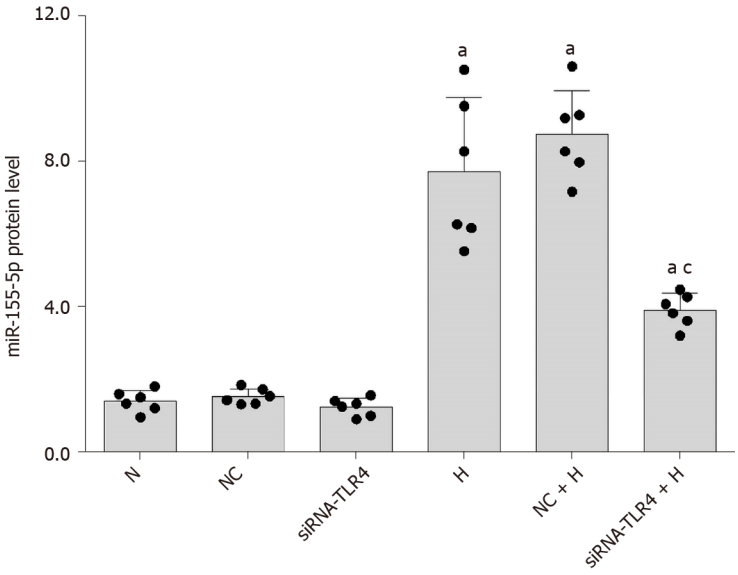


Figure 8 Silencing of Toll-like receptor-4 expression inhibits miR-155-5p expression. Rat mesangial cells (RMCs) were transfected with siRNA-TLR4 to silence Toll-like receptor-4 (TLR4) expression and siRNA-NC for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations. The expression of miR-155-5p was quantified using real-time polymerase chain reaction. The results were normalized to the expression of the U6 mRNA and are presented as the mean ± SD of six independent experiments. TLR4: Toll-like receptor-4; N: Normal control; NC: Negative control; H: High-glucose.

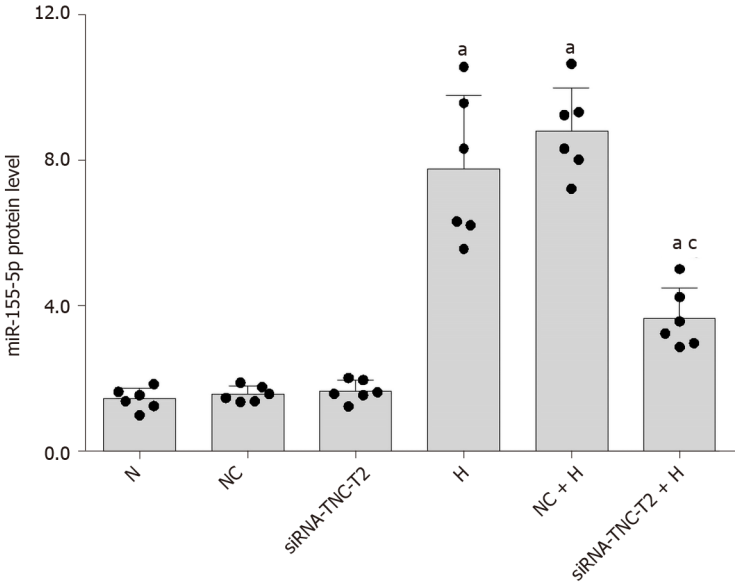
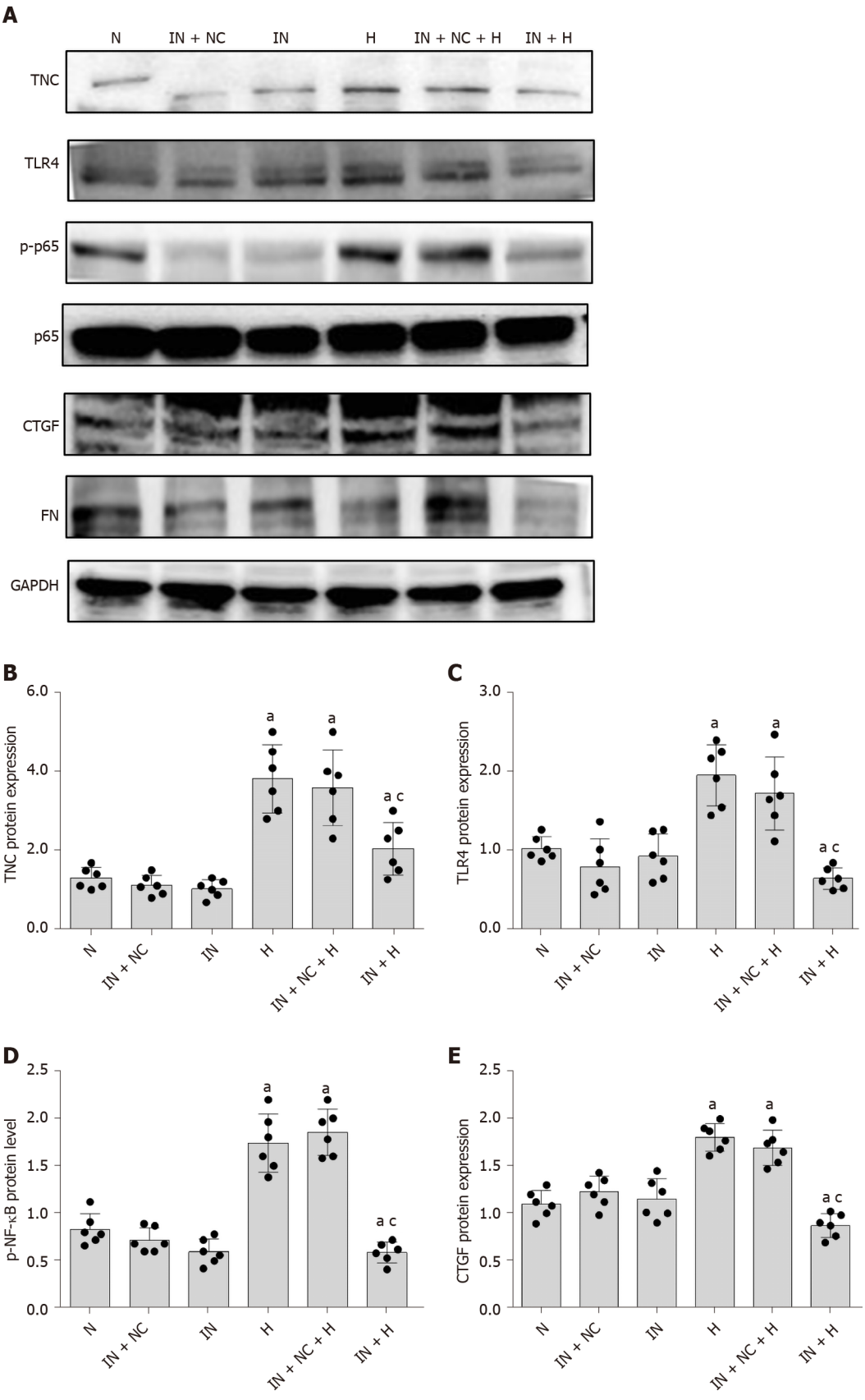


Figure 9 Silencing of tenascin-C expression inhibits miR-155-5p expression. Rat mesangial cells (RMCs) were transfected with siRNA-TNC-T2 and siRNA-NC for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations. The expression of miR-155-5p was quantified using real-time polymerase chain reaction. The results were normalized to the expression of the U6 mRNA and are presented as the mean ± SD of six independent experiments. N: Normal control; NC: Negative control; H: High-glucose.

as evidenced by Masson's trichrome staining of collagen deposition[37].

The silencing of *TNC* expression might inhibit the proliferation of fibroblasts and alleviate the development of renal fibrosis, indicating an important role for TNC in the development of renal fibrosis. Based on the conclusions drawn from the aforementioned experiments, we conducted the next experiment.

First, we used an miR-155-5p inhibitor to specifically inhibit the expression of miR-155-5p. After transfection with the miR-155-5p inhibitor for 6 h, the medium was changed, and cells were cultured with normal or high glucose concentrations for 24 h. The levels of the fibrogenic factors FN and CTGF were decreased ($P < 0.05$) (Figure 10).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TLR4
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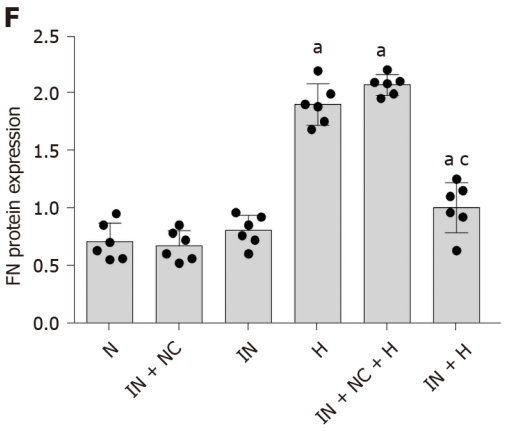


Figure 10 Inhibition of miR-155-5p expression. A: Protein bands; B-F: Protein expression of tenascin-C (TNC), Toll-like receptor-4 (TLR4), phosphorylated nuclear factor-κB p65 (Ser536) (p-NF-κB p65), connective tissue growth factor (CTGF), and fibronectin (FN). Rat mesangial cells (RMCs) were transfected with a miR-155-5p inhibitor to inhibit the expression of miR-155-5p (IN) or non-specific inhibitor (IN-NC) for 6 h and further cultured with normal glucose or high glucose concentrations for 24 h. The inhibition of miR-155-5p reduced the levels of TNC, TLR4, fibrosis factors (CTGF and FN), and p-NF-κB p65. RMCs were transfected with siRNA-IN to inhibit miR-155-5p and siRNA-NC for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) or high-glucose (HG, 30 mmol/L glucose) medium for 24 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations. TNC, TLR4, p-NF-κB p65, NF-κB p65, CTGF, and FN levels were all detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TNC: Tenascin-C; TLR4: Toll-like receptor-4; p-NF-κB p65: Phosphorylated nuclear factor-κB p65 (Ser536); NF-κB p65: Nuclear factor-κB p65; CTGF: Connective tissue growth factor; FN; Fibronectin; N: Normal control; NC: Negative control; H: High-glucose.

Further use of siRNA-TLR4 to inhibit the expression of TLR4 revealed that the silencing of *TLR4* expression decreased not only the phosphorylation of the inflammatory factor NF-κB p65 ($P < 0.05$) (Figure 6) but also the expression of miR-155-5p ($P < 0.05$) (Figure 8) and the fibrosis factors CTGF and FN ($P < 0.05$) (Figure 6) in high-glucose-treated RMCs. Then, siRNA-TNC-T2 was used to specifically silence the expression of TNC. Following the specific silencing of TNC expression, TLR4 expression and phosphorylation of the inflammatory factor NF-κB p65 were decreased ($P < 0.05$) (Figure 5), miR-155-5p expression was decreased ($P < 0.05$) (Figure 9), and the expression of the fibrosis factors CTGF and FN was decreased ($P < 0.05$) (Figure 5) in high-glucose-treated RMCs.

Inhibition of miR-155-5p expression reduces the expression of TNC, TLR4, and NF-κB in RMCs cultured under high-glucose conductions

We transfected RMCs with the miR-155-5p inhibitor to further confirm the effect of miR-155-5p on TNC, TLR4, and NF-κB p65 levels (Table 6). When the expression of miR-155-5p was inhibited, the levels of the TNC, TLR4, and phosphorylated NF-κB p65 proteins in RMCs were significantly reduced in cells stimulated with hyperglycaemia ($P < 0.05$) (Figure 10).

Signaling pathway experiment

TNC inhibitory antibodies and recombinant proteins were used to stimulate rat mesangial cells, respectively, and it was found that inhibitory antibodies significantly reduced the expression of TLR4, CTGF, and FN proteins under high glucose stimulation, while the expression of TLR4, CTGF and FN was significantly increased after the addition of r-TNC protein; the expression of CTGF and FN could be restored when silencing TLR4 under r-TNC stimulation. These results suggested that TNC downregulated inflammatory and fibrosis factors through TLR4/NF-κB ($P < 0.05$) (Figure 11).

Metformin regulates the expression of inflammatory and fibrosis factors in DKD models by regulating TNC levels

Metformin inhibits the inflammatory response of endothelial cells by inhibiting NF-κB through an AMPK-dependent pathway[38]. However, metformin has not been reported to inhibit the inflammatory response through TNC. By reviewing the literature on the effect of metformin on NF-κB, we concluded that metformin may inhibit TNC expression through NF-κB p65. Therefore, we designed the experiment described below.

Table 6 Real-time polymerase chain reaction primer sequences

Primer	Sequence	
miR-155-5p	Forward	5′-GGT GCG GTT AAT GCT AAT TGT G-3′
	Reverse	5′-CAG AGC AGG GTC CGA GGTA-3′
U6	Forward	5′-CGC TTC GGC AGC ACA TAT AC-3′
	Reverse	5′-TTC ACG AAT TTG CGT GTC ATC-3′

In previous studies, colon cancer cells were treated with metformin at concentrations of 0.5, 1, 5, 10, and 50 mmol/L[39]. In a study of the protective effect of metformin on nerve cells, the effect was most pronounced at 20 mmol/L[40]. Based on these studies, we selected the following concentration of metformin to treat cells.

RMCs were divided into the MET, 24H, H+1, H+5, H+10, H+20, and H+50 groups. Western blot showed that increasing concentrations of metformin decreased levels of phosphorylated NF-κB p65 and TNC ($P < 0.05$) (Figure 12). Then, we treated the cells with 20 mmol/L metformin, and observed the levels of downstream inflammatory and fibrosis proteins using Western blot. Metformin significantly reduced the levels of CTGF and FN proteins ($P < 0.05$) (Figure 12).

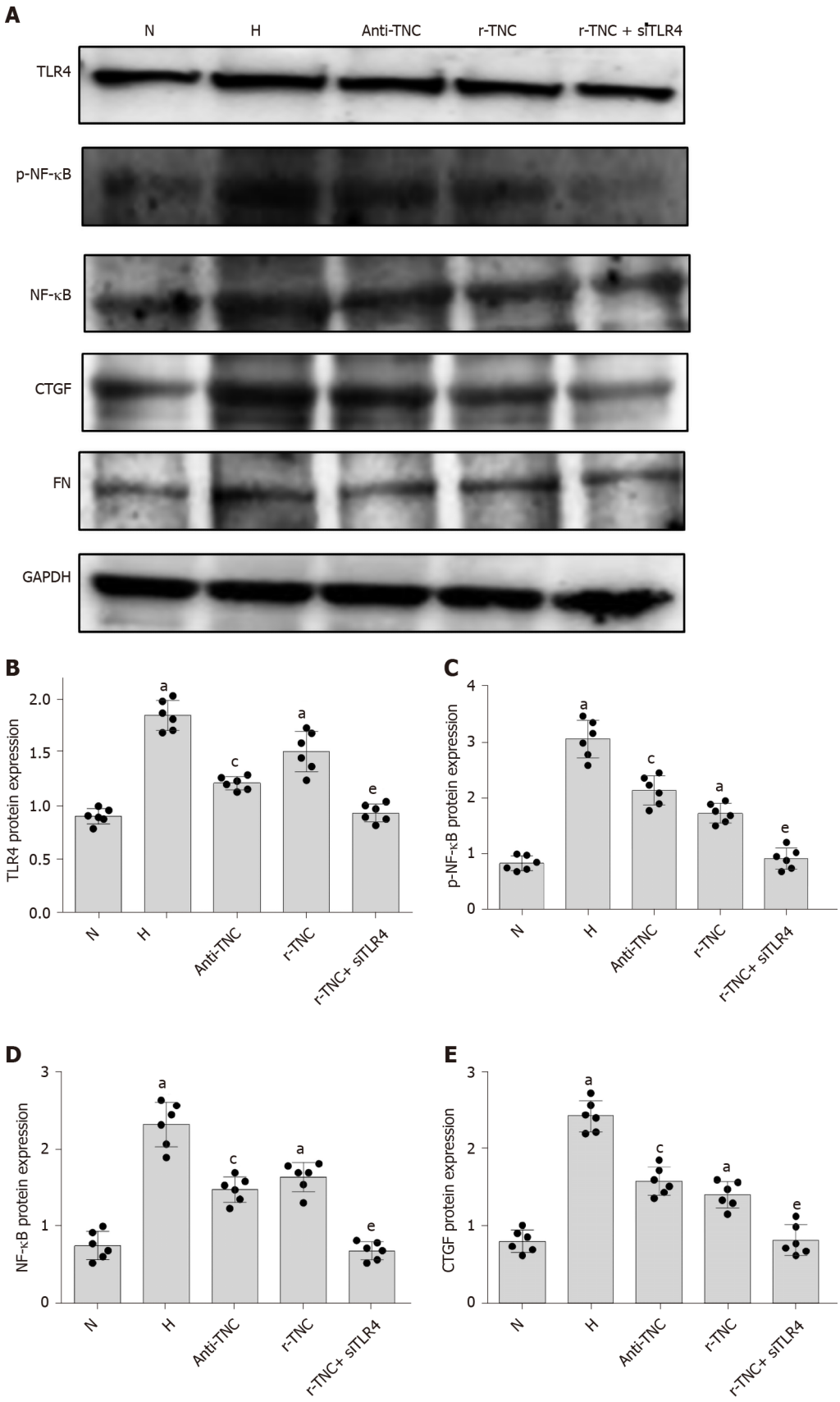
DISCUSSION

The main pathological manifestations of DKD are thickened glomerular basement membrane and increased mesangial matrix synthesis. Degradation of the glomerular filtration barrier leads to the development of proteinuria, an important manifestation of DKD, and proteinuria develops in a dynamic rather than linear manner[15,41,42]. As an ECM protein, TNC activates downstream signalling pathways by binding structural proteins in the ECM and the cell surface receptors EGF and integrin to regulate cell adhesion, migration, and proliferation[37]. According to Mackie *et al*[43], TNKO mice showed increased mesangial cell apoptosis, reduced proliferation, and reduced ECM deposition, while the addition of exogenous TNC reversed the changes.

Serum TNC levels were increased in patients with T2DM patients with an increase in the UACR, and an increased blood pressure further increased the TNC levels in the present study. In addition, serum TNC levels in patients with T2DM were positively correlated with HbA1c levels and BMI. TNC levels were increased significantly in the group with hypertension compared to the control group. As shown in previous studies, TNC is a tensor protein, and thus tension traction and Ang II may lead to increased TNC expression[44]. In addition, Fujimoto *et al*[45] observed that TNC promoted cerebral vasoconstriction through TLR4 through a MAPK-dependent pathway. Because hypertension exacerbates proteinuria, we assayed the hypertensive group to observe the effect of hypertension on the expression of TNC in different groups stratified by urine albumin excretion rates, and hypertension increased serum TNC levels in patients with an increasing UACR. TNC may play a role in the development of proteinuria in individuals with DKD. However, the specific mechanism by which TNC promotes the development of DKD remains unclear. In addition, a significantly higher level of TNC protein was observed in the glomeruli of diabetic rats than in the normal control group. Therefore, we performed *in vitro* cell culture to elucidate this molecular mechanism at the cellular and molecular levels.

Exogenous TNC induces the expression of collagen genes and the transformation of myofibroblasts through TLR4. TLR4 signalling promotes the production and accumulation of TNC in damaged tissues. TNC increases the expression and activity of TLR4 in turn, producing an inflammatory response loop that continuously increases the expression of TNC[46]. TNC and TLR4 not only regulate each other in an inflammatory state but also change cell function through TLR4. For example, TNC stimulation significantly increases the number of osteoclasts, a change that is antagonized by inhibitory antibody against TLR4[47]. In TNKO mice, the degree of fibrosis in cardiomyocytes is significantly reduced compared to control mice. Moreover, TNC regulates the polarization of M1/M2 macrophages through the TLR4 pathway. Furthermore, TNC promotes reactive oxygen species (ROS) production in bone marrow macrophages through TLR4[48].

In this study, it was found that the expression of TNC in the kidney tissues of diabetic rats was significantly increased, and the expression of TNC in the serum of


A
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H
Anti-TNC
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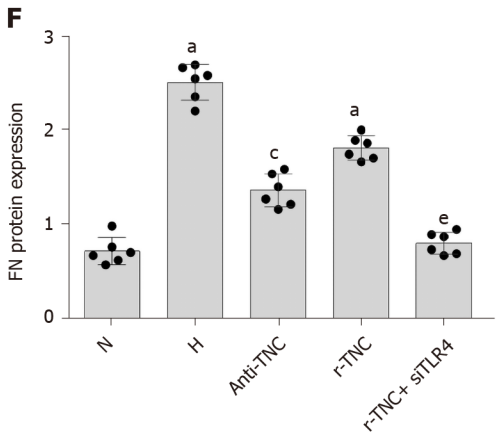

Figure 11 Signaling pathway experiment. A: Protein bands; B-F: Protein expression of Toll-like receptor-4 (TLR4), phosphorylated nuclear factor-κB p65 (Ser536) (p-NF-κB p65), connective tissue growth factor (CTGF), and fibronectin (FN). Rat mesangial cells (RMCs) were treated with TNC inhibitory antibody or TNC recombinant protein with or without transfected with siRNA-TLR4. RMCs cultured with normal glucose concentrations (N, 5.5 mmol/L glucose) or high-glucose (H, 30 mmol/L glucose) medium for 24 h, RMCs were pretreated with 0.5 mg/mL TNC blocking peptide and cultured with high-glucose (Anti-TNC, 30 mmol/L glucose), RMCs were treated with 2.5 ug/mL recombinant TNC (r-TNC), RMCs were transfected with siRNA-TLR4 to silence TLR4 expression for 6 h, and the media were then replaced with normal-glucose (NG, 5.5 mmol/L glucose) and treated with 2.5 ug/mL r-TNC(r-TNC+siTLR4). All RMCs were treated for 24 h and collected for subsequent experiments. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations; $^{e}P < 0.05$ compared with RMCs treated with r-TNC. TLR4, p-NF-κB p65, NF-κB p65, connective tissue growth factor, and fibronectin levels were all detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TLR4: Toll-like receptor-4; p-NF-κB p65: Phosphorylated nuclear factor-κB p65 (Ser536); NF-κB p65: Nuclear factor-κB p65; CTGF: Connective tissue growth factor; FN: Fibronectin; N: Normal control; H: High-glucose.

diabetic rats was increased and had a positive correlation with urea nitrogen and creatinine.

TNC expression was significantly increased in RMCs cultured under high-glucose conditions. TNC has been reported to function as an endogenous activator of TLR4-mediated inflammation, and the functional domain of TNC, the fibrinogen-like globe domain, is the major contributor to TLR4 production[6,23,24]. In our study, TLR4 expression was significantly decreased when TNC was silenced by an siRNA in high glucose-cultured RMCs. We then used an siRNA to silence TLR4 expression and observed a significant decrease in the expression of TNC in cells cultured under high-glucose conditions, confirming the interaction between TNC and TLR4 in an anti-inflammatory reaction loop in high glucose-cultured RMCs.

In the present study, the silencing of TLR4 downregulated the expression of the inflammatory factor NF-κB p65 in high glucose-cultured RMCs. The inhibition of TNC also decreased NF-κB p65 phosphorylation. In addition, the expression of TLR4, CTGF, and FN was reduced when TNC expression was inhibited by blocking antibody in the presence of high glucose, while the expression of TLR4, CTGF, and FN was increased when r-TNC protein was added, and the expression levels of CTGF and FN were restored when knocking down TLR4 under r-TNC stimulation. Based on these results, TNC induces the phosphorylation of NF-κB p65 by TLR4 in high-glucose-cultured RMCs.

LPS stimulation of macrophages increases the expression of miR-155 and decreases the expression of SHIP1, which is believed to negatively regulate TLRs. Activation of TLR4 increases the expression of miR-155 and decreases the expression of the negative regulator SHIP1, thus prolonging TLR4 signalling[27]. MiR-155 is located on human chromosome 21[49]. The specific mechanism of miR-155 in the inflammatory response in individuals with T2DM remains unknown. Some studies reported a significant decrease in the expression of miR-155 in the platelets from patients with T2DM[50]. In contrast to its expression in platelets from patients with T2DM, miR-155 expression is significantly upregulated in skin samples from patients with diabetes, and the inhibition of miR-155 significantly reduces the wound inflammatory response[51]. Similarly, increased expression of miR-155 was observed in the endothelial progenitor cells from another group of patients with diabetes. The overexpression of miR-155 significantly reduces cell activity, migration, tubule formation, and NO production, and promotes LDH release, ROS production, and apoptosis[52].

The level of miR-155 is significantly increased in the urine of patients with DKD. Researchers speculated that the excess miR-155 in the urine of patients with DKD is released into the urine through glomerular ultrafiltration. As shown in the analysis of

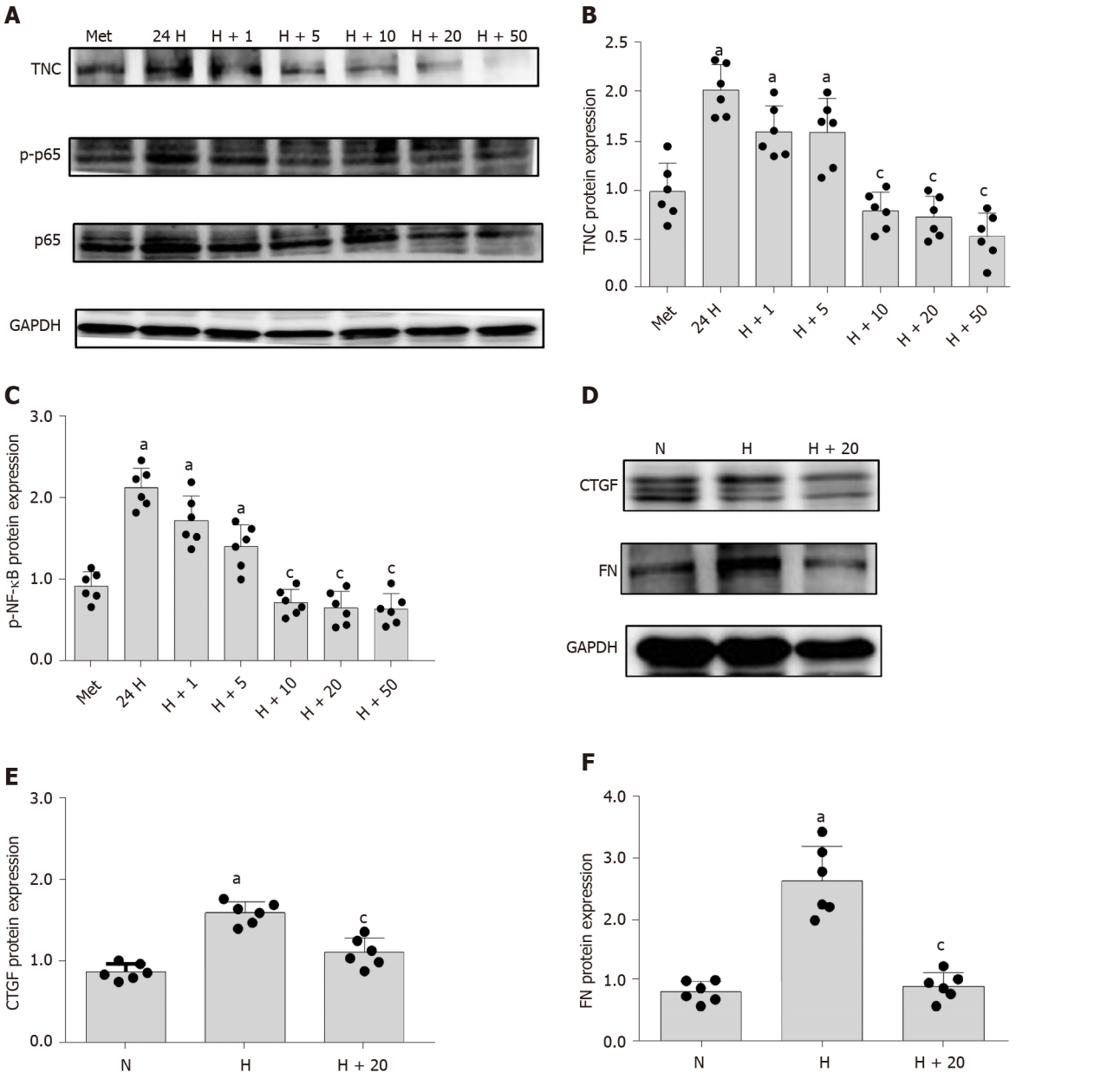

Figure 12 Inhibitory effects of metformin on rat mesangial cells. A-C: Protein bands and protein expression of tenascin-C (TNC) and phosphorylated nuclear factor-κB p65 (Ser536) (p-NF-κB p65). The MET (5.5 mmol/L glucose + 10 μmol/L metformin), 24H (30 mmol/L glucose), H+1 (30 mmol/L glucose + 1 μmol/L metformin), H+5 (30 mmol/L glucose + 5 μmol/L metformin), H+10 (30 mmol/L glucose + 10 μmol/L metformin), H+20 (30 mmol/L glucose + 20 μmol/L metformin), and H+50 (30 mmol/L glucose + 50 μmol/L metformin) groups were cultured with the appropriate medium for 24 h. $^{a}P < 0.05$ compared with rat mesangial cells (RMCs) cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations; D-F: Protein bands and protein expression of connective tissue growth factor (CTGF) and fibronectin (FN). RMCs were divided into normal-glucose (NG, 5.5 mmol/L glucose), high-glucose (HG, 30 mmol/L glucose), and H+20 (30 mmol/L glucose + 20 μmol/L metformin) groups and cultured with the appropriate medium for 24 h. $^{a}P < 0.05$ compared with RMCs cultured with normal glucose concentrations; $^{c}P < 0.05$ compared with RMCs cultured with high glucose concentrations. TNC, p-NF-κB p65, and NF-κB p65 levels were detected using Western blot. The results are presented as the mean ± SD of six independent experiments after normalization to GAPDH levels. TNC: Tenascin-C; p-NF-κB p65: Phosphorylated nuclear factor-κB p65 (Ser536); NF-κB p65: Nuclear factor-κB p65; CTGF: Connective tissue growth factor; FN: Fibronectin; N: Normal control; H: High-glucose.

renal biopsies, the expression of miR-155 is significantly increased in patients with DKD and significantly associated with serum creatinine levels. In addition, the absence of miR-155 significantly alleviates kidney injury and IL-17 expression in mice with DKD induced by streptomycin[18]. Further studies of DKD models showed a significant increase in miR-155-5p expression in HK-2 cells stimulated with high glucose concentrations[53]. Reduced podocyte cell damage was observed in high-glucose-stimulated miR-155 −/− mice[54]. Thus, miR-155 has an important role in DKD. However, with the exception of the studies described above, the specific mechanism of miR-155 in DKD has not been studied.

In our study, high glucose concentrations induced the expression of miR-155-5p in

RMCs in a time-dependent manner. In RMCs cultured under high-glucose conditions, the silencing of TLR4 with an siRNA altered the phosphorylation of the inflammatory factor NF-κB p65, and miR-155-5p expression was significantly reduced after TLR4 silencing. Similarly, after the silencing of TNC, the levels of TLR4, phosphorylated NF-κB p65, and miR-155-5p were decreased, indicating that TNC induced the expression of miR-155-5p through the TLR4/NF-κB pathway. In RMCs cultured under high-glucose conditions, the inhibition of miR-155-5p decreased the expression of TNC and TLR4 and the phosphorylation of NF-κB p65, indicating that miR-155-5p also induces the expression of TNC and TLR4/NF-κB in turn, thereby forming a complete inflammatory response loop that functions together with downstream fibrosis factors and promotes the development of DKD. Although TNC primarily functions as an ECM protein, it produces a series of subsequent inflammatory responses by communicating with TLR4, a membrane receptor and a bridge between the intracellular and extracellular environments, closely linking extracellular TNC with intracellular NF-κB p65 and miR-155-5p.

Metformin has been widely studied as a first-line drug for treating diabetes. It functions by reducing the hepatic production of glycogen and improving the sensitivity to insulin. Based on accumulating evidence obtained recently, metformin not only reduces blood glucose levels but also protect endothelial cells through its anti-inflammatory, anti-apoptotic, and anti-oxidant activities. Metformin significantly inhibits the migration of NF-κB from the cytoplasm to the nucleus, thereby inhibiting the subsequent inflammatory cascade[19]. Sekino *et al*[55] performed immunostaining to determine the intracellular localization of NF-κB. In the control group, NF-κB was localized in the cytoplasm and nucleus, while in the metformin-treated group, nuclear NF-κB expression was significantly decreased. Thus, metformin affects the level of NF-κB in the nucleus, thereby altering the activation of the NF-κB signalling pathway and further modulating downstream inflammatory pathways[55]. Therefore, metformin relieves inflammation by inhibiting the expression of NF-κB.

In the present study, we treated RMCs with different concentrations of metformin and detected the levels of TNC and phosphorylated NF-κB p65 using Western blot. The levels of NF-κB p65 phosphorylation and TNC protein were significantly reduced by increasing metformin concentrations. In addition, metformin not only reduced the levels of TNC and phosphorylated NF-κB p65 proteins but also reduced the levels of CTGF and FN proteins, suggesting that metformin might delay the occurrence of inflammation and fibrosis in individuals with DKD by inhibiting TNC expression.

In conclusion, serum TNC levels were increased in patients with DKD with an increasing UACR, and hypertension further increased the TNC levels. In addition, significantly higher TNC expression was observed in the glomeruli of diabetic rats than in the normal control group. In cell-based experiments, TNC induced the expression of miR-155-5p through the TLR4/NF-κB pathway and miR-155-5p induced the expression of TNC and TLR4/NF-κB in turn, thus forming a complete inflammatory response loop. The components of this loop jointly affect the levels of downstream inflammatory and fibrosis factors. After treatment with metformin, the level of TNC protein was reduced, thereby inhibiting downstream inflammatory and fibrotic responses. We explored the mechanism by which increased serum TNC levels promote the development of DKD at the cellular and molecular levels. Our results are expected to further refine the pathological mechanism of DKD and provide new insights into the treatment of DKD.

CONCLUSION

TNC can promote the occurrence and development of DKD. Interfering with the TNC/TLR4/NF-κB p65/mi-155-5p pathway can become a new target for DKD treatment.

ARTICLE HIGHLIGHTS

Research background

With the increasing incidence of diabetes, the incidence of diabetic kidney disease (DKD) continues to rise, which has become the leading cause of end-stage renal disease. However, current treatments like angiotensin-converting enzyme inhibitors only partially inhibited the DKD progression. Thus, the mechanisms underlying DKD

should be clarified and disclosed, and new strategies for delaying the progression are urgently needed.

Research motivation

By testing the serum tenascin-C (TNC) level in patients with type 2 diabetic mellitus (T2DM), we aim to provide insights into the pathogenesis of the DKD and suggest that TNC can serve as a therapeutic target for this disease.

Research objectives

In the present study, we evaluated the alterations of TNC expression levels in the serum or/and glomeruli of patients with T2DM and rats with streptozotocin-induced diabetes. We also evaluated the diagnose indexes of DKD, including glycosylated hemoglobin (HbA1c) level, body mass index (BMI), systolic blood pressure (SBP), and urinary albumin to creatinine ratio (UACR) in the serum of patients. In addition, we explored the specific molecular mechanism of TNC on DKD by culturing rat glomerular mesangial cells.

Research methods

Diabetes patient serum samples were collected to detect the expression level of TNC in serum and make analysis with other related factors in diabetes. Diabetic rat models were used to observe the expression of TNC in diabetic rat kidney, and enzyme-linked immunosorbent assay was used to detect the expression of TNC in diabetic rat serum, and analyze the associated renal function indexes. SiRNA transfection technique was used in cultured rat glomerular mesangial cells stimulated with high glucose to explore the molecular mechanisms of TNC in DKD.

Research results

Diabetic patients had significantly increased serum levels of TNC, and TNC was positively correlated with UACR, BMI, SBP, and DBP. TNC expression in diabetic rat kidney increased obviously, and diabetic rats had significantly higher serum TNC expression levels compared with normal rats. Urea nitrogen and creatinine were positively correlated with the increase of TNC in diabetic rats. Rat glomerular mesangial cells stimulated with high glucose had significantly increased protein expression of TNC, and TNC can promote inflammation and fibrosis through the Toll-like receptor 4/nuclear factor-κB pathway. Metformin can inhibit the expression of TNC and delay the progress of DKD.

Research conclusions

We demonstrated that increased TNC expression is involved in the cascade of DKD. Importantly, inhibition of TNC delays the development of DKD, indicating that TNC represents a potential therapeutic target in DKD.

Research perspectives

By targeting TNC expression, the occurrence and development of DKD can be delayed.

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ORIGINAL ARTICLE

Case Control Study

Relationship between serum Dickkopf-1 and albuminuria in patients with type 2 diabetes

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Abstract

BACKGROUND

Diabetic kidney disease is a microvascular complication of diabetes with complex pathogenesis. Wingless signaling-mediated renal fibrosis is associated with diabetic kidney disease. Dickkopf-1, a negative regulator of Wingless, has been proven to participate in renal fibrosis, glucose metabolism, and inflammation. However, whether serum Dickkopf-1 levels are associated with diabetic kidney disease remains unclear.

AIM

To assess the relationship between serum Dickkopf-1 levels and albuminuria in individuals with type 2 diabetes.

METHODS

Seventy-three type 2 diabetes patients and 24 healthy individuals were enrolled in this case-control study. Diabetic individuals were separated into normal albuminuria, microalbuminuria, and macroalbuminuria groups based on their urinary albumin/creatinine ratios (UACRs). Clinical characteristics and metabolic indices were recorded. Serum Dickkopf-1 levels were determined by enzyme-linked immunosorbent assay.

RESULTS

No significant difference in serum Dickkopf-1 levels was found between healthy individuals and the normal albuminuria group. However, the levels in the microalbuminuria group were significantly lower than those in the normal albuminuria group ($P = 0.017$), and those in the macroalbuminuria group were the

University.

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lowest. Bivariate analysis revealed that serum Dickkopf-1 levels were positively correlated with hemoglobin A1c level (r = 0.368, P < 0.01) and estimated glomerular filtration rate (r = 0.339, P < 0.01), but negatively correlated with diabetes duration (r = -0.231, P = 0.050), systolic blood pressure (r = -0.369, P = 0.001), serum creatinine level (r = -0.325, P < 0.01), and UACR (r = -0.459, P < 0.01). Multiple and logistic regression showed that serum Dickkopf-1 levels were independently associated with UACR (odds ratio = 0.627, P = 0.021).

CONCLUSION

Serum Dickkopf-1 levels are negatively associated with UACR. Lower serum Dickkopf-1 levels could be a critical risk factor for albuminuria in diabetes.

Key Words: Dickkopf-1; Albuminuria; Diabetic kidney disease; Type 2 diabetic mellitus; Wingless; Microalbuminuria

Core Tip: This study explored the relationship between circulating Dickkopf-1 levels and albuminuria in type 2 diabetic individuals. Multiple characteristics and metabolic indices were collected, and analyses were performed using various statistical methods. The main finding was that circulating Dickkopf-1 levels were negatively correlated with albuminuria and lower Dickkopf-1 could be a risk factor for albuminuria in diabetes.

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INTRODUCTION

Diabetic kidney disease (DKD), the key cause of end-stage kidney disease, is a severe microvascular complication of diabetes[1]. Because of the multiple factors underlying induction of DKD, its pathophysiology remains not fully elucidated[2-4]. Previous studies indicated that hemodynamic changes and metabolic disorders initiate a sequence of events that accelerate the progression of DKD. Activation of the renin-angiotensin-aldosterone system, secretion of proinflammatory factors and cytokines, and dysregulation of multiple intracellular pathways are involved in DKD progression[5].

The Wingless (Wnt) signaling pathway is essential in cell proliferation, cell migration, stem cell maintenance, tissue repair, and embryonic development[6,7]. In recent years, the Wnt signaling pathway has received considerable attention due to its roles in kidney disease, cancer, bone disease, diabetes, rheumatoid arthritis, and Alzheimer's disease[8]. Dysregulation of Wnt signaling has been shown to contribute to abnormal kidney function (*e.g.*, renal fibrosis, ischemic injury, and acute renal failure), leading to podocyte injury, mesangial cell dysfunction, and extracellular matrix deposition[9].

Dickkopf-1, together with dickkopf-2, dickkopf-3, and dickkopf-4, belonging to the dickkopf family, is an antagonist of Wnt signaling. It is a secreted glycoprotein and is widely expressed in various tissues, including the skin, osteocytes, endothelial tissue, and placenta. Dickkopf-1 can bind to lipoprotein-receptor-related protein 5/6 and interrupt the formation of lipoprotein-receptor-related protein and Wnt protein complex to inhibit the canonical Wnt signaling. Thus, it is characterized as a comprehensive regulator of Wnt signaling involved in inflammation, atherogenesis, and regulation of glucose metabolism[10]. As such, Dickkopf-1 has been proposed to influence disease in individuals with diabetes[11]. However, the relationship between serum Dickkopf-1 levels and DKD has not been established thus far. Considering that most individuals with diabetes are type 2 diabetes with complicated patho-

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physiological mechanisms, we aimed to assess the serum Dickkopf-1 levels in type 2 diabetic individuals with different albuminuria stages and further explore the potential relationship between them.

MATERIALS AND METHODS

Participants

This case-control study involved 73 type 2 diabetic individuals who had an age of onset > 18 years and 24 healthy volunteers. Type 2 diabetes was diagnosed based on the 1999 World Health Organization criteria. The exclusion criteria were as follows: (1) Presence of acute complications of diabetes, such as diabetic ketosis, lactic acidosis, hyperglycemia, or hyperosmolarity; (2) Presence of comorbid thyroid disease, adrenal disease, or other endocrine diseases; (3) Diagnosis with osteoporosis; (4) Presence of serious heart, liver, lung, hematological system, autoimmune, neoplastic, or acute cardiovascular diseases; (5) Presence of operation or acute infection; and (6) Presence of other systemic diseases that can induce proteinuria. The study was approved by the Medical Ethics Committee of the Affiliated Hospital of Weifang Medical University. In addition, informed consent was obtained from all individuals. The individuals with diabetes were categorized into normal albuminuria group [urine albumin creatinine ratios (UACR) < 30 mg/g], microalbuminuria group (UACR, 30–300 mg/g), and macroalbuminuria group (UACR, > 300 mg/g). Twenty-four healthy volunteers were included in a control group.

Measurements

Medical history and clinical characteristics (sex, age, height, weight, and blood pressure) were recorded in the morning during patients' clinic visits. Blood samples for analysis of metabolic indices were also collected. Urine samples were obtained for urinary albumin and creatinine testing. Body mass index (BMI), UACR, and estimated glomerular filtration rate (eGFR) were calculated. HOMA-IR [fasting glucose × fasting insulin (μU/mL)/22.5] was used to calculate insulin resistance.

Renal function and lipids were assessed using an autoanalyzer (Cobas 8000, Roche, Basel, Switzerland). Hemoglobin A1c (HbA1c) was detected by using a high-performance liquid chromatography system (Bio-Rad, United States). Fasting insulin and C-peptide levels were tested by chemiluminescence (e601, Roche). Serum Dickkopf-1 concentrations were assayed by ELISA employing a human Dickkopf-1-specific antibody, with a range of 10 to 1000 pg/mL (R&D systems Catalog DKK100, United States). Urinary albumin was measured by immune turbidimetry and creatinine was measured with a chemistry analyzer (AU2700, Olympus, Tokyo, Japan).

Statistical analysis

Parametric variables are presented as the mean (SE), and nonparametric variables (HOMA IR and triglycerides) are expressed as medians (IQR). Logarithmic transformations were applied to the nonparametric variables prior to analysis. One-way analysis of variance (ANOVA) was performed for multiple comparisons, followed by Tukey post hoc comparison. Pearson's correlation was used to examine relationships between variables. Multivariate linear regression models were used to estimate the determinants of Dickkopf-1. Logistic regression analyses indicated the risk factors in diabetic patients with proteinuria. The sample size was calculated using G. Power 3.1 (Germany) with the accepted minimum level of $\alpha = 0.05$ and $\beta = 0.2$ (power = 0.8). IBM SPSS Statistics, version 20.0, was used to perform data analyses. A value of $P < 0.05$ was accepted as statistically significant.

RESULTS

Cohort and clinical characteristics of individuals with diabetes

Normal healthy individuals were matched for age and sex with diabetic individuals. No significant differences were observed regarding sex, age, and BMI among diabetic patients with different stages of albuminuria. Higher blood pressure was found in patients in the macroalbuminuria group ($P < 0.01$). As expected, the fasting plasma glucose and HbA1c levels of diabetic individuals were significantly higher than those of normal controls ($P < 0.0001$ for both). However, with respect to C-peptide or insulin levels, no significant differences among groups were observed. Lipid profile tests

revealed that triglyceride levels were elevated and high-density lipoprotein cholesterol levels were lower in patients with diabetes ($P < 0.05$). Additionally, no significant difference in low-density lipoprotein cholesterol was found among the four groups. Compared with healthy individuals, the microalbuminuria and macroalbuminuria groups exhibited significantly elevated serum urea nitrogen, creatinine, and uric acid levels ($P < 0.05$ for all); they also exhibited lower eGFR ($P < 0.05$).

Comparison of serum Dickkopf-1 levels among the four groups

No significant difference in serum Dickkopf-1 levels between healthy individuals and all diabetic individuals was found (6.63 ± 0.29 ng/mL *vs* 6.13 ± 0.23 ng/mL; $P = 0.2598$). However, among patients with diabetes, the serum Dickkopf-1 level was the lowest in patients with macroalbuminuria (4.73 ± 0.13 ng/mL). Patients with microalbuminuria had a lower mean serum Dickkopf-1 level, compared with patients with normal albuminuria (6.14 ± 0.36 ng/mL *vs* 7.52 ± 0.43 ng/mL; $P = 0.017$) (Table 1).

Univariate correlations with serum Dickkopf-1 levels

Correlation analysis revealed that serum Dickkopf-1 levels were positively correlated with HbA1c ($r = 0.368$, $P = 0.001$) and eGFR ($r = 0.339$, $P = 0.003$), whereas it had negative correlations with diabetes duration ($r = -0.231$, $P = 0.050$), systolic blood pressure ($r = -0.369$, $P = 0.001$), serum creatinine levels ($r = -0.325$, $P = 0.005$), uric acid levels ($r = -0.375$, $P < 0.01$), and UACR ($r = -0.459$, $P < 0.01$). Notably, Dickkopf-1 remained negatively correlated with UACR ($r = -0.268$, $P = 0.029$) in patients with diabetes after being adjusted for sex, age, diabetes duration, HbA1c, eGFR, and uric acid levels. No statistical correlations were found between serum Dickkopf-1 levels and lipids, including triglyceride, total cholesterol, low-density lipoprotein and high-density lipoprotein (Table 2).

Multivariate correlations with serum Dickkopf-1 levels

Predictors of Dickkopf-1 levels were determined among variables that showed significant univariate associations with Dickkopf-1, by means of multivariate linear regression analysis. After adjustments for age, sex, diabetes duration, and HbA1c levels, the analysis revealed that Dickkopf-1levels were independently associated with UACR (beta coefficient = -0.280; $R^2 = 0.395$, $P = 0.025$), but not with systolic blood pressure, serum creatinine level, or uric acid level. Logistic regression analyses indicated that Dickkopf-1 levels were strongly associated with UACR in diabetic individuals (odds ratio = 0.627, $P = 0.021$) (Tables 3 and 4).

DISCUSSION

The present study revealed that serum Dickkopf-1 levels declined as the degree of albuminuria increased in diabetic individuals. Notably, Dickkopf-1 levels were independently and negatively related with UACR. These findings indicate that Dickkopf-1 is independently associated with the occurrence of proteinuria in diabetes patients.

Dickkopf-1 has been suggested to play roles in diabetes and DKD; however, there have been few investigations of these relationships. Although serum Dickkopf-1 levels were similar between healthy individuals and all diabetic individuals in our study, Dickkopf-1 levels were positively associated with HbA1c levels in the further correlation analysis. This is consistent with the conclusion by Franceschi *et al*[12], who showed similar serum Dickkopf-1 levels between children with type 1 diabetes and healthy children[12]. However, Lattanzio *et al*[11] observed elevated serum Dickkopf-1 levels in type 2 diabetic patients; it decreased upon treatment with acarbose or rosiglitazone. Our study suggested that Dickkopf-1 levels were positively correlated with HbA1c levels; thus, we speculate that Dickkopf-1 may participate in diabetes through modulation of glucose metabolism. However, the specific mechanism merits further exploration in a future study.

DKD is a severe microvascular complication of diabetes with characteristic pathological changes comprising glomerular sclerosis, as well as glomerular basement membrane thickness, mesangial cell expansion, and tubular apoptosis. Increasing degrees of albuminuria/proteinuria have been regarded as indicators of DKD progression. The Wnt pathway has been verified to participate in renal fibrosis and play a dichotomous role in DKD pathogenesis. Modulation of Wnt over-activation has been shown to improve albuminuria; downregulation of the Wnt pathway could induce renal injury and fibrosis[13,14]. Exogenous administration of nitric oxide donors

Table 1 Clinical and metabolic characteristics of the four groups

	Normal healthy group	Normal albuminuria group	Microalbuminuria group	Macroalbuminuria group
n	24	24	25	24
Sex (M/F)	7/17	11/13	11/14	15/9
Age (years)	51 ± 3	54 ± 1	57 ± 2	59 ± 2
Diabetes duration (yr)	-	5.19 ± 1.33[a]	9.23 ± 1.38[a]	12.83 ± 1.39[a,b]
BMI (kg/m^2)	23.80 ± 0.70	25.21 ± 0.67	25.52 ± 0.91	26.10 ± 0.72[a]
SBP (mmHg)	111.90 ± 3.12	134.5 ± 4.26[a]	143.5 ± 4.86[a]	156.1 ± 2.64[a,b,c]
DBP (mmHg)	70.92 ± 1.83	85.92 ± 2.11[a]	84.80 ± 3.05[a]	87.50 ± 2.52[a]
FPG (mmoL/L)	4.83 ± 0.09	9.98 ± 0.56[a]	10.10 ± 0.72[a]	9.69 ± 0.86[a]
HbA_{1c} (%)	5.68 ± 0.05	8.90 ± 0.38[a]	8.66 ± 0.36[a]	8.46 ± 0.34[a]
FCP (ng/mL)	1.68 ± 0.14	1.84 ± 0.17	1.88 ± 0.22	1.99 ± 0.28
FINS (uIU/mL)	7.84 ± 0.88	9.67 ± 1.71	8.21 ± 1.34	15.93 ± 5.48
HOMA-IR	1.40 (0.95, 2.38)	2.91 (1.87, 5.88)	3.84 (1.22, 5.12)	2.47 (0.66, 8.01)
TG (mmoL/L)	0.73 (0.42, 1.33)	1.69 (0.87, 2.67)	1.85 (1.07, 2.29)[a]	2.10 (1.31, 5.07)[a]
TC (mmoL/L)	4.67 ± 0.18	5.03 ± 0.21	4.62 ± 0.30	6.18 ± 0.40[a,b,c]
LDL(mmoL/L)	2.73 ± 0.16	2.85 ± 0.18	2.73 ± 0.19	3.43 ± 0.28
HDL(mmoL/L)	1.47 ± 0.06	1.31 ± 0.09	1.10 ± 0.07[a]	1.12 ± 0.09[a]
SCr (umoL/L)	58.43 ± 1.71	58.79 ± 2.40	76.56 ± 5.93[a,b]	106.3 ± 8.53[a,b,c]
BUN (mmoL/L)	4.45 ± 1.71	4.87 ± 0.25	6.46 ± 0.55[a,b]	8.28 ± 0.57[a,b,c]
UA (mmoL/L)	257.0 ± 14.30	270.1 ± 16.08	337.8 ± 21.93[a]	367.7 ± 19.00[a,b]
eGFR (mL/min/1.73 m^2)	126.2 ± 5.34	128.2 ± 5.77	105.2 ± 7.17[a,b]	75.14 ± 7.50[a,b,c]
UACR (mg/g)	6.55 ± 0.59	9.47 ± 1.63	103.7 ± 13.03[a,b]	581.3 ± 46.75[a,b,c]
Dickkopf-1 (ng/mL)	6.63 ± 0.29	7.52 ± 0.43	6.14 ± 0.36[b]	4.73 ± 0.13[a,b,c]

[a]$P < 0.05$ *vs* normal healthy group.
[b]$P < 0.05$ *vs* normal albuminuria group.
[c]$P < 0.05$ *vs* microalbuminuria group. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; HbA_{1c}: Glycosylated hemoglobin $A1_c$; FCP: Fasting C peptide; FINS: Fasting insulin; TC: Total cholesterol; TG: Triglyceride; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; Scr: Serum creatinine; BUN: Blood urea nitrogen; UA: Uric acid; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin/creatinine ratio.

reportedly alleviated mesangial cell apoptosis and proteinuria in diabetic rats through the restoration of Wnt signaling[15]. Spironolactone has been proposed to prevent mesangial cell apoptosis in DKD by upregulating Wnt protein expression[16,17]. Conversely, over-activation of the Wnt pathway has been found to worsen albuminuria by contributing to podocyte injury. Modulation of Wnt pathway over-activation can improve albuminuria, mesangial cell dysfunction, and extracellular matrix deposition[18,19].

As an endogenous inhibitor of the Wnt pathway, Dickkopf-1 has also been reported to contribute to microvascular complications of diabetes. Qiu *et al*[20] first reported that reduced serum Dickkopf-1 levels led to retinal Wnt pathway activation; thus, Dickkopf-1 could serve as an indicator of diabetic retinopathy. Li *et al*[21] found that Dickkopf-1 reduced podocyte apoptosis, which was associated with calcium influx and oxidative stress induced by Wnt signaling in the context of high glucose levels. Wang *et al*[22] showed that Dickkopf-1 suppressed podocyte injury by inhibiting Wnt pathway signaling that had been activated by high glucose-induced expression of β-arrestin1/2. Activation of the ubiquitin C-terminal hydrolase L1 enzyme, triggered by the Wnt pathway, can also be attenuated by Dickkopf-1, thereby reducing podocyte injury[23]. In this study, lower Dickkopf-1 levels were observed in the microalbuminuria and macroalbuminuria groups, compared with healthy individuals. Moreover, serum

Table 2 Correlation analysis between Dickkopf-1 and clinical characteristics

	DKK1	*P* value		DKK1	*P* value		DKK1	*P* value
Age	-0.124	0.425	HbA_{1c}	0.368	0.001[a]	HDL	0.181	0.125
DD	-0.231	0.050	FCP	-0.019	0.875	SCr	-0.325	0.005[a]
BMI	-0.154	0.193	HOMA-IR	-0.004	0.975	BUN	-0.295	0.011[a]
SBP	-0.369	0.001[a]	TG	-0.058	0.625	UA	-0.375	0.001[a]
DBP	-0.173	0.144	TC	-0.136	0.254	eGFR	0.339	0.003[a]
FPG	0.121	0.309	LDL	-0.162	0.171	UACR	-0.459	0.000[a]

[a]$P < 0.05$. DD: Diabetes duration; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; HbA_{1c}: Glycosylated hemoglobin A1$_c$; FCP: Fasting C peptide; TG: Triglyceride; TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; Scr: Serum creatinine; BUN: Blood urea nitrogen; UA: Uric acid; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin/creatinine ratio.

Table 3 Unstandardized and standardized β-coefficients for associations of Dickkopf-1 with clinical characteristics in multivariate analysis

DKK1	B	S.E.	β	t	*P* value	95%CI
Age	0.014	0.024	0.068	0.580	0.564	-0.034-0.061
DD	-0.031	0.030	-0.115	-1.027	0.308	-0.091-0.029
SBP	-0.017	0.010	-0.190	-1.818	0.074	-0.036-0.002
HbA_{1c}	0.318	0.113	0.281	2.804	0.007	0.091-0.544
UA	-0.005	0.002	-0.236	-1.953	0.055	-0.009-0.000
eGFR	-0.002	0.007	-0.047	-0.318	0.751	-0.017-0.012
UACR	-0.002	0.001	-0.280	-2.303	0.025	-0.004--0.000

DD: Diabetes duration; SBP: Systolic blood pressure; HbA_{1c}: Glycosylated hemoglobin A1$_c$; UA: Uric acid; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin/creatinine ratio.

Table 4 Multivariate logistic analysis of risk factors for proteinuria

Risk factor	B	S.E.	Wals	*P* value	OR	95%CI
Age	-0.010	0.043	0.052	0.820	0.990	0.910-1.077
DD	0.096	0.054	3.207	0.073	1.101	0.991-1.224
SBP	0.020	0.017	1.425	0.233	1.020	0.987-1.054
HBA_{1c}	0.253	0.223	1.291	0.256	1.289	0.832-1.995
eGFR	-0.019	0.015	1.697	0.193	0.981	0.953-1.010
DKK1	-0.468	0.203	5.329	0.021	0.627	0.421-0.932
UA	0.004	0.005	0.615	0.433	1.004	0.995-1.013

DD: Diabetes duration; SBP: Systolic blood pressure; HbA_{1c}: Glycosylated hemoglobin A1$_c$; UA: Uric0020acid; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin/creatinine ratio.

Dickkopf-1 levels successively decreased as UACR increased. It was speculated that decreased Dickkopf-1 level induced abnormal Wnt signaling pathway activation, thus leading to aggravated renal cell damage and increased albuminuria production. Taken together, these results indicated that lower Dickkopf-1 is a risk factor for proteinuria.

Besides, the analysis showed that Dickkopf-1 was positively correlated with HbA1c level and eGFR, but negatively correlated with disease course, systolic blood pressure, serum creatinine, and UACR. Consistent with the findings by Qiu *et al*[20], we speculate

that various factors (*e.g.*, hyperglycemia) may lead to abnormal secretion of Dickkopf-1; diminished Dickkopf-1 levels may over-activate the Wnt signaling pathway and upregulate angiogenic factors (*e.g.*, vascular endothlial growth factor), thereby promoting renal damage, neovascularization, and proteinuria[20]. Additionally, Dickkopf-1 has been found to inhibit cell fibrosis, suggesting that Dickkopf-1 may participate in the modulation of fibrosis during DKD progression[24]. Further analyses are required to elucidate the underlying mechanisms in the pathophysiology of DKD.

Our study also found that long diabetes duration, higher blood pressure, and lower eGFR were strongly correlated with UACR. This is due to the well-known pathophysiology that long-term/chronic hyperglycemia causes hemodynamic changes, including glomerular hyperfiltration, high perfusion, and excess pressure; these changes lead to upregulation of the renin-angiotensin-aldosterone system, overproduction of cytokines, and dysregulation of the redox homeostasis and multiple intracellular signaling pathways, which worsen DKD[25]. Thus, the positive correlation between Dickkopf-1 and eGFR, together with the negative correlation between Dickkopf-1 and diabetes duration, systolic blood pressure, serum creatinine level, and UACR in our study convinced a relationship of Dickkopf-1 and DKD.

Our study has certain limitations. As a cross-sectional study without follow-up, the significance of changes in serum Dickkopf-1 levels and DKD development remains unknown. Although decreased Dickkopf-1 was found in patients with DKD patients, further studies are required to investigate how Dickkopf-1 is involved in this shedding process. Finally, a small number of participants and selection bias also affect the limitation of the conclusion.

CONCLUSION

In summary, this study revealed that circulating Dickkopf-1 concentrations are associated with UACR and successively decrease with the progression of albuminuria in type 2 diabetic individuals. The results imply that Dickkopf-1 participates in the development of DKD. However, large-scale follow-up studies are warranted to confirm the findings and elucidate the underlying mechanism.

ARTICLE HIGHLIGHTS

Research background

Diabetic kidney disease (DKD) is a microvascular complication of diabetes with complex pathogenesis. Wingless signaling-mediated renal fibrosis is associated with DKD. Dickkopf-1, a negative regulator of Wingless, has been proven to be participating in renal fibrosis, glucose metabolism, and inflammation. However, whether serum Dickkopf-1 levels are associated with diabetic kidney disease remains unclear.

Research motivation

Are there any correlations between serum Dickkopf-1 levels and glucose levels or albuminuria in type 2 diabetic individuals? Answering this question will provide significant insight into understanding the roles of Dickkopf-1 in DKD.

Research objectives

In this study, we assessed the relationship between serum Dickkopf-1 levels and albuminuria in individuals with type 2 diabetes. This will be helpful for the exploration of the mechanism of Dickkopf-1 in DKD.

Research methods

Seventy-three type 2 diabetes and 24 healthy individuals were enrolled in this case-control study. Diabetic individuals were separated into normal albuminuria, microalbuminuria, and macroalbuminuria groups based on their urinary albumin/creatinine ratios (UACR). Clinical characteristics and metabolic indices were recorded. Serum Dickkopf-1 levels were determined by enzyme-linked immunosorbent assay.

Research results

No significant difference in serum Dickkopf-1 levels was found between healthy individuals and the normal albuminuria group. However, the levels in the microalbuminuria group were significantly lower than those in the normal albuminuria group, and those in the macroalbuminuria group were the lowest. Bivariate analysis revealed that serum Dickkopf-1 levels were positively correlated with hemoglobin A1c levels and estimated glomerular filtration rate, but negatively correlated with diabetes duration, systolic blood pressure, serum creatinine level, and UACR. Multiple and logistic regression showed that serum Dickkopf-1 levels were independently associated with UACR.

Research conclusions

We have identified that serum Dickkopf-1 levels are negatively associated with UACR. Lower serum Dickkopf-1 levels could be a critical risk factor for albuminuria in diabetes.

Research perspectives

Dickkopf-1, as an endogenous inhibitor of the Wnt pathway, mediates various effects on the microvascular complications of diabetes, including DKD. The value of the study allows scientists to better understand the mechanisms of DKD for treatment in the future.

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ORIGINAL ARTICLE

Retrospective Study

Dyslipidemia and cardiovascular disease risk factors in patients with type 1 diabetes: A single-center experience

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Abstract

BACKGROUND

Type 1 diabetes (T1D) contributes to altered lipid profiles and increases the risk of cardiovascular disease (CVD). Youth with T1D may have additional CVD risk factors within the first decade of diagnosis.

AIM

To examine risk factors for dyslipidemia in young subjects with T1D.

METHODS

Longitudinal and cross-sectional retrospective study of 170 young subjects with T1D (86 males; baseline mean age 12.2 ± 5.6 years and hemoglobin A1c 8.4% ± 1.4%) were followed in a single tertiary diabetes center for a median duration of 15 years. Predictors for outcomes of lipid profiles at last visit (total cholesterol [TC], triglycerides [TGs], low-density lipoprotein-cholesterol [LDL-c], and high-density lipoprotein-cholesterol [HDL-c]) were analyzed by stepwise linear regression models.

RESULTS

At baseline, 79.5% of the patients had at least one additional CVD risk factor (borderline dyslipidemia/dyslipidemia [37.5%], pre-hypertension/hypertension [27.6%], and overweight/obesity [16.5%]) and 41.6% had multiple (≥ 2) CVD risk factors. A positive family history of at least one CVD risk factor in a first-degree relative was reported in 54.1% of the cohort. Predictors of elevated TC: family

takes responsibility for the integrity of the data and accuracy of the data analysis; All authors approved the final version.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at Rabin Medical Center, approval No. 0075-17-RMC.

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history of CVD (β[SE] = 23.1[8.3], *P* = 0.006); of elevated LDL-c: baseline diastolic blood pressure (DBP) (β[SE] = 11.4[4.7], *P* = 0.003) and family history of CVD (β[SE] = 20.7[6.8], *P* = 0.017); of elevated TGs: baseline DBP (β[SE] = 23.8[9.1], *P* = 0.010) and family history of CVD (β[SE] = 31.0[13.1], *P* = 0.020); and of low HDL-c levels: baseline DBP (β[SE] = 4.8[2.1], *P* = 0.022]).

CONCLUSION

Our findings suggest that elevated lipid profiles are associated with DBP and a positive family history of CVD. It is of utmost importance to prevent and control modifiable risk factors such as these, as early as childhood, given that inadequate glycemic control and elevation in blood pressure intensify the risk of dyslipidemia.

Key Words: Type 1 diabetes; Children and adolescents; Cardiovascular disease risk factors; Dyslipidemia; Hypertension; Family history of cardiovascular disease risk factors

Core Tip: Co-occurrence of type 1 diabetes (T1D) and cardiovascular disease (CVD) risk factor clustering (overweight/obesity, hypertension, family history of CVD and dyslipidemia) may contribute to early-onset CVD. Our findings demonstrated that most T1D patients already had at least one CVD risk factor during childhood, with dyslipidemia being the most prevalent. It is noteworthy that clustering of CVD risk factors was observed in approximately one-half of the cohort and that there was a positive family history of at least one CVD risk factor in more than 50% of the patients. The number and distribution of CVD risk factors were similar for males and females.

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease in children and adolescents, with a steady global increase in the number of diagnosed children[1-3]. This chronic disease is associated with well-documented, life-long increases in morbidity and mortality. Over the past several decades, there has been a marked increase in the available data on T1D, resulting in a broad understanding of many aspects of the disease including its genetics, epidemiology, and disease burden. Although a number of methods to improve clinical disease management have been assessed, wide gaps still exist in the ability to standardize clinical care and decrease disease-associated complications and burdens.

One study of the natural history of the development of atherosclerosis clearly showed a possible origin of the lesions in childhood and adolescence[4]. In recent decades, numerous studies have shown that children and adolescents with T1D exhibit subclinical cardiovascular disease (CVD) abnormalities after 10 years of disease duration[5-10]. CVD is a major complication among subjects with T1D, which may lead to a higher incidence of mortality and morbidity than found in the general population. Abnormalities in serum lipid concentrations and composition are commonly associated with both T1D and type 2 diabetes (T2D) and are believed to contribute to excess CVD risk in adults[11].

Childhood obesity and overweight have significantly increased during the last 20 years and have become a major worldwide health concern[12-15]. This trend towards increased body weight is also apparent in the T1D population[16,17] and it has recently been reported that being overweight has a significant effect on the glycemic control of

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T1D patients[18]. Moreover, dyslipidemia has been documented among T1D youth aged 10-22 years, with a substantial proportion found to have lipid levels outside the recommended target range[19]. Dyslipidemia is a significant and modifiable risk factor contributing to the increased risk of atherosclerotic CVD in diabetes[20-22]. Epidemiological data on the prevalence of dyslipidemia and phenotype distribution in youth with T1D are scarce, with only limited longitudinal data on serum lipids in this population[23,24].

Co-occurrence of T1D and CVD risk factor clustering (overweight/obesity, hypertension, family history of CVD and dyslipidemia) may contribute to early-onset CVD. In this retrospective longitudinal study, we examined the association between CVD risk factors in childhood and dyslipidemia in young adulthood, and determined the prevalence of CVD risk factor clustering among T1D patients.

MATERIALS AND METHODS

Subjects

The study population included 170 young patients with T1D (86 males) followed in the National Center for Childhood Diabetes, Schneider Children's Medical Center of Israel, during the years 1998-2013. Inclusion criteria were: children/adolescents aged less than 18 years at diabetes onset, T1D diagnosis prior to 1998, and regular clinical follow-up at our diabetes center. Exclusion criteria were: Patients lost to follow-up, those with a lipid profile not available at the predetermined time points, and those with concomitant diseases likely to interfere with lipid metabolism.

The study was approved by the ethics committee of our institution, which waived the need to obtain informed consent.

Materials and methods

This longitudinal and cross-sectional retrospective cohort study was based on data collected from medical records of patients treated in our national diabetes center in accordance with the principles of Good Clinical Practice. The following data were retrieved from medical files: Sociodemographic parameters (date of birth, sex, ethnicity), anthropometric measurements (height, weight, calculated body mass index [BMI]) pubertal stage, blood pressure measurements, and diabetes-related parameters (age at diagnosis of T1D, diabetes duration, glycemic control as expressed by levels of glycosylated hemoglobin [HbA1c]), and serum lipid profile. The clinical and laboratory data were extracted from the medical files at four points in time (1998, 2003, 2008 and 2013) and a medical interview was conducted in 2017.

The routine clinical practice followed for T1D patients in our center has been quarterly clinic visits, with follow-up every 3-6 mo for weight (in light clothing using a standard calibrated scale) and height (using a commercial Harpenden-Holtain stadiometer, until adult height). BMI was calculated as weight in kilograms divided by height in meters squared. BMI-standard deviation scores (SDS) were calculated according to the recommendations of the Centers for Disease Control and Prevention[25].

The medical team (nurses and physicians) routinely questioned patients about cigarette smoking and alcohol consumption, and self-reported responses were documented in the medical files. Regular smoking was defined as smoking at least one cigarette once a week and regular alcohol consumption as drinking at least one alcoholic beverage once a week. Female patients were routinely questioned about their menstrual cycle (whether menses were absent or present and whether the cycle was regular or irregular) as well as their use of oral contraceptives.

HbA1c was routinely tested at each visit at 3-4 mo intervals. Capillary HbA1c values were measured by an automated immunochemical technique (DCA 2000; Siemens Medical Solutions Diagnostics, Tarrytown, NY, United States; 95% confidence interval [CI] 4.3%-5.7%). Our routine policy is to screen T1D patients for autoimmune thyroid disease, celiac disease, and pernicious anemia at diagnosis and annually thereafter and to screen for dyslipidemia annually. Routine screening for microvascular complications was generally initiated in pubertal patients during the first year after diagnosis, with subsequent annual assessment[26]. Screening for microvascular complications included an ophthalmologic examination, testing of urine for albumin secretion, and neurological examination (bedside Neuropathy Disability Score) to screen for distal polyneuropathy[27].

Data collection through childhood and early adulthood

BMI was calculated using the anthropometric measurements documented in the medical files. In childhood and adolescence, BMI values were converted to age- and sex-specific percentiles according to the CDC2000[25]. In adulthood, BMI values were converted according to the reference data of the National Health and Nutrition Examination Survey and National Center for Health Statistics in 2003-2006[28]. Weight status was categorized as: Obese, ≥ 95th percentile; overweight, ≥ 85th to < 95th percentiles; normal weight, ≥ 5th to < 85th percentiles and underweight, < 5th percentile[29]. Blood pressure was measured according to the recommendations of the National High Blood Pressure Education Program (NHBPEP)[30]. In childhood, percentiles for systolic blood pressure (BP) and diastolic BP were calculated according to height, sex, and age[31]. Normal BP, prehypertension, and hypertension, were defined according to the NHBPEP[30].

Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and triglycerides (TGs) were converted to age- and sex-specific percentiles according to American Academy of Pediatrics (AAP) criteria for children aged 5-19 years[32]. In adults, hypercholesterolemia was defined when: TC levels were > 240 mg/dL; LDL-c levels were > 130 mg/dL; TG levels were > 150 mg/dL and HDL-c levels were < 40 mg/dL in males and < 50 mg/dL in females. CVD risk factors in T1D included: Overweight/obesity, pre-hypertension/hypertension, dyslipidemia (elevated LDL-c/elevated TG/low HDL-c) and positive family history for cardiometabolic diseases. Patients under 5 years of age were not included in the CVD risk factor analysis since lipid level reference values are less established.

Data collection in adulthood through structured interview

In accordance with IRB approval, former T1D patients no longer treated in our center were each sent a letter explaining the general goal of the study and asking permission for a detailed phone-call interview. In 2017, a structured telephone interview was carried out by a pediatric endocrinology fellow, who explained the purposes of the study, the anonymity of responses, and participants' rights. The interview contained questions pertaining to current medical status and updated family history in first-degree relatives (parents and/or siblings), as follows: (1) Habitual behavior - smoking, and if yes, age at initiation and number of cigarettes; and regular physical activity, type (aerobic/muscle-strengthening) and duration (weekly hours); (2) Autoimmune co-morbidities (thyroid disease, celiac disease, pernicious anemia), age at diagnosis; (3) Diabetes complications (retinopathy, microalbuminuria, nephropathy, neuropathy); (4) CVD risk factors (hypertension, dyslipidemia); (5) Current and past medications; (6) In females - age at menarche, regularity of menses, polycystic ovary disease, and oral contraceptive use, and if yes, indication; and (7) family history of cardio-metabolic diseases (diabetes, hypertension, dyslipidemia, CVD, and cerebrovascular episodes).

Statistical analysis

The data were analyzed using IBM SPSS statistical software (release 25.0; IBM SPSS Statistics for Windows, Armonk, NY, United States). All statistical tests were performed as two-sided. The Kolmogorov-Smirnov *z*-test was performed to test the null hypothesis that the variable has a normal distribution. Data are expressed as the mean and standard deviation (SD) for normal distribution median, interquartile range for skewed distribution, and number and percent for discrete variables. Pearson's chi-square test was used for analysis of between-group differences in discrete variables. Independent-samples *t*-tests or Mann–Whitney *U* test were used to compare between groups for continuous variables, with normal or skewed distributions, respectively. Predictors for long-term outcomes of lipid profile (TC, TGs, LDL-c, and HDL-c) were analyzed by a stepwise linear regression model. The independent variables included in all four linear model analyses were potential predictors and confounders (sex, ethnicity, Tanner stage, BMI-SDS, systolic and diastolic BP, and HbA1c levels, age at diagnosis, T1D duration, and family history of cardiometabolic diseases). $P \leq 0.05$ was considered statistically significant.

RESULTS

The baseline characteristics of the 170 young subjects with T1D (86 males) at a mean age 12.2 ± 5.6 years are presented in Table 1. At the first evaluation, 46.5% (79) patients were prepubertal (Tanner stage 1), while 24.7% (42) were in puberty (Tanner stage 2-4) and 28.8% (49) were fully pubertal. At baseline, mean HbA1c was 8.4% ± 1.4%, 61.2%

Table 1 Baseline characteristics of the study cohort (1998)

	All, *n* = 170	Males, *n* = 86	Females, *n* = 84	*P* value
Age in yr	12.6 ± 5.6	12.4 ± 6.0	12.6 ± 5.1	0.854
Age at diabetes diagnosis	8.1 ± 4.4	8.0 ± 4.7	8.2 ± 4.1	0.735
Diabetes duration	4.4 ± 4.0	4.4 ± 4.2	4.3 ± 3.8	0.828
HbA1c, %	8.4 ± 1.4	8.6 ± 1.4	8.2 ± 1.4	0.064
HbA1c in mmol/L	68.3	70.5	66.1	
Pubertal stage, *n* (%)				
Tanner 1	79 (46.5)	47 (54.7)	32 (38.1)	0.291
Tanner 2	11 (6.5)	4 (4.7)	7 (8.3)	
Tanner 3	19 (11.2)	8 (9.3)	11 (13.1)	
Tanner 4	12 (7.1)	5 (5.8)	7 (8.3)	
Tanner 5	49 (28.8)	22 (25.6)	27 (32.1)	
Menarche		NA	48 (57.1)	
Age at menarche		NA	13.3 ± 1.3	
Ethnicity, *n* (%)				
Ashkenazi Jew	102 (60.0)	47 (54.7)	55 (65.5)	0.120
North African Jew	19 (11.2)	7 (8.1)	12 (14.3)	
Oriental Jew	19 (11.2)	10 (11.6)	9 (10.7)	
Yemenite Jew	16 (9.4)	12 (14)	4 (4.8)	
Ethiopian Jew	4 (2.4)	2 (2.3)	2 (2.4)	
Arab	10 (5.9)	8 (9.3)	2 (2.4)	
Cardiovascular disease risk factors in patients, *n* (%)				
Overweight	20 (11.8)	9 (10.5)	11 (13.1)	0.864
Obesity	8 (4.7)	4 (4.7)	4 (4.8)	
Systolic pre-hypertension, ≥ 90th to the 95th percentile	15 (8.8)	8 (9.3)	7 (8.3)	0.336
Systolic stage 1 hypertension, ≥ 95th to the < 99th percentile	19 (11.2)	12 (14)	7 (8.3)	
Systolic stage 2 hypertension, ≥ 99th percentile	8 (4.7)	5 (5.8)	3 (3.6)	
Diastolic pre-hypertension, ≥ 90th to the 95th percentile	3 (1.8)	2 (2.3)	1 (1.2)	0.670
Diastolic stage 1 hypertension, ≥ 95th to the < 99th percentile	7 (4.1)	3 (3.5)	4 (4.8)	
Diastolic stage 2 hypertension, ≥ 99th percentile	1 (0.6)	1 (1.2)	0 (0)	
Pre-hypertension, systolic and/or diastolic ≥ 90th to the 95th percentile	15 (8.8)	9 (10.5)	6 (7.1)	0.426
Stage 1 hypertension, systolic and/or diastolic ≥ 95th to the < 99th percentile	23 (13.5)	12 (14)	11 (13.1)	
Stage 2 hypertension, systolic and/or diastolic ≥ 99th percentile	9 (5.3)	6 (7)	3 (3.6)	
Lipid profile[1]	*n* = 144	*n* = 71	*n* = 73	
LDL-c, *n* (%)				0.049
< 75th percentile	63 (43.8)	27 (38)	36 (49.3)	
75th-90th percentile	47 (32.6)	21 (29.6)	26 (35.6)	

Borderline elevated 90th-95th percentile	10 (6.9)	7 (8.1)	3 (4.1)	
Elevated > 95th	24 (16.7)	16 (18.6)	8 (11)	
Triglycerides, *n* (%)				0.393
< 75th percentile	31 (21.5)	12 (16.9)	19 (26)	
75th-90th percentile	59 (41)	30 (42.3)	29 (39.7)	
Borderline elevated 90th-95th percentile	16 (11.1)	7 (9.9)	9 (12.3)	
Elevated > 95th	38 (26.3)	22 (30.9)	16 (21.9)	
HDL-c, *n* (%)				0.728
Normal level > 10th percentile	127 (88.2)	62 (87.3)	65 (89%)	
Borderline low 5th-10th percentile	9 (6.2)	4 (5.6)	5 (6.8)	
Low level < 5th percentile	8 (5.5)	5 (6.9)	3 (4.1)	
Positive family history of cardiovascular risk factors in a first-degree relative, *n* (%)				
Cardiovascular disease risk factors				
Type 2 diabetes	51 (30)	23 (26.7)	28 (33.3)	0.349
Premature coronary artery disease	24 (14.1)	10 (11.6)	14 (16.7)	0.346
Dyslipidemia	32 (18.8)	17 (19.8)	15 (17.9)	0.750
Hypertension	33 (19.4)	16 (18.6)	17 (20.2)	0.778
Cerebrovascular accident	4 (2.4)	4 (4.7)	0 (0)	0.035

Data are expressed as the number (percent) or as mean ± standard deviation. *P* values are between males and females. Highlighted in bold are significant values.

[1]Lipid profiles were available for 144 patients (71 males, 73 females). Only one female patient used statins. First-degree relatives included parents and siblings. Premature coronary artery disease (*i.e.* heart attack, treated angina, interventions for coronary artery disease, stroke, or sudden cardiac death) in male relatives < 55 years or female relatives < 65 years. HbA1c: Hemoglobin A1c; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol.

were treated by multiple daily insulin injections (the rest treated with insulin pump), and 14.1% (24/170) had co-existent autoimmune thyroid disease.

Positive family history of at least one CVD risk factor (T2D, premature coronary artery disease, dyslipidemia, hypertension or cerebrovascular accident) in a first-degree relative was reported in 54.1% (92/170) of the cohort. At baseline 128/161 patients (79.5%) already had an additional CVD risk factor in addition to the diabetes. Occurrence of multiple CVD risk factors (2 or more) was found in 67/161 patients (41.6%). The number and distribution of multiple CVD risk factors is presented in Figure 1, with no significant differences between males and females (P = 0.210). CVD risk factors in descending order of frequency were borderline dyslipidemia/dyslipidemia (37.5%), pre-hypertension/hypertension (27.6%), and overweight/obesity (16.5%), with no significant differences between males and females.

The characteristics of the study cohort at last visit at a mean age of 26.3 ± 5.7 years are presented in Table 2. Smoking was reported in 7.6% (13/170) of the cohort. 13.1% (11/84) of females reported oral contraceptive use. The number and distribution of multiple CVD risk factors at young adulthood is presented in Figure 2, with no significant differences between males and females (P = 0.275). CVD risk factors in descending order of frequency were borderline dyslipidemia/dyslipidemia (66.5%), overweight/obesity (39.9%), and pre-hypertension/hypertension (24.3%), with no significant differences between males and females.

Predictors for dyslipidemia are presented in Table 3. Predictors for elevated TC: Family history of CVD (β[SE] = 23.1[8.3], P = 0.006); elevated LDL-c: baseline diastolic blood pressure (DBP) (β[SE] = 11.4[4.7], P = 0.003) and family history of CVD (β[SE] = 20.7[6.8], P = 0.017); elevated TGs: baseline DBP (β[SE] = 23.8[9.1], P = 0.010) and family history of CVD (β[SE] = 31.0[13.1], P = 0.020); low HDL-c levels: baseline DBP (β[SE] = 4.8[2.1], P = 0.022).

Table 2 Characteristics of the study cohort at the last visit

	All, *n* = 170	Males, *n* = 86	Females, *n* = 84	*P* value
Age in yr	26.3 ± 5.7	26.5 ± 6.0	26.0 ± 5.5	0.540
Diabetes duration in yr	18.2 ± 5.6	18.6 ± 5.5	17.8 ± 5.8	0.375
Duration follow-up in yr	13.7 ± 3.7	14.1 ± 3.6	13.4 ± 3.9	0.222
HbA1c, %	8.0 ± 1.4	8.2 ± 1.6	7.9 ± 1.1	0.164
HbA1c in mmol/L	63.9	66.1	62.8	
Smoker, *n* (%)	13 (7.6)	7 (8.1)	6 (7.1)	0.807
Oral contraceptive use, *n* (%)			11 (13.1)	
Cardiovascular disease risk factors in patients[1],*n* (%)	**All, *n* = 149**	**Males, *n* = 74**	**Females, *n* = 75**	
Diabetes only	13 (8.7)	5 (6.8)	8 (10.7)	0.578
+ 1	41 (27.5)	21 (28.4)	20 (26.7)	
+ 2	62 (41.6)	28 (37.8)	34 (45.3)	
+ 3	27 (18.1)	16 (21.6)	11 (14.7)	
+ 4	6 (4.0)	4 (5.4)	2 (2.7)	
Cardiovascular disease risk factors in patients, *n* (%)				
Overweight/obesity, *n* = 158	63/158 (39.9)	32 (40)	31 (39.7)	0.974
Hypertension, *n* = 152	37 (24.3)	26 (35.1)	11 (14.1)	0.003
Systolic BP > 130 mmHg	28 (18.4)	22 (29.7)	6 (7.7)	< 0.001
Diastolic BP > 80 mmHg	21 (13.8)	16 (21.6)	5 (6.4)	0.007
Dyslipidemia	113 (66.5)	57 (66.3)	56 (66.7)	0.957
LDL-c > 100 mg/dL	83 (48.8)	43 (50.0)	40 (47.6)	0.641
Triglycerides > 150 mg/dL	27 (15.9)	11 (12.7)	16 (19.0)	0.279
HDL-c < 40 mg/dL (males) and < 50 mg/dL (females)	39 (22.9)	15 (17.4)	24 (28.5)	0.099
Statin use[2]	29 (18.2)	18 (22.5)	11 (13.9)	0.316

Data are expressed as number (percent) or as mean ± standard deviation. *P* values are between males and females. Significant values are highlighted in bold.

[1]Data on cardiovascular disease risk factors were available for 149 patients (74 males, 75 females).

[2]Data on medications other than insulin (statins) were documented in the medical records of 159 patients (80 males, 79 females). BP: Blood pressure; HbA1c: Hemoglobin A1c; HDL-c; High-density lipoprotein cholesterol; LDL-c; Low-density lipoprotein cholesterol.

DISCUSSION

CVD is a leading cause of increased morbidity and mortality in subjects with T1D[33]. Co-occurrence of T1D and CVD risk factor clustering (overweight/obesity, hypertension, family history of CVD and dyslipidemia) may contribute to early-onset CVD. Goldberg *et al*[34] recently reported that clustering of cardiometabolic risk factors was more prominent in young adults diagnosed with T1D in early childhood, thus placing them at risk for premature cardiovascular morbidity and mortality. Our findings demonstrate that most T1D patients already had at least one CVD risk factor during childhood, with dyslipidemia being the most prevalent. It is noteworthy that clustering of CVD risk factors was observed in approximately one-half of the cohort and that there was a positive family history of at least one CVD risk factor in many patients. Number and distribution of CVD risk factors were similar for males and females.

Weight gain is a clinical concern in patients with T1D. The insulin resistance in overweight and obese individuals with T1D may be associated with an increased risk of vascular complications[35,36]. Over the 15-year observation period of this study, we found a marked increase in the percentage of T1D individuals with overweight/obesity, from 16.5% to 40% of the cohort. This increase in BMI may mirror the

Table 3 Factors associated with dyslipidemia (1998-last visit)

	β (SE)	*P* value	95%CI
Low-density lipoprotein cholesterol			
Diastolic blood pressure 1998	11.4 (4.7)	0.003	2.0, 20.7
Positive family history of cardiovascular disease	20.7 (6.8)	0.017	7.2, 34.1
Triglycerides			
Diastolic blood pressure 1998	23.8 (9.1)	0.01	5.8, 41.8
Positive family history of cardiovascular disease	31.0 (13.1)	0.02	5.0, 57.0
High-density lipoprotein cholesterol			
Diastolic blood pressure 1998	-4.8 (2.1)	0.022	-8.9, -0.7
Total cholesterol			
Positive family history of CVD	23.1 (8.3)	0.006	6.6, 39.5

The following variables were entered into each of the four stepwise linear regression models for long-term outcomes of the lipid profile (total cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol): sex, age at diagnosis, ethnicity, positive family history of type 2 diabetes, positive family history of cardiovascular disease (CVD), positive family history of dyslipidemia, positive family history of hypertension, body mass index-standard deviation scores 1998, diastolic and systolic blood pressure categories according to percentiles 1998, Tanner pubertal stage 1998, duration of diabetes 1998, and the mean HbA1c from 1998 to the last visit.

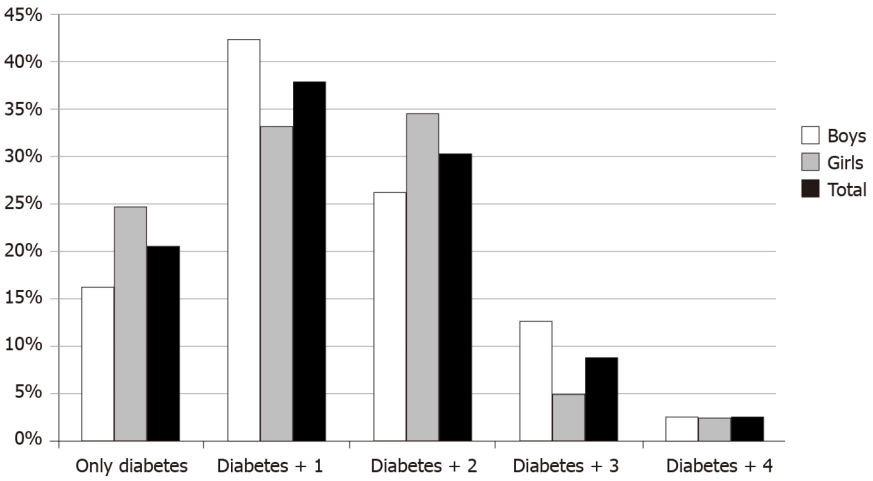

Figure 1 Number and distribution of multiple cardiovascular disease risk factors (overweight/obesity, pre-hypertension/hypertension, dyslipidemia [elevated low-density lipoprotein-cholesterol/elevated triglyceride/low high-density lipoprotein-cholesterol] and positive family history for cardiometabolic diseases) categorized by sex in childhood and adolescence. No significant differences between males and females (P = 0.210). Bar graphs in black represent the entire cohort, males in white, and females in gray.

increased prevalence of overweight/obesity in the general Israeli population with progression of age[37,38]. Our findings are in line with other reports from the United States, Europe, and Australia[16,39,40]. Since all T1D individuals followed in our institute receive medical nutrition therapy, the high prevalence of overweight/obesity is surprising. The excessive weight gain may perhaps be partially attributable to the intensive insulin therapy[41,42] or reflect the increase in overweight/obesity in the general Israeli population[37]. Although BMI is a good predictor of weight status, it is not a direct measure of adiposity and may slightly overestimate weight status in individuals with a relatively high muscle mass. It is therefore plausible that the rate of overweight/obesity is overestimated in our cohort. Unfortunately, body composition analysis was not available.

Hypertension is a co-morbid condition of T1D that contributes to the onset and progression of both microvascular and macrovascular complications of the disease. Studies in adults with T1D also have increased mortality rates when systolic and DBPs

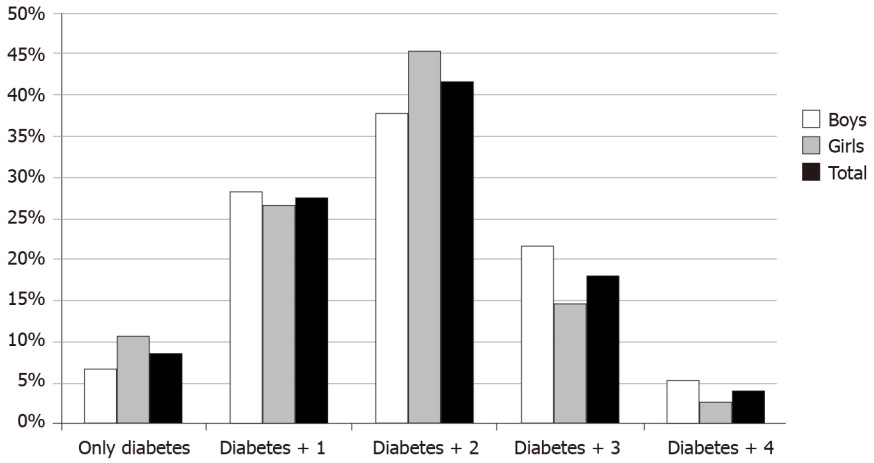

Figure 2 Number and distribution of multiple cardiovascular disease risk factors (overweight/obesity, pre-hypertension/hypertension, dyslipidemia [elevated low-density lipoprotein-cholesterol/elevated triglyceride/low high-density lipoprotein-cholesterol] and positive family history for cardiometabolic diseases) categorized by sex at young adulthood. No significant differences between males and females (P = 0.275). Bar graphs in black represent the entire cohort, males in white and females in gray.

are elevated[43,44]. Moreover, elevated BP is independently associated with an increased risk of stroke in individuals with T1D[45]. In children with T1D, the prevalence of elevated BP is reportedly as high as 4%-16%[23,46,47]. Our data show a prevalence of hypertension in approximately 25% of the cohort, both at first evaluation and at last visit, which is higher than previously reported. However, one should keep in mind that there is a known under-diagnosis of hypertension in children with T1D.

T1D and dyslipidemia are both risk factors for CVD. International guidelines recommend lifestyle modifications and then consideration of statin pharmacotherapy, depending on an individual's age and the severity of CVD risk based on LDL-c level and other risk factors[22,48,49]. Previous studies have reported a high frequency of dyslipidemia among pediatric and young adult patients with T1D[19,50,51], with a prevalence rate between 26%-72% and the highest prevalence (72%) in a Brazilian study[52]. Similarly, we found a relatively high prevalence of dyslipidemia already during childhood in slightly more than one-third of our study population, rising to about 60% at adulthood. In 2008 the American Academy of Pediatrics endorsed pharmacologic intervention for children with diabetes when LDL concentration is > 130 mg/dL[33]. Our findings suggest that, although statin therapy is recommended from the age of 8 years, physicians and patients are reluctant to initiate therapy in childhood and adolescence.

BP and cholesterol are major modifiable CVD risk factors and key components of risk prediction algorithms[53]. We found that elevated lipid levels were associated with DBP in childhood and a positive family history of CVD. An atherogenic lipid profile (specifically, elevated LDL-c in adulthood) was associated with both a positive family history of CVD and DBP in childhood; low HDL-c in adulthood was associated with DBP in childhood. In a recent report on pooled data from six large prospective United States cohort studies (of over 36000 participants), young adult exposures to elevated DBP and LDL-c were associated with incident congestive heart disease, and young adult exposure to elevated SBP and DBP was associated with incident heart failure, independent of later adult exposures[52]. These findings suggest that intervention to control modifiable risk factors during childhood, adolescence and young adulthood may reduce the future burden of CVD. Furthermore, since a family background of CVD risk factors plays such a pivotal role in the cardiometabolic health of patients, it is important to update medical files over time.

The strengths of this study lie in the fact that all the patients in our cohort received a similar standard of clinical care, as provided by the same team in a tertiary care center, and the relatively long follow-up period (median of 15 years) from childhood through adolescence to young adulthood. It should be noted that young T1D patients are referred to our center from all over the country and thus serve as a representative sample of all sectors of the Israeli population, including patients of various ethnic origins and socioeconomic status. This study had some limitations, including the single-center experience, the small sample size, and importantly, the lack of an intermediate outcome measure of CV risk/damage (*i.e.* cardiovascular risk score, intimal media thickness). Although patients were advised to perform fasting prior to

lipid profile testing, there was no guarantee that the lipid profile was taken after fasting. In the study population, there was an underrepresentation of the Arab population. Another limitation was the lack of precise data on lifestyle, including physical activity levels and dietary habits. Finally, there may be limits to the generalization of our findings, which are based on T1D patients in our country, and may differ from T1D patients in other countries. Despite these limitations, this study provides important data regarding which factors are associated with elevated CVD risk in young T1D patients.

CONCLUSION

In conclusion, our findings suggest that an elevated lipid profile is associated with DBP and positive family history of CVD. It is of utmost importance to prevent and control these types of modifiable risk factors as early as childhood, given that inadequate glycemic control and elevation in blood pressure intensify the risk for dyslipidemia. The clustering of CVD risk factors is recognized as being more prominent in patients whose TID is poorly controlled, further emphasizing the importance of rapid and intense intervention when required.

ARTICLE HIGHLIGHTS

Research background

Type 1 diabetes (T1D) contributes to altered lipid profiles and increased cardiovascular disease (CVD) risk.

Research motivation

Co-occurrence of T1D and CVD risk factor clustering (overweight/obesity, hypertension, family history of CVD and dyslipidemia) may contribute to early-onset CVD.

Research objectives

We examined the association between CVD risk factors in childhood and dyslipidemia in young adulthood and determined the prevalence of CVD risk factor clustering among T1D patients.

Research methods

Longitudinal and cross-sectional retrospective study of 170 young subjects with T1D followed in a single tertiary diabetes center for a median duration of 15 years.

Research results

Our findings demonstrate that most T1D patients already had at least one CVD risk factor during childhood, with dyslipidemia being the most prevalent. It is noteworthy that clustering of CVD risk factors was observed in approximately one-half of the cohort and that there was a positive family history of at least one CVD risk factor in many patients. The number and distribution of CVD risk factors were similar for males and females.

Research conclusions

Our findings suggest that an elevated lipid profile is associated with diastolic blood pressure and positive family history of CVD.

Research perspectives

It is of utmost importance to prevent and control modifiable risk factors as early as childhood, given that inadequate glycemic control and elevation in blood pressure intensify the risk for dyslipidemia.

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ORIGINAL ARTICLE

Observational Study

Impact of diabetes mellitus and cardiometabolic syndrome on the risk of Alzheimer's disease among postmenopausal women

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Abstract

BACKGROUND

In spite of an increase in the incidence and prevalence of diabetes mellitus (DM) and Alzheimer's disease (AD) in the aging population worldwide, limited attention has been paid to their potential association.

AIM

To investigate the association of DM and cardiometabolic syndrome (CMS, a precursor to DM) with risk of incident AD among postmenopausal women.

METHODS

Postmenopausal women aged 50-79 (n = 63117) who participated in the U.S. Women's Health Initiative Observational Study (WHIOS), recruited in 1993-1998, without baseline AD and followed up through March 1, 2019, were analyzed. AD was classified by participant-reported history of doctor-diagnosis of incident AD in the WHIOS. DM was defined by participant-report or treated because of diabetes or serum glucose concentrations ≥ 126 mg/dL. CMS was defined as having ≥ 3 of five CMS components: large waist circumference, high blood pressure, elevated triglycerides, elevated glucose, and low high-density lipoprotein cholesterol. The associations of DM and CMS with AD were analyzed using Cox's proportional hazards regression analysis.

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RESULTS

During a median follow-up of 20 years (range: 3.36 to 23.36 years), of 63117 participants, 8340 developed incident AD. Women with DM had significantly higher incidence of AD [8.5, 95% confidence interval (CI): 8.0-9.0 per 1000 person-years (PY)] than those without DM (7.1, 95%CI: 6.9-7.2 per 1000 PY). Multivariate Cox's regression analysis indicated that women with DM or CMS had a significantly higher risk of AD than those without DM or CMS. The corresponding hazard ratios [HR (95%CI)] were 1.22 (1.13-1.31, $P < 0.001$) in subjects with DM, and 1.18 (1.09-1.27, $P < 0.001$) in subjects with CMS. The HRs diminished with age and became non-significant in the oldest age group.

CONCLUSION

During a median follow-up of 20 years, DM and CMS were significantly associated with the risk of AD among postmenopausal women. More specifically, women aged 50-69 with DM or CMS *vs* those without these conditions had significantly higher relative risks of AD than the relative risks of AD in those aged 70-79 with DM or CMS *vs* those without DM or CMS.

Key Words: Epidemiology; Diabetes mellitus; Cardiometabolic disorders; Alzheimer's disease; Aging population

Core Tip: Data from population-based studies on the association of diabetes and cardiometabolic syndrome (CMS) with risk of Alzheimer's disease (AD) was limited. This study, using data from one of the largest population-based cohort studies in the United States women aged 50-79 at baseline to test a hypothesis that diabetes and CMS are significantly associated with the risk of AD. This analysis is one of the first studies to prospectively test this hypothesis using a large-scale longitudinal cohort data. Findings from the study add new evidence to the body of research literature and provide new insights into the prevention of AD through control of diabetes and CMS.

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INTRODUCTION

Diabetes mellitus (DM) and Alzheimer's disease (AD, an irreversible, progressive brain disorder, which is the most common type of dementia among older adults) pose serious public health problems in the United States and worldwide. Today, about 34.1 million adults aged 18 years or older have DM (13.0% of United States adults). The prevalence rate of DM is 4.2% in United States adults aged 18-44 years old, 17.5% in adults aged 45-64, and 26.8% among the elderly aged 65 and older[1]. Similar to DM, increased incidence and prevalence of AD have been observed in the United States. An estimated 5.4 million Americans suffered from AD in 2016 and that number is likely to increase to 13.8 million by 2050[2-4]. AD affects one in nine people aged ≥ 65. The risk of AD is one out of three in ages ≥ 85. Meanwhile, about two thirds of patients diagnosed with AD are women, which is partly attributable to women having a longer life expectancy than men[5]. Furthermore, an estimated 121000 Americans died due to AD in 2017[3]. AD is the nation's sixth leading cause of death. In 2015, the direct cost of treating the disease totaled $236 billion, and economists estimate that the unpaid labor provided by caregivers to AD's patients amounted to another $220 billion in 2015[3,4]. AD mainly affects older people and it was traditionally viewed as a predominately inherited disease for many years. However, recent studies suggest that cardiovascular and metabolic disorders including hypertension, dysglycemia, central adiposity and dyslipidemia may play a pivotal role in the development of AD[6,7]. The cluster of these

risk factors is commonly called cardiometabolic syndrome (CMS or called metabolic syndrome)[8,9]. DM and CMS are the established risk factors for cardiovascular diseases (CVD)[10-14]. Consequently, whether AD or AD related dementia are similar to CVD as a brain complication of diabetes has been hypothesized and studied in the recent years[6,7,15,16]. Most of these results of the association of DM and CMS with risk of AD or AD related dementia were reported from hospital-based or small sample size of population-based observational studies[4,6,7,16-21]. Given the complex status of disease in participants in hospital-based studies and small sample sizes of the previous population-based studies, selection bias due to nonrepresentative participants and potential misclassification attributable to multiple comorbidities may occur using data from hospital-based studies and loss of statistical power in hypothesis testing from studies with small sample sizes. Furthermore, although two-thirds of patients with AD are women, only few studies were conducted in women. Studies of the associations between cardiometabolic disorders and AD are in their infancy compared to other areas of studies of diabetic complications. In the study, we hypothesize that women with DM or CMS have significantly higher risk of AD than those without DM or CMS. To test this hypothesis, we have two specific aims: (1) To examine the long-term effect of DM and CMS on the risk of incident AD, and (2) To examine whether there is a potential modifying effect of age on AD risk using data from a large-scale population-based observational cohort study among women aged 50 to 79 years.

MATERIALS AND METHODS

Study design and population

The study used data from the U.S. Women's Health Initiative Observational Study (WHIOS) with a prospective analysis design[22-24]. The WHIOS, launched in 1993 is a longitudinal population-based observational study. The study design and materials were approved by institutional review boards at each survey center. Details of the scientific rationale, eligibility requirements and survey designs have been published[25]. In brief, a total of 93676 postmenopausal women aged 50-79 were enrolled from October 1, 1993 to December 31, 1998 at 40 United States clinical centers nationwide and participated in the study to examine risk factors for cardiovascular disease, cancers, diabetes and osteoporotic fractures. The inclusion criteria of the participants were those who planned to reside in the survey area for at least 3 years and had medical conditions predictive of survival more than 3 years, and no complicating conditions such as alcoholism, drug dependency and no baseline AD and dementia. Participants received annual follow-ups *via* mailings sent from the Clinical Coordinating Center (CCC) and were invited to have Clinical Center visits at year 3 after enrollment to update selected baseline data, obtain additional risk factor data, and collect a blood specimen. The annual mailing follow-ups consisted of a cover letter, a self-administered medical history update (*i.e.*, health outcomes) and assessment of exposures to risk factors. Non-responders received two additional mailings and telephone contacts by clinical center staff. When these efforts were not successful, clinical center staff contacted proxies to determine the location and status of the participant and to collect information on health outcomes. The initial WHIOS ended in March 2005. Then it continued to follow up the study participants who stay in the survey cohort, that it is designed as the WHIOS extension studies, with its Extension 1 from 2005 to 2010, and Extension 2 from 2010 to 2020. In the Extension studies, participants' health outcomes are followed by mails using self-administrated survey questionnaires. The assessments of incident AD started from the WHIOS Extension 1 and continued to its Extension 2[23,24]. Starting from the Extension 1, there were 63231 participants who had the assessments of AD status using a self-administered survey instrument. In the analysis, we used the recently available ended-follow-up data through March 1, 2019. This study was reviewed and approved by Drexel University Institutional Review Board and data was obtained from the U.S. National Heart, Lung, and Blood Institute (NHLBI) Biologic Specimen and Data Repository. In the analysis, of 63231 participants, 36 with prevalent AD and 78 without information on DM or CMS were excluded. The final analysis sample size is 63117 in the report (listed in the rectangular box of Figure 1).

Measures of outcomes and comorbid conditions

In the WHIOS, AD and chronic conditions, including hypertension, hypercholesteremia, diabetes, heart attack, coronary heart disease, heart failure and AD were classified by self-administered standard survey instruments[23,24]. These chronic

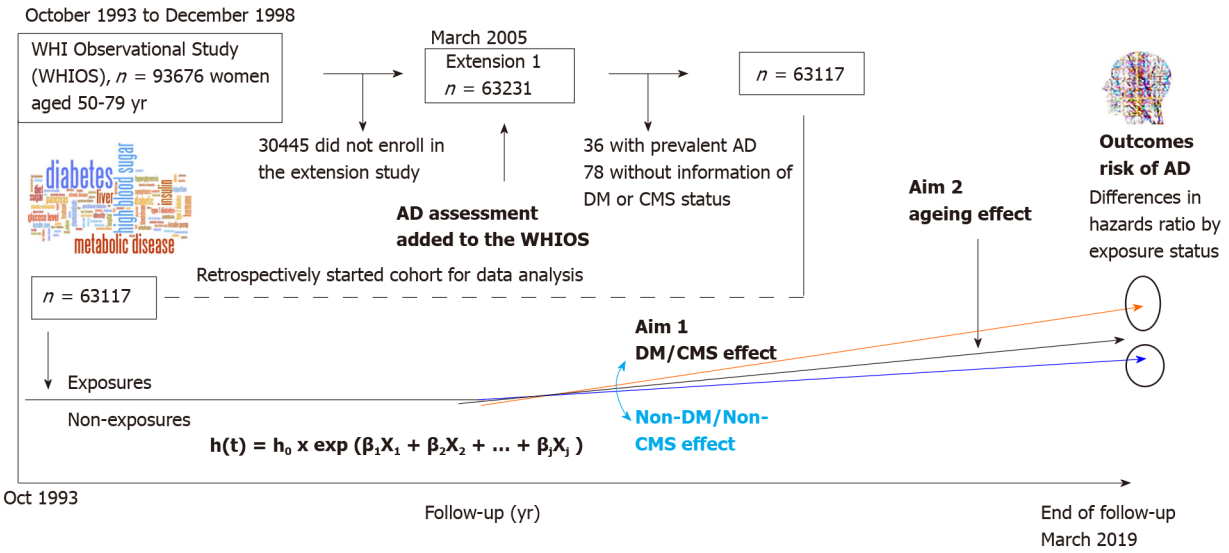


Figure 1 Study sample size and analysis framework. Cox proportional hazards regression model: h(t) is the hazard function determined by a set of Xj covariates.

conditions were determined by the WHIOS participants' self-reported answers to questions "have you been diagnosed or treated because of the disease?" An answer "yes" was classified as having the disease. The survey assessment of incident AD started from WHIOS Extension 1 (2005) and ongoing. Annual survey self-administered questionnaires were mailed to the WHIOS participants' households to collect major health outcomes data through participants self-report or a family member or those who knew the study participants' health status. The assessment of AD was classified by a question that asked, "has a doctor told you for the first time that you have moderate or severe memory problems, for example, dementia or Alzheimer's?" Of the total classified incident AD cases, 40% were reported by the WHIOS participants (*i.e.*, self-report), 13% by a family member or friend of the WHIOS study participants, 2% by a healthcare provider of the WHIOS study participants, and 45% by any others who knew the participants' AD status. Duration (days) of follow-up for each participant was calculated from the participant's enrollment in the survey to the date of their first physician-diagnosis of AD or to the date of the end of the follow-up for those who had no AD or for those who ended the survey earlier due to any other reasons, whatever which came first[26].

Predictors

Diabetes was determined by WHIOS participants' self-reported answer to, "have you been diagnosed or treated because of diabetes?" The answer "yes" was classified as having diabetes. In addition, subjects who had a blood sample with fasting glucose ≥ 126 mg/dL or those using glucose-lowering medication were also classified as DM. For CMS, there are several definitions[8,9,27,28]. We used the criteria proposed by the U.S. National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) with a minor modification by the American Heart Association (AHA), because this definition is most frequently used in the United States[28]. The AHA defines CMS as having ≥ 3 of the following five components: (1) Central adiposity (assessed by waist circumference > 88 cm in women); (2) Elevated fasting blood triglyceride (TG ≥ 150 mg/dL); (3) Reduced blood high-density lipoprotein cholesterol (HDL-C < 50 mg/dL in women); (4) Elevated blood pressure (systolic blood pressure ≥ 130 or diastolic blood pressure ≥ 85 mmHg) or those who are taking antihypertensive medications; and (5) Elevated blood fasting glucose (≥ 110 mg/dL) or those who had self-reports of physician-diagnosis of pre-diabetes or who have glucose-lowering medication use. In the WHIOS participants with AD status assessments, blood TG, HDL-C and glucose were tested in a subsample of 4443 participants. To estimate the total prevalent CMS, we applied the WHIOS participants' self-reports of physician-diagnosis of hypercholesterolemia as a surrogate for the estimate of elevated TG among those who had no blood sample measures of TG[29,30]. Meanwhile, the WHIOS participants' self-reported angina was applied to serve as a surrogate for the estimate of reduced HDL-C for those who had no blood sample measures of HDL-C because there is a well-

established association between reduced HDL-C and angina risk[29]. This method of using a surrogate to estimate CMS has been applied and accepted by several large-scale observational population-based studies[9,29,30].

Covariates

Covariates included participants' age (years), race/ethnicity (self-reported, White, African American, the other race/ethnicity group), current marital status (never or separated, widowed, or married), educational attainments (grouped as ≤ high school, > higher school or completed associate degree, and ≥ college), average annual family income (grouped as < 35000, 35000-49999, 50000-74999, and ≥ 75000 US $/year), health insurance status (including Medicare and pre-paid private health insurance), hormone therapy use (yes/no), smoking (defined as never, former (irrespective of the time since quitting) and current)), and self-reported physician-diagnosis of chronic conditions (myocardial infarction, stroke and peripheral artery disease).

Statistical analyses

To test the study hypothesis, we conducted a serial analysis. In the first analysis, baseline characteristics of participants were described by DM and CMS status. Differences in demographic characteristics, socioeconomic status, smoking, and medical history of chronic conditions by DM and CMS status were tested using Student *t*-tests for continuous variables, and Chi-square tests for categorical variables. In the second analysis, the incidence rates of AD per 1000 person-years of follow-up, and 95% confidence intervals (95%CI) of the incidence rates, were estimated using SAS Proc GEMOND. Differences in incident AD rates between participants with DM or with CMS and those without DM or without CMS were tested using Wald Chi-square tests[31,32]. In the third analysis, multivariate-adjusted Cox's proportional hazards (PH) regression was applied to estimate the associations of DM and CMS with the risk of AD. Cox's PH assumption were tested by using Kaplan-Meier curves [*i.e.*, assessed by the graph of the log(-log(survival) *vs* log of survival time)], and including time dependent covariates in the Cox models[33,34]. Hazard ratios (HRs) and their 95%CI of DM and CMS associated with the risk of incident AD were estimated in three multivariate-adjusted models for key covariates because these covariates are strongly correlated with both the study exposures and the outcomes. Model 1 adjusted for age, race/ethnicity, and marital status. Model 2 adjusted for covariates that were included in Model 1 plus education, family income, health insurance, smoking, and history of hormone therapy use. Model 3 adjusted for covariates that were included in Model 2 plus medical history of myocardial infarction, stroke, and peripheral arterial disease.

To test whether there are modifying effects of age differences on the associations of DM and CMS with risks of AD, stratification analyses were performed by three 10-year age groups (50-59, 60-69, and 70-79). Interaction effects between age stratum (the 3 10-year groups), DM and CMS (age × DM and age × CMS) on the risk of AD were examined based on a 1-degree-of-freedome test using maximum likelihood estimates using the total combined sample dataset. The analysis framework is shown in Figure 1.

Sensitivity analysis: We performed sensitivity analyses to take into consideration the accuracy of the assessment of AD by repeating the analysis separately for the data sources who completed the self-administered survey questionnaire: (1) For those who completed the survey questionnaire themselves, (2) For those with surveys completed by a family member or a friend of the WHIOS study participants, (3) For those with surveys completed by a health care provider for the WHIOS study participants, and (4) For those with surveys completed by others who knew the participant's health conditions. Of them, groups 2 and 3 had relatively small number of AD reports.

All statistical analyses were performed using SAS software version 9.4 (SAS Institute, Cary, NC 2018). A two-sided *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of participants

Of 63117 participants, Table 1 shows that participants with DM (n = 7255) had a significantly higher mean age than those without DM (n = 55862), (ages 63.2 *vs* 62.8 years old, P < 0.001). African Americans had the highest prevalence of DM (24.4%) followed by the "other" race/ethnicity group (16.5%) and White (10.3%) (P < 0.001). Married participants, those who had educational attainments of college or higher, or those who had the highest family average incomes had the lowest prevalence of DM

Table 1 Baseline characteristics of participants by diabetes mellitus status

	By DM status				
	Non-DM		DM		
	n	mean, %	*n*	mean, %	*P* value
Age, mean (SD), yr	55862	62.9 (7.2)	7255	63.2 (7.0)	< 0.001
Age group, No., %					< 0.001
50-59	19398	89.4	2295	10.6	
60-69	25068	87.9	3467	12.2	
70-79	11396	88.4	1493	11.6	
Race/ethnicity, No., %					< 0.001
White	49927	89.7	5745	10.3	
Africa American	2706	75.7	871	24.4	
Others	3229	83.5	639	16.5	
Marital status[1], No., %					< 0.001
Never or separated	10645	87.1	1579	12.9	
Widowed	8139	86.3	1296	13.7	
Presently married	36869	89.5	4341	10.5	
Education level[2], No., %					< 0.001
≤ High School	9484	84.7	1707	15.3	
> HS and Associate	19398	87.1	2873	12.9	
≥ College	26589	91.1	2608	8.9	
Annual family income (US$), No., %					< 0.001
< 35000	17099	84.8	3073	15.2	
35000-49999	10809	88.5	1401	11.5	
50000-74999	11685	89.9	1306	10.1	
≥ 75000	12902	92.4	1055	7.6	
Medicare, No., %					< 0.001
No	35403	88.9	4402	11.1	
Yes	20034	87.8	2787	12.2	
Pre-paid private insurance, No., %					0.13
No	32403	88.4	4269	11.6	
Yes	23034	88.7	2920	11.3	
Hormone usage status, No., %					< 0.001
Never used	20552	86.6	3180	13.4	
Past user	7901	87.7	1104	12.3	
Current user	27368	90.2	2963	9.8	
Smoking status, No., %					0.46
Never	28078	88.6	3609	11.4	
Past	24222	88.5	3140	11.5	
Current	2908	87.9	401	12.1	
Medical history, No., %					
Hypertension	20494	81.8	4566	18.2	< 0.001
Myocardial infarction	1746	78.9	467	21.1	< 0.001

Angina	1080	79.1	285	20.9	< 0.001
Stroke	1802	81.1	421	18.9	< 0.001
Peripheral artery disease	766	75.6	247	24.4	< 0.001

[1]Never/separated includes divorced; presently married includes marriage-like relationship.
[2]Associate: Included vocational or training school, some college or associate degree. DM: Diabetes mellitus; SD: Standard deviation.

compared to their counterparts (P < 0.001). Participants with Medicare (a federal health insurance program for adults aged 65 and older in the United States) had higher prevalence of DM than those without Medicare (12.2% *vs* 11.1%, P < 0.001). Women with current hormone therapy use had the lowest prevalence of DM compared to those who never used or used before. Participants with hypertension, myocardial infarction, angina, stroke or peripheral artery disease had significantly higher prevalence of DM than those without these conditions. Similar to DM, there were significant differences in mean age and the other factors between participants with CMS and those without CMS (Table 2).

Incidence of Alzheimer's disease

With a median follow-up of 20 years, of 63117 participants who had no AD at baseline, 8340 women reported that they had incident AD. The cumulative incidence rate (95%CI) of AD was 8.5 (8.0-9.0) per 1000 person-years in participants with DM and 7.1 (6.9-7.2) per 1000 person-years in those without DM (P < 0.001). Similar to DM, women with CMS had significantly higher incidence rates of AD (8.6 per 1000 person-years, 95%CI: 8.1-9.1) than those without CMS (7.0 per 1000 person-years, 95%CI: 6.9-7.2), P < 0.001.

The cumulative incidence rates (95%CI) of AD were 2.7 (2.5-2.9), 8.1 (7.8-8.3), and 14.5 (14.0-15.0) per 1000 person-years among women ages 50-59, 60-69, and 70-79 respectively (age differences in AD rates, P < 0.001).

Multivariate-adjusted Cox regression analyses

Figure 2 shows that DM was significantly associated with risk of AD. After adjusting for all the study covariates, Model 3 indicates that women with DM had 22% higher risk of AD than those without DM. The hazard ratio (95%CI) was 1.22 (1.13-1.31, P < 0.001). Women aged 50-59 at baseline had the largest hazard ratio (HR) of DM *vs* absence of DM for AD (HR = 1.52, 95%CI: 1.27-1.82, P < 0.001), followed by 1.26 (1.14-1.39, P < 0.001) and 1.04 (0.92-1.19, P = 0.53) in those aged 60-69 and 70-79. Women with CMS had 18% higher risk of AD than those without CMS, with corresponding HR (95%CI) of 1.18 (1.09-1.27, P < 0.001). Of five CMS components, Model 3 indicates that high BP (HR = 1.07, 95%CI: 1.02-1.13, P = 0.004), elevated TG (1.14, 1.07-1.21, P < 0.001), and elevated glucose concentrations (1.21, 1.13-1.30, P < 0.001) were significantly associated with increased risks of AD. Similar to DM, the relative risk of CMS *vs* absence of CMS for AD were greater in the younger age groups than that in the older. No significant association between CMS and AD risk was observed in the oldest age group (ages 70-79), with HR (95%CI) of 1.00 (0.87-1.14, P = 0.97). No significant interaction effects of age with DM, and age with CMS on the risk of AD were observed.

Figure 3 shows that overall increase in age was significantly associated with an increased incidence rate of AD in individuals with or without DM. Individuals with diabetes had significantly higher relative risk (*i.e.*, hazards) of AD in those aged 50-59 and 60-69 as compared to their corresponding counterparts without diabetes (Figure 3A). The incidence rates (95%CI) of AD for DM *vs* non-DM individuals were 4.3 (3.7-4.8) *vs* 2.5 (2.4-2.8) per 1000 person-years, P < 0.001 in those aged 50-59, and 9.3 (8.6-10.1) *vs* 7.9 (7.7-8.2) per 1000 person-years, P < 0.001 in those aged 60-69. This difference in AD by DM status was not significant among those aged 70-79 [14.4 (12.9-16.0) *vs* 14.5 (13.9-15.0) per 1000 person-years, P = 0.88, Figure 3B)]. Similar to DM, the relative risks of those with CMS *vs* non-CMS for incident AD were higher among the younger age groups (data not shown).

Sensitivity analysis

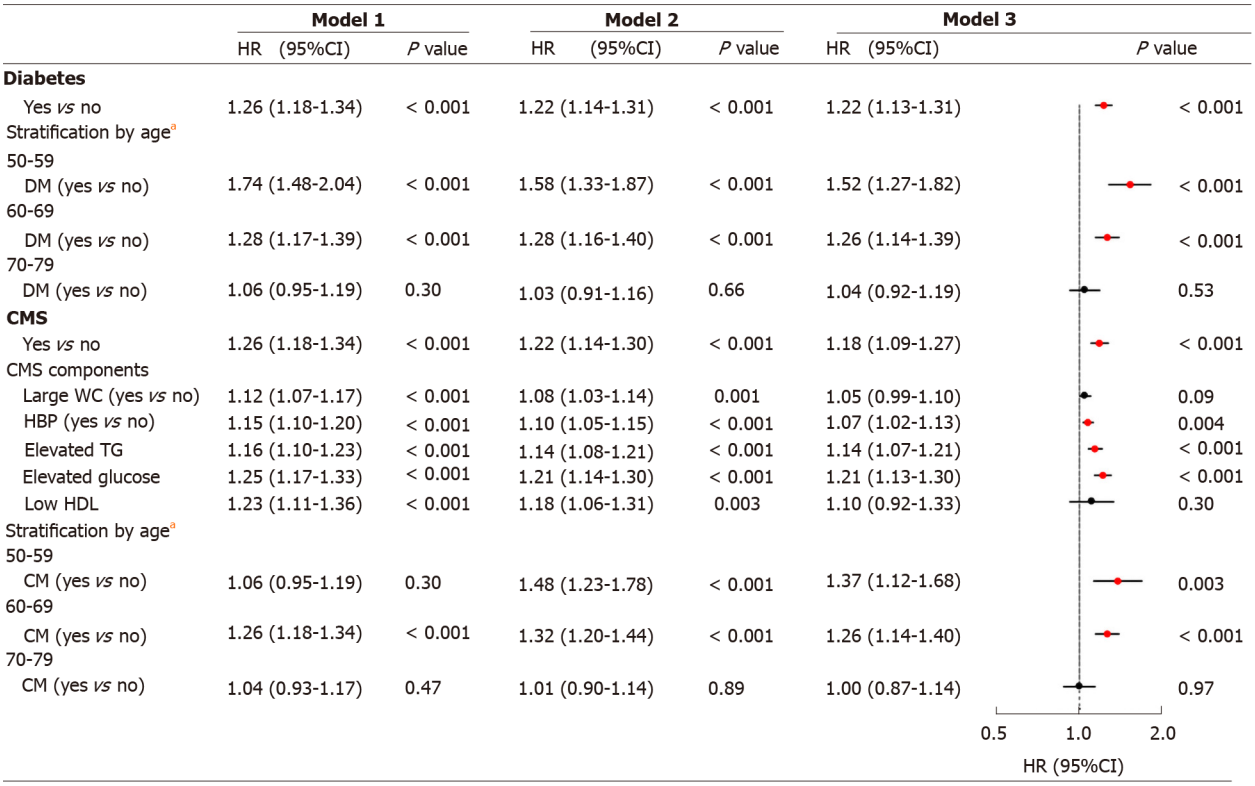
Supplementary Table 1 shows that the HRs (95%CI) remained statistically significant for those who completed the survey instrument on AD conditions by themselves (HR = 1.33, 95%CI: 1.19-1.49, P < 0.001, Model 3) and those whose AD status was reported

Table 2 Baseline characteristics of participants by cardiometabolic syndrome status

	By CMS status				
	Non-CMS		CMS		
	n	mean, %	*n*	mean, %	*P* value
Age, mean (SD), yr	55485	62.8 (7.2)	7632	63.7 (6.9)	< 0.001
Age group, No., %					< 0.001
50-59	19522	90.0	2171	10.0	
60-69	24771	86.8	3764	13.2	
70-79	11192	86.8	1697	13.2	
Race/ethnicity, No., %					< 0.001
White	50415	90.6	5257	9.4	
Africa American	1996	55.8	1581	44.2	
Others	3074	79.5	794	20.5	
Marital status[1], No., %					< 0.001
Never or separated	10472	85.7	1752	14.3	
Widowed	7946	84.2	1489	15.8	
Presently married	36852	89.4	4358	10.6	
Education level[2], No., %					< 0.001
≤ High School	9207	82.3	1984	17.7	
> HS and Associate	19253	86.4	3018	13.6	
≥ College	26641	91.2	2556	8.8	
Annual family income (US$), No., %					< 0.001
< 35000	16660	82.6	3512	17.4	
35000-49999	10710	87.7	1500	12.3	
50000-74999	11734	90.3	1257	9.7	
≥ 75000	13056	93.5	901	6.5	
Medicare, No., %					< 0.001
No	35329	88.8	4476	11.2	
Yes	19741	86.5	3080	13.5	
Pre-paid private insurance, No., %					0.084
No	32178	87.7	4494	12.3	
Yes	22892	88.2	3062	11.8	
Hormone usage status, No., %					< 0.001
Never used	20136	84.8	3596	15.2	
Past user	7802	86.6	1203	13.4	
Current user	27502	90.7	2829	9.3	
Smoking status, No., %					0.027
Never	27885	88.0	3802	12.0	
Past	24092	88.0	3270	12.0	
Current	2861	86.5	448	13.5	
Medical history, No., %					
Diabetes	2740	37.8	4515	62.2	< 0.001
Hypertension	18928	75.5	6142	24.5	< 0.001

Myocardial infarction	1695	76.6	518	23.4	< 0.001
Angina	629	46.1	736	53.9	< 0.001
Stroke	1758	79.1	465	20.9	< 0.001
Peripheral artery disease	721	71.2	292	28.8	< 0.001

[1]Never/separated includes divorced; presently married includes marriage-like relationship.
[2]Associate: included vocational or training school, some college or associate degree. CMS: Cardiometabolic syndrome; SD: Standard deviation.



Model 1: Adjusted for age, race/ethnicity and marital status.
Model 2: Adjusted for Model 1 plus Medicare, pre-paid private insurance, education, income, smoking status, and hormone therapy use.
Model 3: Adjusted for Model 2 plus medical history of myocardial infarction, stroke and peripheral artery disease.
[a]: Age was not adjusted in the age stratification analysis.

Figure 2 Hazard ratios (95% confidence interval) of diabetes, cardiometabolic syndrome and individual cardiometabolic syndrome components associated with incident Alzheimer's disease. HR: Hazard ratio; CI: Confidence interval; CMS: Cardiometabolic syndrome; DM: Diabetes mellitus; TG: Triglyceride; HDL: High-density lipoprotein.

by others (1.14, 1.02-1.28, P = 0.022). Similar to DM, subjects with CMS had a significantly increased risk of AD among these two subgroups (Model 3). The corresponding HRs (95%CI) are 1.19 (1.05-1.34, P = 0.006) and 1.19 (1.07-1.34, P = 0.002). However, the associations were statistically non-significant among the other two subgroups who completed the survey questionnaire on behalf of the study participants. The corresponding HRs (95%CI) of DM associated with AD are 1.14 (0.93-1.39, P = 0.20) and 1.57 (0.96-2.56, P = 0.07) among those whose survey questionnaires were answered by a family member or friend, or a healthcare provider. The corresponding HRs (95%CI) of CMS associated with AD are 1.09 (0.89-1.33, P = 0.41) and 1.36 (0.82-2.27, P = 0.24) in the two subgroups. It should be noted that restricting to these subgroups greatly decreased the available sample size for these analyses. The resulting estimated HRs had wider 95%CI's, suggesting that further studies with increased sample sizes are needed (Supplementary Table 1).

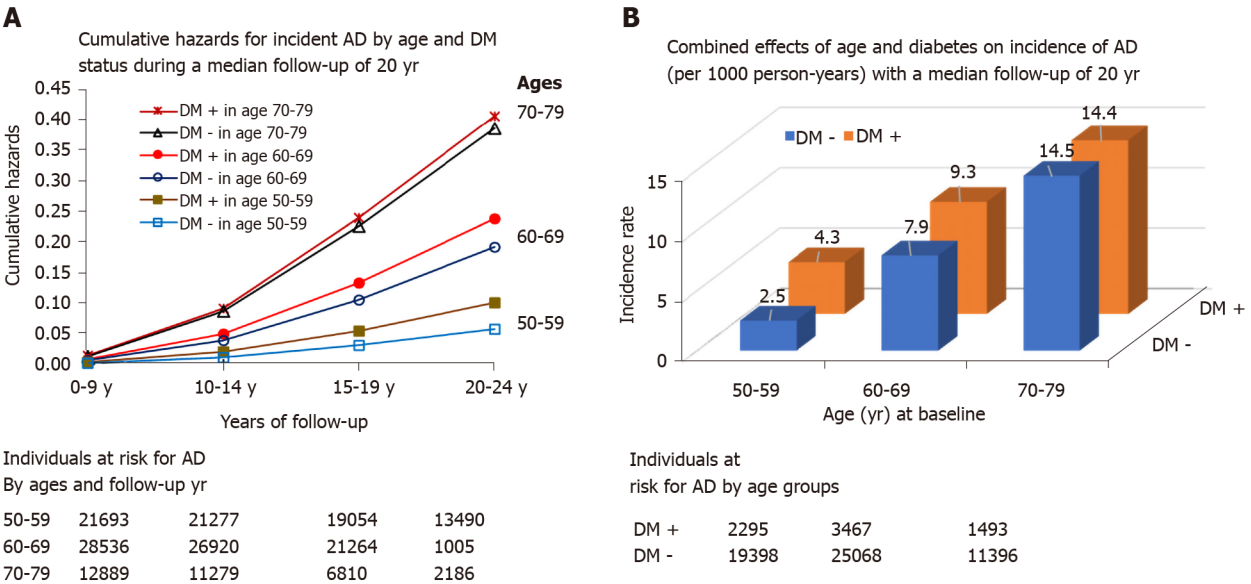


Figure 3 Increase in age was significantly associated with the risk of Alzheimer's disease in subjects with or without diabetes mellitus. A and B: Cumulative hazards for incident Alzheimer's disease by age and DM status during the follow-up (A) and combined effects of age and diabetes on incidence rates of Alzheimer's disease (B).

DISCUSSION

To the best of our knowledge, this study is one of the first reports on the association of DM and CMS with AD risk among postmenopausal women. The main findings indicate that with a median follow-up of 20-years, postmenopausal women aged 50-79 with DM or CMS had an overall significantly higher incidence rate of AD than their corresponding counterparts in the total study participants. Although the absolute risk of AD increased with age, the relative risks of AD with DM or CMS among younger women aged 50-69 with DM or CMS *vs* those without DM or CMS were much stronger than the relative risk of AD among older women aged 70-79.

AD is an irreversible and progressive brain disorder that slowly destroys memory and thinking skills, and eventually the ability to carry out the simplest tasks. Therefore, early prevention of this disease becomes important if any preventable risk factors prior to the development of AD can be identified. In recent decades a rapidly increasing number of studies have examined the association of diabetes, obesity, hypertension, dyslipidemia and dysglycemia with risk of cognitive impairment and AD[20]. For example, the Rotterdam study observed a significant association of diabetes *vs* non-DM with AD risk (relative risk, RR = 1.9, 95%CI: 1.2-3.1) in a prospective population-based cohort study of 6,370 adults aged 55 and older during an average of 2.1 years of follow-up[16,35]. Findings from the Rochester study, a prospective cohort study of 1455 subjects aged 20 and older, indicated a significant association between adult onset diabetes and risk of AD in men (relative risk: 2.27, 95%CI: 1.55-3.31), and a positive but nonsignificant association among women (RR = 1.37, 95%CI: 0.94-2.01)[36]. Findings from the Canadian Study of Health and Aging (CSHA) in participants (n = 5574) aged 65 and over in a 5-year follow-up indicate that the relative risk of DM *vs* non-DM for incident AD was 1.30 (0.83-2.03) in their combined sample of both genders (results are not presented by gender in their report)[16]. Our results are in general consistent with these previous reports, with a 22% higher risk of AD in those with DM *vs* those without DM in women aged 50 and older (HR = 1.22, 95%: 1.13-1.31, P < 0.001). Our study extends the previous studies by using a larger sample size and addressing AD risk by age groups. Of individual CMS components, findings from Nägga *et al*[21] study indicate a significant association between increased midlife triglycerides and risk of beta-amyloid (Aβ) and tau pathology. Several review articles have also examined the associations of CMS and diabetes with the development of AD[20,37,38]. However, studies on the development of AD were limited. Data from the WHIOS fills the gap to assess the metabolic disorders and risk of AD among postmenopausal women. Findings of this study contribute evidence to the body of literature by demonstrating a significant association of DM and CMS with AD. Given the potentially preventable risk of DM and CMS, we expect that the risk of AD can be reduced if we focus on the control and prevention of DM and CMS as early as

possible.

Although the mechanisms by which DM or CMS play a role in the development of AD remain poorly understood, some possible pathways have been proposed. Of these, insulin resistance, an impaired response of the body to insulin resulting in elevated levels of glucose in the blood, has been proposed to be an important cause of DM and its relationship to the development of AD, because severe neuropathological changes are common to diabetes and AD. For example, hyperglycemia in patients with DM can result in glycosylation of various receptors, leading to the formation of "receptors for advanced glycation end products" (RAGE). An affinity between RAGE and beta-amyloid (Aβ) peptides can trigger and propagate chronic brain inflammation[39]. Dyslipidemia and hypertension are also proposed as important risk factors associated with risk of AD. In a longitudinal study of 318 elderly individuals with normal cognition, the investigators found that higher fasting triglyceride levels in midlife were associated with increased risk of brain Aβ and tau pathology 20 years later. This association was independent of age, sex, APOE ε4, and vascular risk factors[21]. The potential pathophysiologic effect of triglycerides on Aβ pathology remains under study. In transgenic AD mouse models blood triglyceride concentrations have been shown to increase prior to Aβ deposition, indicating a direct association between triglycerides and Aβ homeostasis[40]. Lipids may influence membrane fluidity, which could directly affect secretase-mediated Aβ[41]. In a meta-analysis of 18 prospective studies examining the relationship of total cholesterol with risk for AD and vascular dementia, midlife total cholesterol levels were consistently associated with an increased risk of AD and all dementia, whereas no increased risk was observed for late-life total cholesterol[42]. In our study, significant associations of central adiposity (assessed by large waist circumference) and low HDL-C with AD were observed after adjustment for age, race/ethnicity, marital status, insurance, education, family income, smoking and hormone therapy use (Models 1 and 2 of Figure 2). The associations of large WC and low HDL-C with risk of AD became nonsignificant after a further adjustment by including myocardial infarction (MI), stroke and peripheral arterial disease (PAD) (Model 3 of Figure 2). These attenuated associations in model 3 are possibly attributable to an over-adjustment because these cardiovascular conditions are likely on the pathway of the development of AD (large WC and dyslipidemia—cardiovascular diseases—AD). Several studies have indicated that MI, stroke, and PAD predict the risk of cognitive impairment and AD[43-46]. The association between hypertension and AD is complex and may involve direct and indirect effects with other risk factors, such as the fact that hypertension increases risk of arterial stiffness, Aβ accumulation, and has interaction effect with insulin resistance[42,47,48]. Increased ages are significantly associated with increased risks of AD. However, it should be noted that in our study the relative risks (*i.e.*, HRs) of AD in younger women aged 50-69 with DM or CMS *vs* those without DM or CMS were higher compared to the relative risks of AD in older adults aged 70-79 without DM or CMS *vs* those without DM or CMS. The relative risks of DM and CMS *vs* those without DM or CMS for AD became nonsignificant in the older age group. Similar nonsignificant associations between those with and those without DM among the elderly were reported from previous studies as well[21,49]. The potential mechanisms are unclear. One of the explanations is that this nonsignificance in the older age group is possibly attributable to survivor bias. Individuals aged 70-79 may have better healthy conditions than those who died before their ages 70-79. The relative effect of DM or CMS on AD risk may become weaker in the older age group. Meanwhile, individuals aged 70-79 have a higher proportion of comorbidities, such as stroke and hypertension than the youngers. Other, currently-unknown, age-related causes of AD may become more important in older age groups as well. Therefore, DM may have a relatively smaller impact on AD risk in the older patients. Further studies among the older age groups are warranted.

That there are several limitations when interpreting the results. First, the WHIOS included only women who were postmenopausal, which limits the generalizability to women earlier in their life cycle. Second, the classification of AD was based on a self-administered survey questionnaire. We had no access to more definitive measures such as imaging of brain structures (*i.e.*, magnetic resonance imaging and computerized tomography) or measures of the burden of amyloid deposits, and of neurofibrillary tangles in brain (such as Amyloid PET and Tau Pet imaging). Although it is common to define a disease status using a pre-designed self-administered questionnaire in most large-scale observational epidemiological studies, possible information bias may have occurred, which may lead to under- or over-estimate of the incidence of AD. In addition, based on the survey instrument on the question on AD, we thought that the classification of AD in the analysis might have included these with

AD or AD related dementia. In our sensitivity analysis, we tested DM-AD and CMS-AD associations separately for subgroups based on who completed the self-administered questionnaire. It shows that AD reported by a family member or friend or a healthcare provider of the study participant had nonsignificant associations with DM and CMS. However, this subsample analysis is questionable by their much smaller sample sizes (AD cases) reported by these two groups, which is also demonstrated by their wide 95% confidence intervals. Certainly, given the limitation that using a self-administered instrument is subject to bias, findings from this study call for further studies with detailed clinical measures and evaluations. Third, in the analysis, CMS was included for the purpose of examining the associations of CMS and its components with risk of AD. We had to estimate missing values of TG and HDL using their corresponding surrogates. These estimations may lead to under- or overestimate of their true values, which would introduce bias. Nevertheless, as consistent findings of CMS and DM were observed in the study and compared with the others, we would say that the bias due to the estimated approach is minimized. Finally, residual confounding cannot be completely eliminated from epidemiological studies, which could be attributable to the factors that the survey did not include.

Our study also has several strengths. First, the WHIOS is one of the largest observational prospective study among postmenopausal women. With an increase in life expectancy in the nation and worldwide, findings from the study add important evidence to the research literature. Second, of all reported studies related to DM and AD risk, the WHIOS has the longest duration of follow-up, which offers an unique opportunity to test the long-term effect of DM and CMS on the risk of AD. Third, a significantly higher relative risk of AD in younger women with DM or CMS *vs* those without DM or CMS than that in the older age group not only calls for further etiological studies, but also adds evidence to the prevention of AD risk in younger adults with DM or CMS.

CONCLUSION

During a median follow-up of 20 years, DM and CMS were significantly associated with the risk of AD among postmenopausal women. More specifically, women aged 50-69 with DM or CMS *vs* those without these conditions had significantly higher relative risks of AD than the relative risks of AD in those aged 70-79 with DM or CMS *vs* those without DM or CMS.

ARTICLE HIGHLIGHTS

Research background

In spite of an increase in the incidence and prevalence of diabetes mellitus (DM) and Alzheimer's disease (AD) in the aging population, limited attention has been paid to investigate their associations.

Research motivation

To investigate the association of DM and cardiometabolic syndrome with risk of AD among the United States older adults.

Research objectives

To examine the association of DM and cardiometabolic syndrome (CMS, a precursor to DM) with risk of incident AD among postmenopausal women.

Research methods

Postmenopausal women aged 50-79 (n = 63117) who participated in the U.S. Women's Health Initiative Observational Study (WHIOS), recruited in 1993-1998 without baseline AD and followed up through March 1, 2019 were analyzed. AD was classified by participants-reported history of doctor-diagnosis of first-listed AD. DM was defined by participant-report or serum glucose concentrations or those anti-diabetic medication use. CMS was defined as having ≥ 3 of five CMS components: large waist circumference, high blood pressure, elevated triglycerides, elevated glucose, and low high-density lipoprotein cholesterol. The associations of DM and CMS with AD were analyzed using Cox's proportional hazards regression analysis.

Research results

Within a median follow-up of 20 years (range: 3.36 to 23.36 years), of 63117 participants, 8340 had incident AD. Women with DM had significantly higher incidence of AD [8.5, 95% confidence interval (CI): 8.0-9.0 per 1000 person-years (PY)] than those without DM (7.1, 95%CI: 6.9-7.2 per 1000 PY). Multivariate Cox's regression analysis indicates that women with DM or CMS had significantly higher risk of AD than those without DM or CMS. The corresponding hazard ratios [HR (95%CI)] were 1.22 (1.13-1.31, $P < 0.001$) in subjects with DM, and 1.18 (1.09-1.27, $P < 0.001$) in subjects with CMS. The HRs of AD in those with DM or MS *vs* those without DM or CMS diminished with age and became non-significant in the oldest age group.

Research conclusions

Diabetes and cardiometabolic syndrome were significantly associated with risk of Alzheimer's disease.

Research perspectives

Further studies are needed to investigate the mechanisms by which DM and CMS may cause the development of AD.

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META-ANALYSIS

Novel glucose-lowering drugs for non-alcoholic fatty liver disease

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Abstract

BACKGROUND

The efficacy of novel glucose-lowering drugs in treating non-alcoholic fatty liver disease (NAFLD) is unknown.

AIM

To evaluate the efficacy of glucose-lowering drugs dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 receptor agonists (GLP-1 RAs), and sodium-glucose cotransporter 2 (SGLT2) inhibitors in treating NAFLD and to perform a comparison between these treatments.

METHODS

Electronic databases were systematically searched. The inclusion criteria were: Randomized controlled trials comparing DPP-4 inhibitors, GLP-1 RAs, or SGLT2 inhibitors against placebo or other active glucose-lowering drugs in NAFLD patients, with outcomes of changes in liver enzyme [alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST)] from baseline.

RESULTS

Nineteen studies were finally included in this meta-analysis. Compared with placebo or other active glucose-lowering drug treatment, treatment with DPP-4 inhibitors, GLP-1 RAs, and SGLT2 inhibitors all led to a significant decrease in ALT change and AST change from baseline. The difference between the DPP-4 inhibitor and SGLT2 inhibitor groups in ALT change was significant in favor of

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DPP-4 inhibitor treatment ($P < 0.05$). The trends of reduction in magnetic resonance imaging proton density fat fraction and visceral fat area changes were also observed in all the novel glucose-lowering agent treatment groups.

CONCLUSION
Treatment with DPP-4 inhibitors, GLP-1 RAs, and SGLT2 inhibitors resulted in improvements in serum ALT and AST levels and body fat composition, indicating a beneficial effect in improving liver injury and reducing liver fat in NAFLD patients.

Key Words: Non-alcoholic fatty liver disease; Glucose-lowering drug; Meta-analysis; Dipeptidyl peptidase-4 inhibitor; Sodium-glucose cotransporter 2 inhibitor; Glucagon-like peptide-1 receptor agonist

Core Tip: The efficacy of novel glucose-lowering drugs in treating non-alcoholic fatty liver disease is unknown. The results of this meta-analysis showed that treatment with dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor agonists, and sodium-glucose cotransporter 2 inhibitors resulted in improvements in serum alanine aminotransferase and aspartate aminotransferase levels, indicating a beneficial effect in the improvement of liver injury.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of liver disease worldwide. The prevalence of NAFLD has increased rapidly over the past two decades. Approximately 24% of the adult population are reported suffering from NAFLD worldwide[1]. NAFLD is characterized by the presence of > 5% hepatic fat accumulation without alcohol abuse, virus infection, autoimmunity, or other secondary causes of liver disease[2], leading to inflammation and subsequent disruption of hepatic function[3]. There is growing evidence that NAFLD is not limited to liver-related morbidity and mortality, but also known to be a multisystem disease. NAFLD increases the risk of type 2 diabetes mellitus (T2DM), chronic kidney disease, heart failure, cancer, and cardiovascular disease[4].

Nowadays, the effective treatment for NAFLD is limited. Lifestyle modification is the standard management, including calorie restriction, body weight control, and adequate physical activity[5]. Based on the common pathophysiological pathways shared by T2DM and NAFLD including insulin resistance, lipotoxicity, inflammation, and oxidative stress[6], pharmacotherapies treating T2DM might be effective for NAFLD.

Incretin-based therapies and sodium-glucose cotransporter 2 (SGLT2) inhibitors are novel classes of glucose-lowering drugs used in the management of T2DM. Dipeptidyl peptidase-4 (DPP-4) inhibitors prevent the degradation of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide, while GLP-1 receptor agonists (GLP-1 RAs) directly mimic the action of GLP-1[7-9]. By improving insulin resistance and inflammation, and decreasing dietary fat, incretin-based therapies might offer potential benefits in treating NAFLD[10]. SGLT2 inhibitors are a class of novel glucose-lowering drugs by stimulating glucose excretion in the urine. SGLT2 inhibitors not only improve hyperglycemia, but also obesity, oxidative stress, and inflammation[11-13].

Although accumulated evidence suggests that these novel glucose-lowering drugs are promising in the treatment of NAFLD and nonalcoholic steatohepatitis (NASH)[14-16], there is conflicting evidence and no convincing consensus on their long-

term efficacy and outcome. In addition, there have been few head-to-head clinical trials comparing these novel glucose-lowering drugs directly. In this context, we carried out this meta-analysis to further evaluate the efficacy of novel glucose-lowering drugs in treating NAFLD and to perform a comparison between these treatments.

MATERIALS AND METHODS

Data sources and searches

The inclusion criteria for this meta-analysis were: (1) Randomized controlled trials conducted in patients with NAFLD; (2) Studies comparing DPP-4 inhibitors, GLP-1 RAs, or SGLT2 inhibitors against placebo or other active glucose-lowering drugs; and (3) The primary outcome was the change of liver enzyme [alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST)] from baseline. The secondary outcomes were the changes of magnetic resonance imaging proton density fat fraction (MRI-PDFF), visceral fat area (VFA), body weight, body mass index (BMI), and/or glycated hemoglobin A1c (HbA1c) from baseline. Case reports, animal studies, and case-control studies were excluded. The study selection followed the Preferred Reporting Items for systematic reviews and meta-analyses flow diagram[17].

Study selection

Two investigators (ZDF and XLC) independently conducted systematic searches of the databases MEDLINE, Cochrane central register of controlled trials, and Embase for studies up to May 30, 2020 according to the Cochrane handbook for systematic reviews for meta-analysis. The following terms were searched: Dipeptidyl peptidase 4 inhibitor, DPP-4 inhibitor, DPP-IV inhibitor, sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin, dutogliptin, teneligliptin, anagliptin, evogliptin, retagliptin, omarigliptin, glucagon-like peptide-1 receptor agonist, GLP-1 receptor agonist, GLP-1 RA, GLP1RA, liraglutide, semaglutide, albiglutide, lixisenatide, taspoglutide, exenatide, dulaglutide, sodium-glucose cotransporter 2 inhibitor, SGLT2 inhibitor, SGLT-2 inhibitor, empagliflozin, dapagliflozin, canagliflozin, luseogliflozin, ipragliflozin, tofogliflozin, sotagliflozin, ertugliflozin, nonalcoholic fatty liver disease, NAFLD, fatty liver, NASH, nonalcoholic steatohepatitides, NASH, NAFLD, non-alcoholic steatohepatitis, and non-alcoholic steatohepatitides. To identify additional relevant data of eligible published trials, we also searched ClinicalTrial.gov.

Data extraction and quality assessment

Two independent reviewers (ZDF and XLC) extracted data from each publication, including sources of publication, title, year, first author, study design, baseline characteristics of study population (sample size, age, assessment of diagnosis, body weight, BMI, and HbA1c), treatment allocation, duration of treatment, and changes of ALT, AST, MRI-PDFF, VFA, body weight, BMI, and HbA1c from baseline. If the above relevant data was not reported in the published trial, the registry report of ClinicalTrials.gov was further extracted.

The quality of all included studies was assessed using the Cochrane risk of bias tool. The following aspects were included: Selection bias, performance bias, detection bias, attrition bias, reporting bias, and others.

Statistical analysis

The continuous outcomes in this meta-analysis were evaluated by computing the weighted mean differences (WMDs) and 95% confidence intervals (CIs). The I^2 statistic was analyzed to assess the heterogeneity. If $I^2 > 50\%$ and the P value of the $\chi^2 < 0.10$, representing statistical heterogeneity across studies, the random-effects model was used. The fixed-effects model was used with low levels of heterogeneity. Publication bias was assessed *via* visual inspection of funnel plot. All analyses were conducted with Review Manager, version 5.3 (Nordic Cochrane Centre, Copenhagen, Denmark). Meta-regression analyses were conducted with STATA statistical software package (Version 13.1, Stata Corp, College Station, TX, United States). A P value < 0.05 was considered statistically significant.

RESULTS

Characteristics of enrolled studies

After literature search, 19 studies were included in the meta-analysis (Table 1). Among these 19 studies, 7 compared DPP-4 inhibitors with placebo or other active glucose-lowering drugs[18-24], 8 compared GLP-1 RAs with placebo or other active glucose-lowering drugs[24-31], and 5 compared SGLT2 inhibitors with placebo or other active glucose-lowering drugs[32-36]. The process of study selection is presented in Figure 1. The risks of bias of the included studies are presented in Supplementary Figure 1.

Changes in ALT and AST

Overall, compared with placebo or other active glucose-lowering drug treatment, DPP-4 inhibitor treatment led to a significant decrease in ALT (WMD = -9.48 IU/L, 95%CI: -15.61 to -3.36 IU/L, $P < 0.01$) and AST from baseline (WMD = -8.36 IU/L, 95%CI: -14.86 to -1.85 IU/L, $P = 0.01$) in NAFLD patients (Figure 2A and B). Compared with placebo or other active glucose-lowering drug treatment, GLP-1 RA treatment led to a significant decrease in ALT (WMD = -8.73 IU/L, 95%CI: -13.29 to -4.18 IU/L, $P < 0.01$) and AST from baseline (WMD = -2.65 IU/L, 95%CI: -5.14 to -0.16 IU/L, $P = 0.04$) (Figure 2C and D). Compared with placebo or other active glucose-lowering drug treatment, SGLT2 inhibitor treatment also led to a significant decrease in ALT (WMD = -3.44 IU/L, 95%CI: -5.08 to -1.81 IU/L, $P < 0.01$) and AST from baseline (WMD = -1.47 IU/L, 95%CI: -2.65 to -0.30 IU/L, $P = 0.01$) (Figure 2E and F).

When DPP-4 inhibitor treatment was compared with SGLT2 inhibitor treatment, the difference between groups in AST change was significant in favor of DPP-4 inhibitors treatment ($P = 0.04$). When GLP-1 RA treatment was compared with SGLT2 inhibitor treatment, the difference between groups in ALT change and AST change was not significant ($P > 0.05$). While comparisons between DPP-4 inhibitor treatment and GLP-1 RA treatment also indicated no significant difference between groups in ALT or AST change relative to baseline ($P > 0.05$).

Changes in body weight and BMI

The absolute body weight change relative to baseline after DPP-4 inhibitor treatment was -1.20 kg (95%CI: -1.44 to -0.95 kg, $P < 0.01$) when compared with placebo or other active glucose-lowering drug treatment, while the absolute BMI change relative to baseline showed no significant difference. When GLP-1 RA treatment was compared with placebo or other active glucose-lowering drug treatment, the difference between groups in body weight and BMI were both significant (WMD = -3.61 kg, 95%CI: -5.74 to -1.48 kg, $P < 0.01$; WMD = -1.46 kg/m^2, 95%CI: -2.23 to -0.69 kg/m^2, $P < 0.01$, respectively). When SGLT2 inhibitor treatment was compared with placebo or other active glucose-lowering drug treatment, the difference between groups in body weight and BMI were both significant (WMD = -2.53 kg, 95%CI: -3.35 to -1.71 kg, $P < 0.01$; WMD = -1.72 kg/m^2, 95%CI: -2.68 to -0.75 kg/m^2, P < 0.01, respectively) (Table 2).

The body weight change from baseline and BMI change from baseline were significantly greater with GLP-1 RA treatment and SGLT2 inhibitor treatment when compared with DPP-4 inhibitor treatment ($P < 0.05$). No significant difference was observed in the change of body weight or BMI from baseline between the GLP-1 RA treatment and SGLT2 inhibitor treatment groups ($P > 0.05$).

Change in HbA1c

The majority of the included studies evaluated the change in HbA1c from baseline. The results showed that DPP-4 inhibitor treatment, compared with placebo or other active glucose-lowering drug treatment, had a significant reduction in HbA1c level from baseline (WMD = -0.425%, 95%CI: -0.58% to -0.25%, $P < 0.01$). While GLP-1 RA and SGLT2 inhibitor treatment showed no statistical difference in HbA1c reductions (WMD = -0.29, 95%CI: -0.85% to 0.26%, $P < 0.30$; WMD = -0.36, 95%CI: -0.80% to 0.08%, $P = 0.11$, respectively) (Table 3). Subgroup analyses only comprising studies that evaluated the studied drugs against placebo showed that HbA1c levels decreased significantly in the DPP-4 inhibitor, GLP-1 RA, and SGLT2 inhibitor treatment groups (Table 3). The difference of HbA1c change from baseline between all these three treatment groups was not significant ($P > 0.05$).

Changes in MRI-PDFF and VFA

Two studies provided information on MRI-PDFF change in the comparison between the DPP-4 inhibitor and control groups. The results showed no significant reduction in MRI-PDFF, with a WMD of -2.31% (95%CI: -4.92% to 0.29%, $P = 0.08$). The only one

Table 1 Summary of included studies

Ref.	Study duration	Population	Assessment of NAFLD	Intervention	No. of participants
Alam *et al*[18], 2018	52 wk	NAFLD	Biopsy	Sitagliptin 100 mg QD + lifestyle modification	20
				Lifestyle modification	20
Cui *et al*[19], 2017	24 wk	NAFLD with prediabetes or early diabetes	MRI	Sitagliptin 100 mg QD	25
				PBO	25
Hussain *et al*[20], 2016	12 wk	NAFLD + dyslipidemia	Ultrasound	Vildagliptin 50 mg Bid	29
				PBO	29
Macauley *et al*[21], 2015	6 mo	T2DM with hepatic steatosis	Imaging (CT or ultrasound)	Vildagliptin 50 mg Bid	22
				PBO	22
Joy *et al*[22], 2017	24 wk	NASH	Biopsy	Sitagliptin 100 mg QD	6
				PBO	6
Li *et al*[23], 2019	24 wk	T2DM with NAFLD	Imaging or biopsy	Saxagliptin 5 mg QD	31
				Glimepiride 2 mg QD	33
				Glimepiride 2 mg QD + polyenephosphatidylcholine 456 mg TID	31
Yan *et al*[24], 2019	26 wk	T2DM with NAFLD	MRI	Liraglutide 1.8 mg + metformin	24
				Sitagliptin + metformin	27
				Glargine 0.2 IU/Kg/d + metformin	24
Armstrong *et al*[25], 2016	48 wk	T2DM with NASH	Biopsy	Liraglutide 1.8 mg QD	26
				PBO	26
Cusi *et al*[26], 2018	6 mo	T2DM with NAFLD/NASH	Laboratory liver tests	Duraglutide 1.5 mg QW	971
				PBO	528
Fan *et al*[27], 2013	12 wk	T2DM with NAFLD	Ultrasound	Exenatide	49
				Metformin	68
Feng *et al*[28], 2017	24 wk	T2DM with NAFLD	Ultrasound	Metformin 1000 mg Bid	29
				Gliclazide 120 mg/d	29
				Liraglutide 1.8 mg QD	29
Khoo *et al*[29], 2017	26 wk	NAFLD	MRI	Liraglutide 3.0 mg QD	12
				Diet and exercise	12
Shao *et al*[30], 2014	12 wk	T2DM with NAFLD	Ultrasound	Exenatide 10 μg bid + glargine	30
				Intensive insulin therapy	30
Tian *et al*[31], 2018	12 wk	T2DM with NAFLD	Ultrasound	Liraglutide 0.6-1.2 mg QD	52
				Metformin 1000-1500 mg/d	75
Eriksson *et al*[32], 2018	12 wk	T2DM with NAFLD	MRI	Dapagliflozin 10 mg QD	21
				PBO	21
				Dapagliflozin 10 mg QD + OM-3CA	22

				OM-3CA 4 g	20
Ito *et al*[33], 2017	24 wk	T2DM with NAFLD	Clinical laboratorytests or imaging	Ipragliflozin 50 mg QD	32
				Pioglitazone 15–30 mg QD	34
Kuchay *et al*[34], 2018	20 wk	T2DM with NAFLD	MRI	Empagliflozin 10 mg QD	22
				Control	20
Shibuya *et al*[35], 2018	6 mo	T2DM with NAFLD	Imaging (CT or ultrasound)	Luseogliflozin 2.5 mg QD	16
				Metformin 1500 mg QD	16
Shimizu *et al*[36], 2018	24 wk	T2DM with NAFLD	Transient elastography	Dapagliflozin 5 mg QD	33
				Control	24

NAFLD: Non-alcoholic fatty liver disease; MRI: Magnetic resonance imaging; T2DM: Type 2 diabetes mellitus; NASH: Nonalcoholic steatohepatitis; CT: Computed tomography.

Table 2 Comparison of body weight and body mass index changes from baseline in different treatment groups

	Studies	Participants	WMD	95%CI		*P* value
Body weight change						
DPP-4 inhibitors	4	140/138	-1.20	-1.44	-0.95	< 0.0001
GLP-1 RAs	8	1222/821	-3.61	-5.74	-1.48	0.0009
SGLT2 inhibitors	5	146/135	-2.53	-3.35	-1.71	< 0.0001
BMI change						
DPP-4 inhibitors	5	144/146	-0.59	-1.80	0.61	0.33
GLP-1 RAs	7	251/293	-1.46	-2.23	-0.69	0.0002
SGLT2 inhibitors	4	114/101	-1.72	-2.68	-0.75	0.0005

WMD: Weighted mean difference; CI: Confidence interval; DPP-4: Dipeptidyl peptidase-4; GLP-1 RA: Glucagon-like peptide-1 receptor agonist; SGLT2: Sodium-glucose cotransporter 2; BMI: Body mass index.

Table 3 Comparison of hemoglobin A1c change from baseline in different treatment groups

	Studies	Participants	WMD	95%CI		*P* value
HbA1c change						
DPP-4 inhibitors	6	166/166	-0.42	-0.58	-0.25	< 0.0001
GLP-1 RAs	7	1210/809	-0.29	-0.85	0.26	0.30
SGLT2 inhibitors	5	146/135	-0.36	-0.80	0.08	0.11
HbA1c change (placebo-controlled studies only)						
DPP-4 inhibitors	5	102/102	-0.38	-0.71	-0.05	0.02
GLP-1 RAs	3	1009/566	-0.88	-1.56	-0.19	0.01
SGLT2 inhibitors	1	21/21	-0.50	-0.84	-0.16	0.004

HbA1c: Hemoglobin A1c; WMD: Weighted mean difference; CI: Confidence interval; DPP-4: Dipeptidyl peptidase-4; GLP-1 RA: Glucagon-like peptide-1 receptor agonists; SGLT2: Sodium-glucose cotransporter 2.

study used the indicator of MRI-PDFF in the comparison between the GLP-1 RA and control groups showed a significant -3.20% reduction (95%CI: -5.98% to -0.42%, P = 0.02). MRI-PDFF was observed in two studies with SGLT2 inhibitor treatment, and the results suggested that SGLT2 inhibitor treatment decreased MRI-PDFF with marginal significance in the comparison with the control group (WMD = -1.18%, 95%CI: -2.38% to 0.03%, P = 0.06) (Table 4). The difference of MRI-PDFF change from baseline between all these three treatment groups was not significant (P > 0.05).

One study comprising sitagliptin and liraglutide arms reported the effect of VFA change in the comparison with glargine insulin treatment. The results of this study indicated that both sitagliptin and liraglutide could obviously decrease VFA (WMD -23.10 cm^2, 95%CI: -38.84 to -7.36 cm^2, P = 0.004; WMD -30.40 cm^2, 95%CI: -46.86 to -13.94 cm^2, P < 0.001; respectively). Although only two studies used the indicator of VFA in the included studies comparing the SGLT2 inhibitor and control groups, both of which showed that SGLT2 inhibitors could reduce it (WMD -23.48 cm^2, 95%CI: -25.85 to -21.12 cm^2, P < 0.001) (Table 4). The difference of VFA change from baseline between all these three treatment groups was not significant (P > 0.05).

Association between changes in transaminase, body fat composition, and weight loss

Meta-regression analyses indicated that the weight change difference was not associated with the difference of ALT, AST, MRI-PDFF, and VFA between antidiabetes drugs and controls (Table 5).

DISCUSSION

In the present meta-analysis, we found that compared with placebo or other active glucose-lowering agents, pooling of results from the included studies revealed significant improvements in serum ALT and AST levels following treatment with the novel agents (DPP-4 inhibitors, GLP-1 RAs, and SGLT2 inhibitors) in NAFLD patients. VFA and MRI-PDFF are quantitative biomarkers that can accurately estimate liver fat content. Although there were just four studies, all of them showed that novel glucose-lowering agents could decrease VFA. Furthermore, the trends of reduction in MRI-PDFF changes were also observed in all the novel glucose-lowering agent treatment groups. Therefore, all these novel glucose-lowering agents showed a beneficial effect in improving liver injury and reducing liver fat in NAFLD patients.

Apart from the proven effect on glucose control, GLP-1 RAs have several metabolic functions, including decreasing insulin resistance and lipotoxicity, enhancing liver glucose uptake, and improving peripheral insulin sensitivity[37]. DPP-4 inhibitors act on the enzyme DPP-4, which are expected to prolong the action of GLP-1. SGLT2 inhibitors also appear to have a beneficial hepatic effect in patients with NAFLD and T2DM[38]. The incretin-based therapies and SGLT2 inhibitors act in numerous potential ways in the pathogenesis of NAFLD: (1) Weight loss is a recognized predictor of reductions in liver fat[39]. The amount of weight loss is considered to be a determinant of histologic improvements in liver injury[40]. Clearly, the evidence that GLP-1 RAs and SGLT2 inhibitors lead to significant weight loss has been proven in many clinical trials conducted in various populations, although through different mechanisms. In the current meta-analysis, significant reductions in BMI and body weight were also observed in NAFLD patients treated with GLP-1 RAs and SGLT2 inhibitors; (2) Improvement of inflammation and oxidation: DPP-4 inhibitors were shown to affect inflammatory pathways in animal models of NASH, including reduced expression of proinflammatory mediators and attenuation of endoplasmic reticulum stress and hepatocyte apoptosis[41,42]. In an animal model of non-obese NASH, GLP-1 RAs were shown to inhibit hepatic inflammation through inhibition of hepatic free fatty acid influx and oxidative stress[43]. In addition, GLP-1 RAs can act directly on Kupffer cell function to reduce the influx of macrophages to the liver[44]. SGLT2 inhibitor treatments were also shown to attenuate the development of NASH through anti-inflammatory and oxidation effects in rodent models[11,45]; (3) Hyperglycaemia plays an important role in the process of liver lipogenesis by activating the key modulator-carbohydrate-responsive element-binding protein transcriptional factor[46]. Therefore, improvement in glycaemic control observed in the current meta-analysis might also contribute to the amelioration of NAFLD; and (4) The above effects help to ameliorate the condition of insulin resistance, which is a common and key feature of NAFLD that contributes to its pathogenesis[47]. All these effects provide the possibility for the treatment of NAFLD with incretin-based therapies and SGLT2 inhibitors.

Table 4 Comparison of magnetic resonance imaging proton density fat fraction and visceral fat area change from baseline in different treatment groups

	Studies	Participants	WMD	95%CI		*P* value
MRI-PDFF change						
DPP-4 inhibitors	2	52/49	-2.31	-4.92	0.29	0.08
GLP-1 RAs	1	24/24	-3.20	-5.98	-0.42	0.02
SGLT2 inhibitors	2	65/61	-1.18	-2.38	0.03	0.06
VFA change						
DPP-4 inhibitors	1	27/24	-23.10	-38.84	-7.36	0.004
GLP-1 RAs	1	24/24	-30.40	-46.86	-13.94	0.0003
SGLT2 inhibitors	2	48/50	-23.48	-25.85	-21.12	< 0.0001

MRI-PDFF: Magnetic resonance imaging proton density fat fraction; VAF: Visceral fat area; WMD: Weighted mean difference; CI: Confidence interval; DPP-4: Dipeptidyl peptidase-4; GLP-1 RA: Glucagon-like peptide-1 receptor agonist; SGLT2: Sodium-glucose cotransporter 2.

Table 5 Meta-regression analyses of association between weight change difference and difference of alanine aminotransferase, aspartate aminotransferase, magnetic resonance imaging proton density fat fraction, and visceral fat area between antidiabetes drugs and controls (ß coefficient value, *P* value, and 95%CI are shown)

	ß	*P* value	95%CI
ALT	0.064	0.267	-0.053-0.181
AST	0.020	0.735	-0.104-0.144
MRI-PDFF	0.308	0.701	-2.678-3.294
VFA	1.776	0.343	-11.723-15.275

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MRI-PDFF: Magnetic resonance imaging proton density fat fraction; VFA: Visceral fat area.

In this meta-analysis, we also tried to figure out the difference of these novel drugs' effects on NAFLD. To the best of our knowledge, there is no head-to-head study comparing their effect. The serum ALT is a specific indicator of hepatic inflammation, which is recommended as a means of monitoring disease improvement[48]. The indirect comparison result from our meta-analysis suggested that GLP-1 RAs can better improve serum ALT level when compared with SGLT2 inhibitors. Regarding VFA and MRI-PDFF, no significance difference was observed between these treatment groups. As mentioned above, the incretin-based therapies and SGLT2 inhibitors shared several targets in the pathogenesis of NAFLD. However, GLP-1 RAs and SGLT2 inhibitors also possess additive benefits, respectively. Ketogenesis plays an important role in the pathogenesis of NAFLD. It has been found that SGLT2 inhibitors could enhance ketone body metabolism by upregulating transporters and ketogenic enzymes in the liver[49]. Besides, the benefits of GLP-1 RAs in amelioration of lipotoxicity were observed in several clinical trials[25,50]. In addition, it is worth noting that heterogeneity existed between different treatment groups. Therefore, we should explain the results with caution, and it is still hard to judge which treatment is more effective. Individualized treatment options for patients with different characteristics might be more appropriate. More head-to-head studies are needed to further illuminate the underlying difference between these treatments.

As a meta-analysis, this study has several potential limitations. First, as mentioned above, this meta-analysis included studies with different treatment durations, baseline characteristics, and predefined outcomes, which might result in bias. Second, the numbers of participants with available reports of VFA and MRI-PDFF were limited, which might weaken the statistical power. Third, the diagnostic criteria for NAFLD were different, leading to heterogeneity across studies. Additionally, the included trials were relatively short in duration compared to the timescale of progression of NAFLD. As we know, the histological progression or resolution is recognized as the

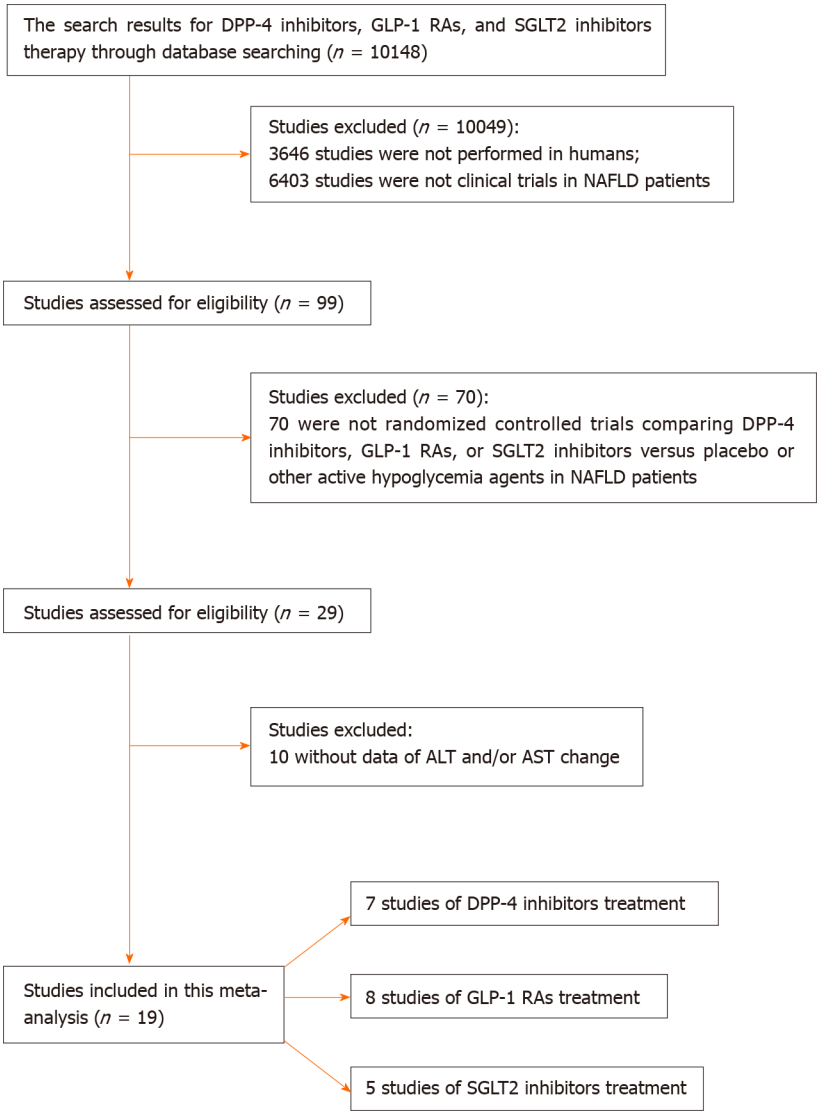


Figure 1 Flow chart of included studies.

"gold standard" of disease evaluation. However, a search of the previous literature showed that controlled trials with histological outcomes were limited. In this context, we used the indirect quantified measures (changes in ALT and AST levels) as the primary outcome measures.

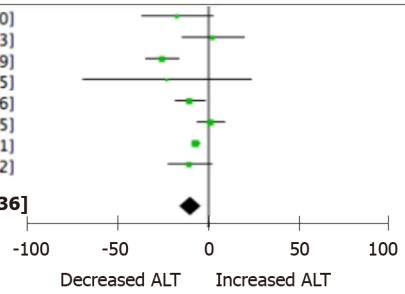
CONCLUSION

According to this study, treatment with DPP-4 inhibitors, GLP-1 RAs, and SGLT2 inhibitors resulted in improvements in serum ALT and AST levels and body fat composition, indicating that all these novel glucose-lowering agents have a beneficial effect in improving liver injury and reducing liver fat in NAFLD patients.

A

Study or subgroup	DPP-4 inhibitors Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, Random, 95%CI
Alam 2018	-38.5	38.13	20	-21.2	23.39	20	6.9%	-17.30 [-36.90, 2.30]
Cui 2017	-9	30.23	25	-11.5	30.85	25	8.3%	2.50 [-14.43, 19.43]
Hussain 2016	-29.6	17.64	29	-4	18.13	29	15.1%	-25.60 [-34.81, -16.39]
Joy 2017	-21	46.1	6	2	35.69	6	1.6%	-23.00 [-69.65, 23.65]
Li 2019-1	-9.64	17.48	31	0.64	15.98	33	16.3%	-10.28 [-18.50, -2.06]
Li 2019-2	-9.64	17.48	33	-10.94	12.7	31	17.2%	1.30 [-6.15, 8.75]
Macauley 2015	-6.9	2.58	22	0	3.72	22	22.5%	-6.90 [-8.79, -5.01]
Yan 2019-1	-11.2	25.7	27	-0.8	18.2	24	12.1%	-10.40 [-22.52, 1.72]
Total (95%CI)			**193**			**190**	**100%**	**-9.48 [-15.61, -3.36]**

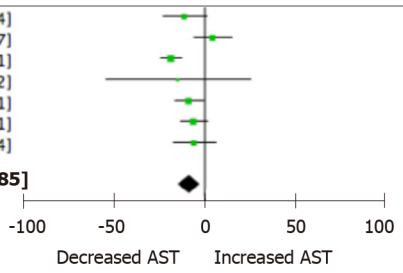
Heterogeneity: Tau^2 = 42.51; Chi^2 = 23.94, df = 7 (P = 0.001); I^2 = 71%
Test for overall effect: Z = 3.03 (P = 0.002)



B

Study or subgroup	DPP-4 inhibitors Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, Random, 95%CI
Alam 2018	-17.9	23.25	20	-6.8	15.07	20	13.1%	-11.10 [-23.24, 1.04]
Cui 2017	-1	19.62	25	-5.5	17.74	25	15.0%	4.50 [-5.87, 14.87]
Hussain 2016	-22	11.03	29	-3.2	11.84	29	20.2%	-18.80 [-24.69, -12.91]
Joy 2017	-58	38.3	6	-43	32.55	6	2.4%	-15.00 [-55.22, 25.22]
Li 2019-1	-15.54	18.71	31	-6.85	13.71	33	17.6%	-8.69 [-16.77, -0.61]
Li 2019-2	-15.54	18.71	33	-9.45	11.18	31	18.3%	-6.09 [-13.59, 1.41]
Yan 2019-1	-8.7	19.2	27	-2.9	23.4	24	13.4%	-5.80 [-17.64, 6.04]
Total (95%CI)			**171**			**168**	**100%**	**-8.36 [-14.68, -1.85]**

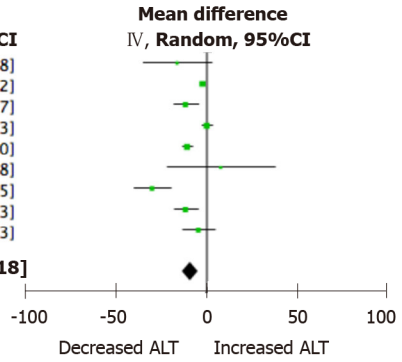
Heterogeneity: Tau^2 = 45.51; Chi^2 = 17.80, df = 6 (P = 0.007); I^2 = 66%
Test for overall effect: Z = 2.52 (P = 0.01)



C

Study or subgroup	GLP-1 RA Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, Random, 95%CI
Armstrong 2016	-26.6	34.4	26	-10.2	35.8	26	4.3%	-16.40 [-35.48, 2.68]
Cusi 2018	-8.8	1.5	971	-6.7	1.8	528	17.1%	-2.10 [-2.28, -1.92]
Fan 2013	-25.92	17.95	49	-14.33	20.01	68	12.3%	-11.59 [-18.51, -4.67]
Feng 2017-1	-22.31	5.31	29	-22.57	5.45	29	16.1%	0.26 [-2.51, 3.03]
Feng 2017-2	-22.31	5.31	29	-11.63	5.09	29	16.1%	-10.68 [-13.36, -8.00]
Khoo 2017	-34	27	12	-42	46	12	2.0%	8.00 [-22.18, 38.18]
Shao 2014	-127.03	17.7	30	-96.56	22.06	30	9.3%	-30.47 [-40.59, -20.35]
Tian 2018	-25.92	17.95	52	-14.33	20.01	75	12.5%	-11.59 [-18.25, -4.93]
Yan 2019-2	-5.2	12.5	24	-0.8	18.2	24	10.4%	-4.40 [-13.23, 4.43]
Total (95%CI)			**1222**			**821**	**100%**	**-8.73 [-13.29, -4.18]**

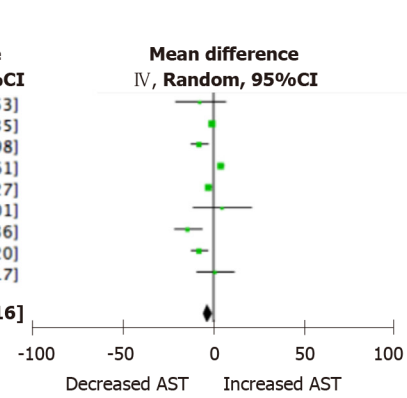
Heterogeneity: Tau^2 = 31.63; Chi^2 = 89.98, df = 8 (P < 0.00001); I^2 = 91%
Test for overall effect: Z = 3.76 (P = 0.0002)



D

Study or subgroup	GLP-1 RA Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, Random, 95%CI
Armstrong 2016	-15.8	21.8	26	-8.6	28.3	26	2.8%	-7.20 [-20.93, 6.53]
Cusi 2018	-4.3	1.3	971	-3.8	1.5	528	20.3%	-0.50 [-0.65, -0.35]
Fan 2013	-10.31	11.63	49	-2.74	13.61	68	12.0%	-7.57 [-12.16, -2.98]
Feng 2017-1	-7.2	2.35	29	-11.45	2.89	29	19.1%	4.25 [2.89, 5.61]
Feng 2017-2	-7.2	2.35	29	-4.73	2.33	29	19.4%	-2.47 [-3.67, -1.27]
Khoo 2017	-18	15	12	-23	24	12	2.2%	5.00 [-11.01, 21.01]
Shao 2014	-92.9	15.17	30	-78.96	14.77	30	7.1%	-13.94 [-21.52, -6.36]
Tian 2018	-10.35	11.63	52	-2.74	13.61	75	12.4%	-7.61 [-12.02, -3.20]
Yan 2019-2	-1.8	9.3	24	-2.9	23.4	24	4.7%	1.10 [-8.97, 11.17]
Total (95%CI)			**1222**			**821**	**100%**	**-2.65 [-5.14, -0.16]**

Heterogeneity: Tau^2 = 7.98; Chi^2 = 90.06, df = 8 (P < 0.00001); I^2 = 91%
Test for overall effect: Z = 2.08 (P = 0.04)



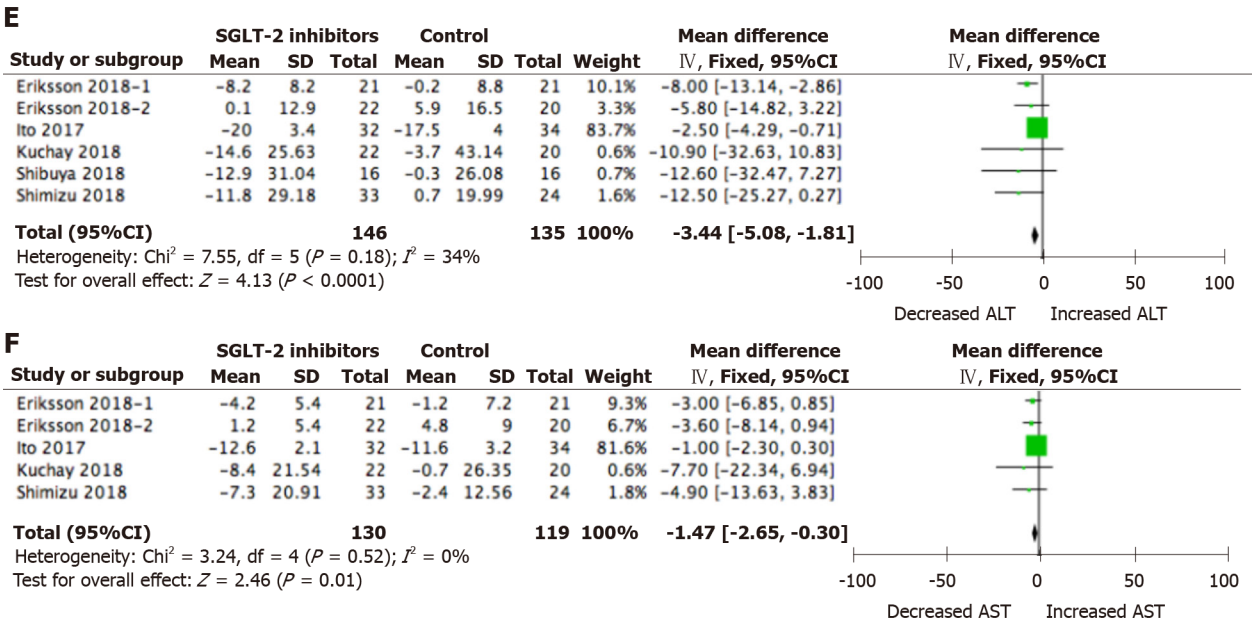


Figure 2 Alanine aminotransferase and aspartate aminotransferase changes from baseline in non-alcoholic fatty liver disease patients treated with novel glucose-lowering drugs. A: Alanine aminotransferase (ALT) changes with dipeptidyl peptidase-4 inhibitor treatment; B: Aspartate aminotransferase (AST) changes with dipeptidyl peptidase-4 inhibitor treatment; C: ALT changes with glucagon-like peptide-1 receptor agonist treatment; D: AST changes with glucagon-like peptide-1 receptor agonist treatment; E: ALT changes with sodium-glucose cotransporter 2 inhibitor treatment; F: AST changes with sodium-glucose cotransporter 2 inhibitor treatment.

ARTICLE HIGHLIGHTS

Research background

The efficacy of novel glucose-lowering drugs in treating non-alcoholic fatty liver disease (NAFLD) is unknown.

Research motivation

Although accumulated evidence suggests that these novel glucose-lowering drugs are promising in the treatment of NAFLD and nonalcoholic steatohepatitis, there is conflicting evidence and no convincing consensus on their long-term efficacy and outcome. In addition, there have been few head-to-head clinical trials comparing these novel glucose-lowering drugs directly.

Research objectives

We carried out this meta-analysis to evaluate the efficacy of novel glucose-lowering drugs in treating NAFLD.

Research methods

Electronic databases were systematically searched. The inclusion criteria were: Randomized controlled trials comparing dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 receptor agonists (GLP-1 RAs), or sodium-glucose cotransporter 2 (SGLT2) inhibitors against placebo or other active glucose-lowering drugs in NAFLD patients, with outcomes of changes in liver enzyme [alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST)] from baseline.

Research results

Treatment with DPP-4 inhibitors, GLP-1 RAs, and SGLT2 inhibitors resulted in improvements in serum ALT and AST levels. The trends of reduction in magnetic resonance imaging proton density fat fraction and visceral fat area changes were also observed in the DPP-4 inhibitor, GLP-1 RA, and SGLT2 inhibitor treatment groups.

Research conclusions

Treatment with DPP-4 inhibitors, GLP-1 RAs, and SGLT2 inhibitors resulted in improvements in serum ALT and AST levels and body fat composition, indicating a

beneficial effect in improving liver injury and reducing liver fat in NAFLD patients.

Research perspectives

In this meta-analysis, we made a comprehensive evaluation of the efficacy of novel glucose-lowering drugs in treating NAFLD.

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